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Luminal-derived stimuli as triggers of irritable bowel syndrome-like states in the rat: role of neurotrophic factors

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"Above all, don't fear difficult moments. The best comes from them"

– Rita Levi-Montalcini

Italian neurologist who won the Nobel Prize in 1986 for the discovery of nerve growth factor

INDEX

ABBREVIATIONS

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Actb: B-Actin

AUC: Area under the curve

BDNF: Brain-derived neurotrophic factor

BSA: Bovine serum albumin

CCh: Carbachol

CGRP: Calcitonin gene-related peptide

CPA: Metalloprotease carboxypeptidase A

Ct: Threshold cycle

CTMC: Connective tissue mast cell

Cy3: Carbocyanine 3

DRG: Dorsal root ganglion

EMS: Electrical mucosal stimulation

ENS: Enteric nervous system

FGD: Functional gastrointestinal disorder

FISH: Fluorescence *in situ* hybridization

GCM: Gut commensal microbiota

GDNF: Glial cell line-derived neurotrophic factor

GFRα: GDNF family receptor α

GMC: Giant migrating contraction

HFLA: High-frequency and low-amplitude contraction

IBD: Inflammatory bowel disease

IBS: Irritable bowel syndrome

IFN: Interferon

IgE: Type E immunoglobulin

IL: Interleukin

iNOS: Inducible nitric oxide synthase

LFHA: Low-frequency and high-amplitude contraction

L-NNA: N^G-nitro-L-arginine

LPS: Lipopolysaccharides

LS: Lumbosacral

MC: Mast cell

MMC: Mucosal mast cell

NGF: Nerve growth factor

NO: Nitric oxide

NT-3: Neurotrophin-3

NT-4/5: Neurotrophin-4/5

NTF: Neurotrophic factor

OVA: Ovalbumin

PAR-2: Protease-activated receptor-2

PI: Post-infection

PI-IBS: Post-infectious-irritable bowel syndrome

RELM: Resistin-like molecule

RMCP: Rat mast cell protease

RMCPII: Rat mast cell protease II

RMCPVI: Rat mast cell protease VI

RPC: Rhythmic phasic contraction

RT-qPCR: Reverse transcription-quantitative polymerase chain reaction

TC: Tonic contraction

TL: Thoracolumbar

Trk: Tropomyosin-receptor-kinase

TrkA: Tropomyosin-receptor-kinase A

TrkB: Tropomyosin-receptor-kinase B

TRPV1: Transient receptor potential vanilloid channel-1

SUMMARY

In the gastrointestinal tract, luminal-derived stimuli have been implicated in the pathophysiology of irritable bowel syndrome (IBS). Although the pathways through which luminal factors contribute to the onset/maintenance of IBS-like symptoms remain unclear, a role for a neuroimmune circuitry implicating mast cells (MCs) and neurotrophic factors (NTFs) has been suggested. This work aims to characterize the ability of luminal-derived stimuli, including gut commensal microbiota (GCM), intestinal infections and food antigens, to trigger IBS-like alterations in the colon of the rat, with special emphasis on the potential role of NTFs. For this, we used two accepted models of mucosal MC (MMC)-dependent intestinal dysfunction in rats: chronic exposure to oral ovalbumin (OVA) and infection with *Trichinella spiralis*. Both, exposure to oral OVA for a 6-week period and infection with *T. spiralis* activated MMCs of the colon, although cell recruitment was only observed during *T. spiralis* infection. A 6-week, but not 1-week, exposure to oral OVA resulted in a colonic contractile dysfunction, observed both *in vivo* and *in vitro*. Nevertheless, short (1-week) OVA exposure led to similar colonic motor alterations when combined with a dysbiotic-like state (treatment with bacterial lipopolysaccharides), thereby indicating a facilitatory interaction between microbiota and food-derived antigens. Treatment with K252a, a n antagonist of tropomyosin-receptor-kinase (Trk) receptors, decreased spontaneous colonic motility and prevented part of the motor alterations associated to OVA exposure, thus suggesting that the neurotrophins-Trk receptors axis participates in the maintenance of basal contractility in the rat colon and in the contractile dysfunction associated to exposure to oral OVA. In the presence of an infectious-related luminal-derived stimulus (*T. spiralis* infection), changes in thoracolumbar dorsal root ganglia (DRG) neurons' morphology and NTFs content was observed, pointing to an extended afferent remodeling through the gastrointestinal tract. Moreover, in infected animals, altered responses of DRGs (down-regulation of TRPV1 receptors) after colonic afferent stimulation with capsaicin indicated a persistent change in afferent sensitivity. Alterations in afferent functionality during *T. spiralis* infection coincided with time- (early vs. late phases) and site-specific (jejunum vs. colon) changes in NTFs expression. Within the jejunum, an early increase in nerve growth factor (NGF), glial cell line-derived NTF and artemin levels was observed. In the colon, however, a general down-regulation of NTFs was observed between days 6-14 post-infection. These regional differences might be related to the local degree of inflammation; since, compared to the jejunum, the colon showed an attenuated inflammatory response. Within the colon, NGF was located in the epithelium and the enteric nervous system, but not in MMCs. Nevertheless, MMCs expressed TrkA and treatment with K252a tended to increase the levels of rat mast cell protease II, thus indicating that colonic MMCs are a target for NGF. In summary, this work shows that the axis luminal-derived stimuli-MMCs-NTFs is implicated in the colonic functional alterations observed in food antigen- and infection-related models of IBS in rats. These results highlight a potentially important role for NTF-related pathways in the onset/maintenance of the symptomatology in, at least, a subset of IBS patients in which the disease is related to luminal dietary- or infectious-related factors. NTFs represent a therapeutic target for the treatment of gastrointestinal disorders characterized by altered colonic sensitivity and dysmotility.

En el tracto gastrointestinal, los estímulos de origen luminal se han implicado en la patofisiología del síndrome del intestino irritable (IBS). Aunque los mecanismos por los cuales contribuyen al inicio/mantenimiento de la sintomatología permanecen poco definidos, se ha propuesto la implicación de un circuito neuroinmune dependiente de mastocitos (MCs) y factores neurotróficos (NTFs). Este trabajo profundiza en la capacidad de los estímulos luminales, incluyendo factores relacionados con la microbiota comensal, las infecciones intestinales y los antígenos alimentarios, para producir alteraciones de tipo IBS en el colon de la rata, con especial énfasis en la implicación de los NTFs. Con este propósito, se han utilizado dos modelos de disfunción intestinal dependientes de mastocitos de mucosa (MMCs): la exposición oral a ovoalbúmina (OVA) y la infección por *Trichinella spiralis*. Tanto la exposición oral a OVA durante 6 semanas como la infección por *T. spiralis* activaron los MMCs del colon, si bien el reclutamiento celular sólo tuvo lugar durante la infección. La exposición a OVA durante 6 semanas alteró la contractilidad colónica, tanto *in vivo* como *in vitro*, un efecto que no se observó en tratamientos cortos (1 semana). Sin embargo, la exposición durante una semana a OVA combinada con un estado de disbiosis, inducido por el tratamiento con lipopolisacáridos bacterianos, produjo alteraciones motoras similares, sugiriendo una interacción facilitadora entre la microbiota y los antígenos alimentarios. El tratamiento con K252a, un antagonista de los receptores tropomiosina-receptor-quinasa, disminuyó la motilidad espontánea del colon y previno parte de las alteraciones motoras asociadas a OVA. Estos resultados apuntan a que, en la rata, el eje neurotrofinas-receptores Trk participa en el mantenimiento de la contractilidad basal del colon y en la disfunción contráctil asociada a OVA. En presencia de un estímulo luminal de origen infeccioso (*T. spiralis*), se observaron cambios morfológicos y en el contenido de NTFs en las neuronas de los ganglios de la raíz dorsal (DRGs) de los segmentos espinales toracolumbares, indicando una remodelación difusa de las vías aferentes entéricas. Los animales infectados presentaban, además, alteraciones en la respuesta a la estimulación aferente (disminución de TRPV1 en neuronas de los DRGs), sugiriendo cambios persistentes en la sensibilidad. Estas alteraciones coincidieron con cambios temporales (fases tempranas vs. tardías) y regionales (yeyuno vs. colon) en la expresión de NTFs. En el yeyuno, se observó un incremento temprano en la expresión de factor de crecimiento nervioso (NGF), de NTF derivado de la línea celular glial y de artemina. Por el contrario, en el colon se observó un descenso general en la expresión de NTFs entre los días 6-14 post-infección. Estas diferencias regionales podrían estar relacionadas con el grado de inflamación local ya que el colon, comparado con el yeyuno, mostró una respuesta inflamatoria atenuada. En el colon, se detectó la presencia de NGF en el epitelio y en el sistema nervioso entérico pero no en los MMCs. Sin embargo, la expresión de receptores TrkA sí se localizó en MMCs. Además, el tratamiento con K252a tendió a aumentar los niveles de la proteasa mastocitaria de rata de tipo II, sugiriendo que los receptores TrkA mastocitarios son funcionales. Por tanto, los MMCs podrían representar una diana para el NGF. Este trabajo demuestra que el eje estímulos luminales-MMCs-NTFs está implicado en las alteraciones funcionales colónicas observadas en modelos de IBS en la rata relacionados con antígenos alimentarios y procesos infecciosos. Las vías dependientes de NTFs podrían desempeñar un papel clave en el inicio/mantenimiento de la sintomatología del IBS, al menos en aquellos pacientes cuya enfermedad esté relacionada con factores dietéticos o infecciosos. Los NTFs representan una diana terapéutica para el tratamiento de los desórdenes gastrointestinales caracterizados por alteraciones sensoriales y motoras.

INTRODUCTION

Functional Organization of the Colon

The large intestine is the final part of the digestive tract and can be divided in two parts: cecum and colon. From a morphological point of view, the development of these two parts shows high species-related variability (1). Nevertheless, cytokinetic and histologic studies reveal many similarities between the colon of rats and humans at the ultrastructural level (2). In both species, the colon is composed by four broad layers, including the mucosa, the submucosa, the muscularis externa, and the serosa (or adventitia) (3,4).

In contrast to the small intestine, the colonic mucosa has no villi although it shows numerous invaginations of the epithelium known as crypts of Lieberkühn. It is lined by an epithelium composed of simple columnar cells with a thin brush border (colonocytes) and numerous interspersed goblet cells (Fig. 1). Colonocytes are the most abundant cells of the epithelium and are implicated in the terminal digestion and absorption of water and nutrients. Goblet cells produce components of the mucus layer, which protects and lubricates the mucosal surface. Dispersed among these cell types, the colonic epithelium also contains enteroendocrine cells. These are secretory cells that produce regulatory substances such as serotonin (5-hydroxytryptamine, 5-HT), vasoactive intestinal polypeptide (VIP), or somatostatin (Fig. 1) (3,4). Near the base of the colonic crypts, resides a population of intestinal stem cells, which can differentiate into the mature cell types composing the intestinal epithelium, accounting for the continuous epithelial cell renewal within the colon (2).

Supporting the epithelium, there is a layer of reticular connective tissue, highly vascularized and rich in immune cells, that constitutes the lamina propria. Directly beneath the lamina propria and separating it from the submucosa, lies the muscularis mucosae, which is a thin layer of smooth muscle that imparts fine movements to the mucosa (3,4).

The submucosa is an irregular fibroelastic connective tissue layer that contains blood and lymphatic vessels as well as a nerve fiber plexus known as the submucosal plexus (Fig. 1). This plexus, which is part of the enteric nervous system (ENS), is associated to the secretomotor control of the mucosa, including local blood flow control (5).

The colonic muscularis externa, which is responsible for the motor activity of the colon, consists of two layers of smooth muscle: an inner circular coat and an outer longitudinal coat that, in some species, forms three separate longitudinal bands called taenia coli. Between these two muscle layers resides the other plexus of the ENS, the myenteric plexus, involved in the control of the activity of the smooth muscle layers (5).

Finally, depending on the region of the colon, the muscularis externa is bounded by the serosa, a single layer of mesothelial cells together with connective tissue, or the adventitia, which blends with the connective general tissue of the region.

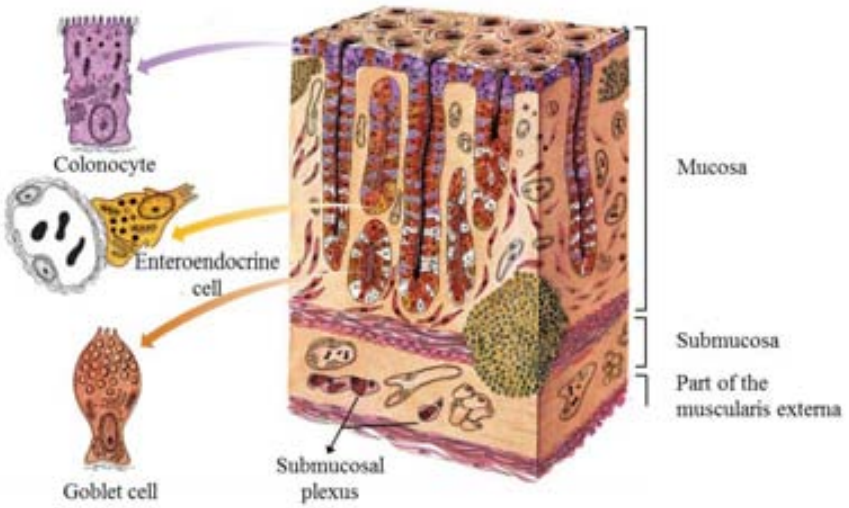

Figure 1. Tissue layers of the colon and epithelial cell types. Image adapted from Gray 2005 (6).

Innervation of the Colon

The colon is innervated by both the ENS and extrinsic projections, including sympathetic and parasympathetic neurons as well as visceral afferents (5).

The neurons of the ENS are usually classified according to their functional properties, thereby distinguishing between motor neurons, interneurons and primary afferent neurons (7,8). The smooth muscle receives projections of both excitatory and inhibitory motor neurons, terms referring to the pattern of neurotransmitters they express. For instance, the excitatory component of the muscle innervation is predominantly due to the

release of acetylcholine and tachykinins while purines (mainly ATP) and nitric oxide (NO) are the main inhibitory transmitters (7,8). In addition, a subgroup of motor neurons project to the mucosa and the local blood vessels and are involved in secremotor and vasomotor control (7,8). As it refers to interneurons, their function is to contribute to the spreading of reflexes up or down the gut, depending if they are orally (ascending interneurons) or anally (descending interneurons) directed (8,9). Finally, within the submucosal and myenteric plexuses, there are also intrinsic primary afferent neurons (IPANs). These are sensory neurons that respond to chemical and mechanical stimuli applied to the mucosa or the muscle layers, thereby initiating gut reflexes (8,9).

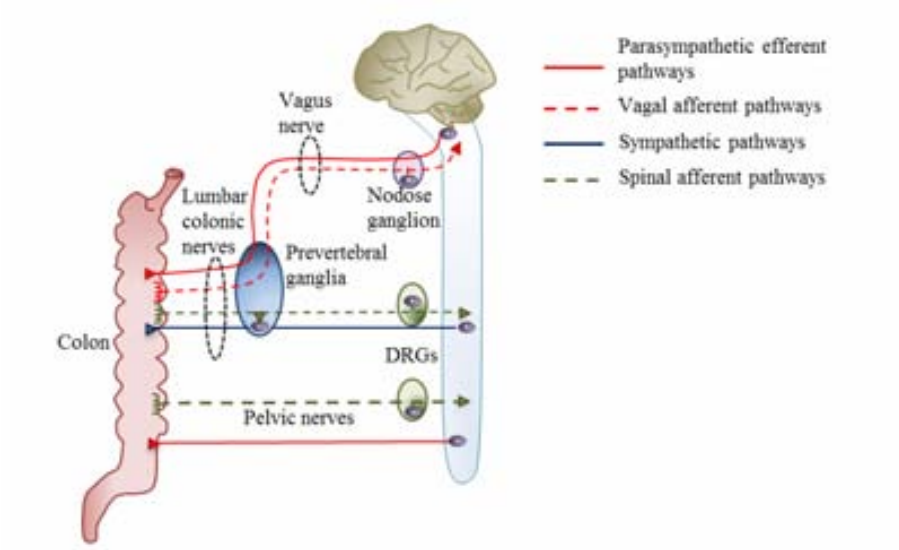

Figure 2. Extrinsic innervation of the human colon. Image modified from Sleisenger 2010 (10).

The activity of the ENS is subjected to modulation by the extrinsic, autonomic innervation (Fig. 2). Sympathetic postganglionic fibers emerge from the prevertebral ganglia and inhibit intestinal motility by acting on the enteric ganglia (Fig.2) (11). As it relates to the parasympathetic innervation, preganglionic fibers descend either via the vagus nerve or the pelvic nerves and synapse on postganglionic neurons located in the myenteric and submucosal plexuses (11) (Fig. 2). Finally, the colon also receives the projections of extrinsic primary afferent neurons (EPANs), with their cell body located either in the nodose (vagal afferents) or dorsal root ganglia (DRGs; spinal and sacral afferents) (12) (Fig. 2). Vagal afferent innervation of the colon is less dense than in the

upper parts of the gastrointestinal tract, with neuronal endings present only in the colonic mucosa, muscular layers and ganglionic plexuses of the proximal part of the colon (13,14). The exact role of these fibers in reflex control and transmission of pain sensation is not clear. In the rat, spinal afferent innervation of the mid and distal colon has been shown to arise mainly from thoracolumbar (TL, T13-L2) and lumbosacral (LS, L6-S1) DRGs (15,16). These fibers are well known for carrying nociceptive information (17).

Colonic Motility

The coordinated contractile activity of the two muscle layers of the colon is required for the water extraction from the non-digestible waste and for defecation (18).

Colonic motor activity is orchestrated mainly by the ENS although, as mentioned above, it is highly modulated by the extrinsic innervation. The circular muscle layer of the colon is the main responsible for the contractility that mixes and propels the content.

Colonic smooth muscle generates three types of contractions: rhythmic phasic contractions (RPCs), giant migrating contractions (GMCs) and tonic contractions (TCs). Colonic RPCs are short-amplitude contractions, usually below 50 mmHg, that depending on their duration can be classified into: short-duration RPCs (2-3 seconds), which occur more frequently, and long-duration RPCs (15-20 seconds), less frequent and normally organized in bursts (19,20). RPCs mix and propel the colonic content at relatively slow rates so that adequate time is available for water absorption (19,20). On the other hand, GMCs are characterized by their large amplitude and long-duration, migrating uninterruptedly over long distances to produce mass movements and defecation (19,20). The frequency of these colonic contractions varies widely between species, occurring between 2-5 times a day in healthy humans while in rodents they show up to 45 times per h (19). Finally, TCs decrease the luminal diameter and, although their effects alone in mixing and propelling the content are insignificant, they potentiate the action of RPCs in propulsion (19).

The Intestinal Immune System

The intestinal mucosa is an enormous surface exposed to the external environment, representing a major gateway for potential pathogens. Thus, its immune system needs to be ready to respond to these threats, at the same time that must remain tolerant to food antigens and the gut commensal microbiota (GCM) (21).

The immediate response to enteric pathogens is mediated by the activation of the innate immune system by pattern recognition receptors (PRRs), which are found both in the intestinal epithelium and immune cells of the lamina propria (22). This innate response of the gut is unspecific and implies the secretion of antimicrobial molecules, the activation of the complement system and the recruitment of phagocytic cells (22). Additionally, the intestine harbors a large number of adaptive immune cells, including lymphocytes T and B, which provide specificity and memory to the intestinal immune response. Depending on the cytokine pattern expressed by T helper (Th) cells after the activation of the adaptive immune system, these responses can be classified into Th1, Th2 or Th17 (23).

The intestinal immune system shows a dynamic equilibrium between the ability to respond rapidly to invading pathogens and the maintenance of tolerance to the GCM and food antigens (24). The breakdown of this tolerance results into an exacerbated and persistent activation of the local immune system, leading to chronic inflammatory states of the gut (24). Thus, it has been suggested that environmental and/or genetic factors affecting these tolerogenic mechanisms are implicated in the induction of inflammatory and functional disorders of the gastrointestinal tract (25).

Functional Gastrointestinal Disorders: Irritable Bowel Syndrome

Functional gastrointestinal disorders (FGDs) are defined as a variable combination of chronic or recurrent symptoms affecting different regions of the gastrointestinal tract and that are not explained by structural or biochemical abnormalities, at least according to the methodologies currently available (26). Irritable bowel syndrome (IBS) is the most frequent FGD and is characterized by abdominal pain, alterations of the bowel habits and visceral hypersensitivity, in the absence of apparent organic alterations (27). IBS is

highly prevalent in industrialized countries, may affect up to 10-20% of the population, particularly women, who are twice as likely to suffer IBS as men, and the symptomatology appears for the first time between the ages of 30 and 50 years (28). As there are no specific diagnostic tests, the identification of IBS patients relies on the application of symptom-based criteria (Rome III criteria), leading to potential diagnostic confusion (27). As a result, IBS therapeutics has a limited development. Therefore, the process has a tremendously important economic burden on health care resources for direct (e.g. diagnosis, therapy) and indirect (e.g. work absenteeism) costs.

Although the underlying pathophysiology of IBS remains unclear, it is accepted that the symptomatology observed arises from a dysregulation of the bidirectional communication between gut and brain (the so-called brain-gut axis), modulated by various psychosocial and environmental factors (e.g. external stressors, dietary constituents, genetics, altered GCM or intestinal infections). In addition, there is evidence that IBS patients consistently present a low grade inflammation in the intestinal mucosa and that IBS-like symptomatology is observed during remission phases of inflammatory bowel disease (IBD) (29,30).

The potential role of adverse food reactions in IBS has been recently reviewed (31). Food ingestion has been related to exacerbation of IBS symptomatology in a large number of IBS patients, thereby making it feasible to hypothesize an implication of food intolerance or alimentary allergy in the onset of IBS symptomatology (32). Supporting this hypothesis, it has been demonstrated an increase in the prevalence of atopic conditions in diarrhea-predominant IBS patients (33). Moreover, the prevalence of IBS is higher in patients with bronchial asthma compared to patients with other pulmonary disorders (34). Finally, several studies demonstrate usefulness of oral disodium cromoglycate, an inhibitor of mast cell (MC) degranulation, and elimination diets in diarrhea-predominant IBS patients, thus suggesting that part of the symptomatology observed could be related to food allergy or food intolerance (33,35). However, the intestinal food allergy-related mechanisms in IBS seem to involve local mucosal responses to dietary antigens rather than classical type-1 hypersensitivity reactions, as revealed by observations showing that IBS patients with positive results to colonoscopic provocation with dietary allergens have low titers of specific type E immunoglobulins (IgEs) in serum (36).

Compelling data demonstrate a strong association between intestinal infections and development of IBS-like symptomatology. Indeed, studies suggest that the probability of developing IBS is increased about six-fold after an acute gastrointestinal infection and remains significantly increased for up to 3 years thereafter (37). Thus, the term post-infectious-IBS (PI-IBS) has been used to refer to those patients that, after undergoing an acute and normally self-limiting gastrointestinal infection, develop chronic IBS-like symptomatology (38). Although the odds of developing PI-IBS have been associated to some risk factors, such as the duration and severity of the illness and the type of pathogen implicated, the exact mechanisms involved in the onset and perpetuation of the symptomatology have not been elucidated (39). Referring to the pathophysiology, increased counts of enterochromaffin cells, lymphocytes and MCs together with high levels of proinflammaory cytokines are common findings in intestinal biopsies of PI-IBS patients, thereby representing an underlying inflammatory basis for the functional alterations observed (38).

Finally, several lines of evidence indicate that GCM-dependent host-bacterial interactions play an important role in the pathogenesis of IBS (40). For instance, it has been reported that patients with IBS have an altered GCM and that treatments targeting GCM can ameliorate the symptomatology (41). However, the underlying pathophysiological mechanisms elicited by GCM in the generation of symptoms in IBS remain unknown.

Animal Models of Irritable Bowel Syndrome

To better understand IBS pathophysiology, results from human and animal studies have to be integrated in a comprehensive manner. The selection of animal models for IBS is limited, in part, because of the large, undefined, spectrum of underlying mechanisms of the disease. Thus, the models described are focused on the development of one or a limited set of symptoms of the disease.

Based on the type of the initiating stimulus, animal models of IBS can de classified mainly in two groups: those induced by a central nervous system (CNS)-directed stimulus (psychosocial) and those induced by a gut-directed stimulus (physical) (42). However, due to the bidirectional interaction of the brain-gut axis, these models are not

restricted to either CNS or the gut but are likely to include both peripheral and central elements.

Animal Models Induced by CNS-Directed Stimuli (Psychosocial)

These models reproduce to some extent the impact of psychosocial stressors as a component related to the induction and/or exacerbation of the symptomatology in patients with IBS.

The models used include early life stress (neonatal maternal separation) (43,44) as well as chronic (45,46) and acute (47,48) stress during adulthood. In either case, long-lasting alterations in the homeostasis of the gastrointestinal tract, with functional changes in epithelial function, motility and visceral sensitivity, similar to those observed in IBS patients, can be observed.

Animal Models Induced by Primary Gut-Directed Etiologies (Physical)

These models are triggered by stimuli (mechanical, chemical or infectious) targeting the intestine and are applied during early life stages or in adulthood (42,49). They have been used to study alterations in the motor and epithelial barrier function of the intestine and, with more emphasis, to explore changes in gut perception (42,49). Frequently, and taking into consideration the multifactorial characteristics of IBS, CNS- and gut-directed stimuli are combined to generate more predictive models, at least from a theoretical point of view (50).

- Chronic Exposure to Oral Ovalbumin in Rats as a Model of Irritable Bowel Syndrome Related to Food Allergens

  In order to study the suggested role for dietary constituents on IBS pathophysiology, it could seem feasible to use validated animal models of food allergy showing functional gastrointestinal alterations similar to those observed in IBS. However, the mechanisms of hypersensitivity to food antigens in IBS seem to be different from those implicated in classical alimentary allergies. Food allergies are IgE-mediated type-I reactions while the aberrant responses of the gut to dietary antigens implicated in IBS are IgE-independent (31,36). Therefore, classical animal models of food allergy are not suitable to study the relationship between food antigens and IBS.

Exposure to oral ovalbumin (OVA) in allergy-prone rats (i.e. Brown Norway) is an accepted model of food allergy with consistent IgE-mediated systemic responses (51-53). From this model, our group has shown that a similar chronic exposure to oral OVA in non-allergy-prone rats (i.e. Sprague-Dawley) induces an IgE-independent intestinal dysmotility related to a state of activation/excitation of intestinal mucosal MCs (MMCs) (54,55). This model, therefore, might represent a valid approach to study the potential role of food allergens in IBS

- Intestinal Infection with *Trichinella spiralis* as a Model of Post-infectious-Irritable Bowel Syndrome

  The experimental infection with *Trichinella spiralis* in rodents is a gut-directed model that has been extensively used to study the pathogenesis of PI-IBS (56-60). The life cycle of the nematode *T. spiralis* begins after the host ingests meat contaminated with larvae cysts. Larvae are released from the cysts by gastric juices of the stomach (61). Subsequently, the parasites invade the enteric epithelium of the small intestine to mature into adult individuals and reproduce (61). Before expulsion by the host immune system, day 14 post-infection (PI) in the rat, females produce a great amount of newborn larvae, which will make their way through the circulatory system of the host to reach the muscle fibers, where they encyst (muscle phase of infection) (61). The enteric phase of the infection in rodents results in long-lasting functional disturbances of the gut, persisting long after the expulsion of the parasites (56,57). At these late stages of the infection, the intestinal inflammation has been resolved except for the presence of a persistent MMC infiltrate in the mucosa (56). In these conditions, a long-term increase in epithelial permeability and contractility dysfunction are observed, reminiscing the functional alterations described in PI-IBS patients (56,59). The presence of sensory alterations related to visceral sensitivity in this model has received little attention.

Mast Cells

MCs are resident granulocytes derived from pluripotent hematopoietic cells that, after an initial development in the bone marrow, enter the blood circulation and complete their differentiation within various tissues (62). The maturation of MCs in the resident tissue is influenced by a variety of cytokines and growth factors, although the most crucial one

is the stem cell growth factor, the ligand for the tyrosine kinase receptor (c-kit) (63). Therefore, depending upon the microenvironment, different MC phenotypes can develop in different tissues, and even in different locations of the same tissue (64).

Although mature MCs are ubiquitous in the body, they are specially located in the proximity to surfaces that interface with the external environment, such as the skin and the mucosa of the gastrointestinal and respiratory tract (65). In particular, within these tissues, MCs reside in close association with structures such as blood and lymphatic vessels and nerves.

Mature MCs are characterized by their ability to synthesize and store in their intracitoplasmatic granules a wide range of neuroimmune mediators that are released upon cell activation (63). Among these mediators, MC specific proteases, including serine proteases (tryptases and chymases) and the metalloprotease carboxypeptidase A (CPA), have been widely used to distinguish between distinct subsets of MC populations and as biomarkers of MC activation (66). For instance, in humans, MCs are classified in two groups, MC_T and MC_{TC}, according to their protease profile. MC_T only express tryptases and are localized in mucosal surfaces. MC_{TC} express all types of MC proteases (tryptases, chymases and CPA) and are located in the skin and intestinal submucosa (67). In rodents, MCs have been divided into two populations defined by their anatomical localization: MMCs, which are preferentially located in mucosal surfaces such as the intestinal mucosa, and connective tissue MCs (CTMCs) located in the skin and the peritoneal cavity (65). MMCs and CTMCs can be differentiated also by their proteinases. In rats, the quimase rat MC protease II (RMCPII) has been used as a specific marker for MMCs while the quimase rat MC protease I (RMCPI) and the tryptase rat MC protease VI (RMCPVI) have been used as specific markers for CTMCs (67).

Activation of Mast Cells

Activation of MCs results in the release into the extracellular environment of a broad array of mediators. MCs mediators can be divided into preformed (stored in the secretory granules and released within minutes upon activation) and synthesized de-novo after cell stimulation (68). Preformed mediators include histamine, MC proteases, proteoglycans and small amounts of tumor necrosis factor alpha (TNF-α), while the

molecules produced upon activation comprise phospolipid metabolites, such as prostaglandin D2 and different leukotrienes, a wide array of cytokines (interleukin-3, 4, 5, 6, 8, 10, 13, IL) and growth factors (69). Upon release, MC mediators are implicated in the induction and regulation of inflammatory processes and in tissue remodeling, including neuronal remodeling.

The best characterized mechanism of MC activation is via crosslinking of IgEs bound to high-affinity receptors for IgE (FcεRI) located on the cell surface (63). IgE-dependent MC activation is particularly important during Th2 allergic disorders or in parasitic responses, which are characterized by high concentrations of circulating IgEs (70). In addition, a large number of IgE-independent triggers have been described to potentially activate MCs, including ligands of Toll-like receptors (TLRs), complement system factors, cytokines, neuropeptides, neurotrophins (mainly nerve growth factor, NGF) and external stressors (71).

Mast Cells in Irritable Bowel Syndrome

Several observations support an involvement of MCs in the pathophysiology of IBS. First, many studies have evidenced a MC infiltration in the colon of IBS patients and high levels of their mediators (mainly proteases) in colonic supernatants and faecal samples (72-75). Second, the number of activated MCs in close proximity to colonic nerve terminals is enhanced in IBS patients compared with healthy controls (72). More interestingly, the severity and the frequency of abdominal pain in IBS patients positively correlate with the apposition MCs-nerves (72). Indeed, supernatants from colonic biopsies of IBS patients, containing a variety of MC mediators, sensitize cultured sensory neurons and elicit functional responses in animals (visceral hypersensitivity and alterations in colonic barrier function), similar to those observed in IBS patients (73-75). Thus, overall, these data point towards an important role of MC-derived mediators and the interaction MCs-nerve fibers on the disturbed secretomotor and sensory functions that characterize IBS.

Neurotrophic Factors

Neurotrophic factors (NTFs) are peptides that act directly on specific neuronal populations to support their survival, maintenance and regeneration, both in the

developing and mature nervous system (76). For instance, the survival of virtually all small-diameter sensory neurons depends on the retrograde transport of NTFs during embryonic life (77). In adulthood, NTFs promote neuronal survival, phenotype maintenance and injury- and degenerative-plasticity, thereby representing a potential therapeutic target for the treatment of some nervous system diseases (78).

NTFs have been grouped into four different families of structurally and functionally related molecules: NGF family, glial cell line-derived NTF (GDNF) family, neurokine family and non-neuronal growth factors family (78).

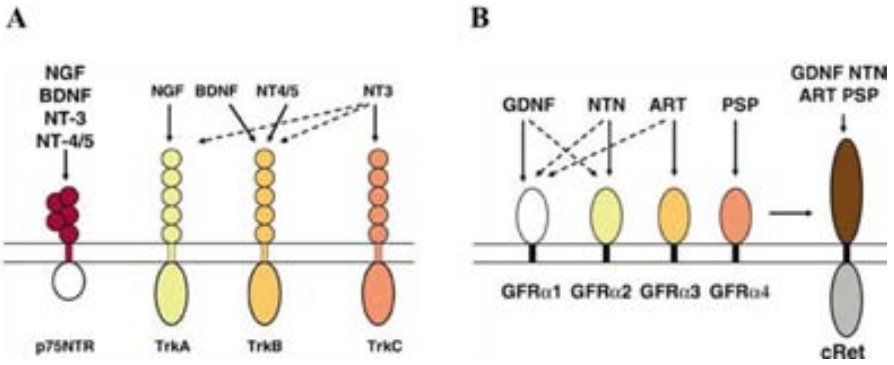

Figure 3. Receptors for the NGF and GDNF family members. A: The members of the NGF family bind specifically to cognate Trk receptors. NT-3 can also potentially interact with TrkB and TrkA receptors. The low-affinity neurotrophin receptor p75 promiscuously binds all of the neurotrophins. B: GDNF family members (GDNF, NTN, ART and PSP) bind a specific GFRα coreceptor and activate the common signaling receptor RET. Solid arrows indicate the preferred functional ligand-receptor interactions, whereas dotted arrows indicate putative crosstalk. ART: Artemin; BDNF: Brain-derived neurotrophic factor; GDNF: Glial cell line-derived neurotrophic factor; GFRα: GDNF family receptor α; NGF: Nerve growth factor; NT-3: Neurotrophin-3; NT-4/5: Neurotrophin-4/5; NTN: Neurturin; PSP: Persephin; Trk: Tropomyosin-receptor-kinase. Image adapted from Krieglstein 2008 (79).

The NGF family includes NGF, brain-derived NTF (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (80). These products interact with two entirely distinct classes of receptors; the low-affinity receptor p75, and the high-affinity tropomyosin-receptor-kinase (Trk) receptors that comprise three subtypes: TrkA, TrkB and TrkC (80) (Fig. 5A). The p75 is a non-selective receptor that binds to all members of the NGF family with similar affinity. On the other hand, Trk receptors are peptide-specific, with NGF

binding TrkA, BDNF and NT-4/5 binding TrkB, and NT-3 binding TrkC (80) (Fig.5A). NGF, the first member of the family to be discovered, has been particularly studied (81). The neurotrophin is produced mainly in target tissues of sensory and sympathetic fibers. NGF interacts with TrkA receptors in axon terminals and the NGF-TrkA complex is internalized and retrogradely transported to the neuronal body, where it is required for the survival and maintenance of the neurons (82). Transgenic mice lacking NGF (NGF knockout) have been reported to suffer severe sympathetic and sensory deficits, thereby reinforcing the role of NGF on modulating the survival of these neuronal populations (83).

The GDNF family includes GDNF and three structurally related members called artemin, neurturin and persephin. These NTFs exert their biological effects by signaling through a unique multicomponent receptor system consisting of a RET tyrosine kinase receptor and a glycosyl-phosphatidylinositol-anchored coreceptor (GDNF family receptor α1–4, GFRα) (84) (Fig.5B). While the RET receptor represents a common signaling component for all the GDNF family members, each member uses a GFRα coreceptor as the preferred ligand-binding protein (84) (Fig.5B). For instance, GNDF, neurturin, artemin and persephin activate GFRα1, GFRα2, GFRα3 and GFRα4, respectively, although some cross-over might occur under certain conditions (82) (Fig.5B). Similarly to NGF, a mechanism of receptor-mediated internalization and retrograde transport to the neuronal soma has been observed for GDNF and neurturin in sensory and motor neurons, but not in sympathetic neurons (85). GDNF and the related family members influence a broad spectrum of neurons, both in the peripheral and CNS (86). Studies in knockout mice for either GDNF or neurturin have demonstrated the crucial role of these NTFs on neuronal-related processes of the gut during development, as these animals lack enteric neurons and show severe reduction of the intestinal parasympathetic cholinergic innervation (87,88).

Neurotrophic Factors in the Gut

Since NTFs are important on neural remodeling, several studies have explored their role in the physiology of highly innervated organs, such as the gut. Wide data support a role for NTFs maintaining extrinsic enteric innervation (89-91); however, the exact source(s) of NTFs within the intestine remains unclear. For instance, NGF-like immunoreactivity has been described in enteric plexuses and also in non-neural cells, such as epithelial and smooth muscle cells (45,92-94). Moreover, results suggest that NGF, or at least a NGF

precursor (pro-NGF), is synthesized and released by MCs (43,92,94). Overall, the exact source(s) of NGF within the intestine remains obscure, with enteric neurons, epithelial cells and resident immune cells (mainly MCs) as the main candidates.

The fact that NGF is detected outside the nervous system could seem surprising as NTFs have been traditionally defined as neural-related molecules. However, growing evidences reveal that NTFs also exert a large broad of effects in non-neural tissues (95). Supporting extra-neuronal effects of NGF, the NGF high-affinity receptor TrkA has been described in non-neuronal structures. In particular, within the intestine, apart from the ENS, TrkA receptors have been described in epithelial and lamina propria cells (92,96,97). *In vitro* studies support the functionality of these receptors, suggesting, overall, an influence of NGF on both neural and non-neural enteric cells (98).

Other NTFs that have been detected in the intestine include GDNF, NT-3 and BDNF (99-101).

Neurotrophic Factors in Inflammatory and Functional Gastrointestinal Disorders

NTFs have been suggested to contribute to the functional alterations observed in inflammatory and FGDs. Results obtained in IBD and IBS patients and relevant animal models reveal an enhanced expression of NTFs, namely, NGF and GNDF family members (58,93,102,103). As NTFs are well known by their ability to sensitize sensory afferents, it has been hypothesized that the increased expression of these mediators could account for the altered visceral hypersensitivity observed in IBS (45,104,105). This is supported by the positive correlation found between abdominal pain and colonic content of BDNF in IBS patients (106). Signaling of NGF and GNDF family members has also been implicated in other key features of inflammatory and FGDs, such as altered epithelial barrier function and intestinal dysmotility (43,58,107) For instance, up-regulated levels of enteric NGF have been associated to the *T. spiralis*–induced jejunal hypermotility and the neonatal maternal separation-related altered gut permeability in rats (43,58). Although these evidences, as mentioned above, the source(s) of intestinal NTFs remains elusive.

HYPOTHESIS AND OBJECTIVES

From the previous background, it is assumed that luminal factors, intestinal mast cells (MCs) and neurotrophic factors (NTFs) participate in the pathophysiological mechanisms implicated in the functional alterations that characterize IBS.

Thus, this work is based on the **HYPOTHESIS** that changes in MCs, NTFs, and their association, are key elements of the neural remodeling observed in abnormal responses of the gut to luminal-derived stimuli, leading to long-term functional changes. In particular, during colonic conditions characterized by an excited-activated state of MCs, NTFs, mainly NGF, would modulate the alterations in the motor and sensory functions of the colon.

In order to demonstrate this hypothesis, two validated animal models of IBS, based on luminal-derived factors as inductive stimuli (chronic exposure to oral OVA and intestinal infection with *T. spiralis)*, together with a new protocol of OVA exposure associated to a dysbiotic-like state, were used in rats. Using these models, potential changes in colonic motor and sensory functions were determined. In addition, the relationship between the observed colonic dysfunction and the dynamics of MCs and NTFs was also explored.

Therefore, the specific **OBJECTIVES** of this work were as follows:

- To characterize the effects of the exposure to the dietary-related luminal antigen OVA on colonic motility, with emphasis on the potential implication of MCs and the neurotrophins-Trk receptors pathway.
- To determine the relationship between colonic MCs and NGF and, in particular, if colonic MCs are a source of NGF.
- To determine if gut microbiota-derived factors interact with oral OVA to mediate functional colonic alterations.
- To characterize plastic changes in the sensory afferent innervation of the colon associated to infectious (*T. spiralis*)-related luminal stimuli.
- To determine if infectious (*T. spiralis*)-related luminal stimuli are able to alter sensory properties of the colon, with emphasis on the potential role of NTFs.

CHAPTER 1

CHAPTER 1

NGF IS INVOLVED IN ORAL OVALBUMIN-INDUCED ALTERED COLONIC CONTRACTILITY IN RATS: EVIDENCE FROM THE BLOCKADE OF TRKA RECEPTORS WITH K252

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Running title: Effects of K252a on colonic contractility

Keywords: Colonic contractility; K252a; Mucosal mast cells; Nerve growth factor; Ovalbumin; TrkA

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Abstract

Background Nerve growth factor (NGF)-mucosal mast cells (MMCs) interaction has been implicated in the remodeling of enteric circuitries and the associated functional changes. We investigated the involvement of NGF and its receptor TrkA in the altered colonic contractile activity observed in the model of oral ovalbumin (OVA)-induced MMC hyperactivity in rats. We also studied the role of colonic MMCs as a source of NGF. **Methods** Rats were exposed to oral OVA, alone or with the TrkA antagonist K252a. Co-expression of NGF/TrkA and rat mast cell protease II (RMCPII) (double immunofluorescence), RMCPII content (ELISA) and expression of NGF, brain-derived neurotrophic factor (BDNF) and TrkA/B (RT-qPCR) were assessed in colon. Colonic contractile activity was determined *in vivo* and *in vitro*. **Key Results** TrkA, but not NGF, was localized in colonic MMCs (RMCPII-positive cells). OVA exposure increased colonic RMCPII levels but did not change the percentage of TrkA-positive MMCs. Neither OVA nor K252a, alone or combined, altered NGF, BDNF or TrkA/B expression. Spontaneous colonic activity *in vivo* and *in vitro* was altered by OVA, an effect prevented by K252a. Electrical stimulation-induced contractile responses *in vivo* and carbachol responses *in vitro* were increased by OVA in a K252a-independent manner. In OVA-treated animals, inhibition of NO synthesis with L-NNA significantly enhanced spontaneous colonic activity *in vitro*, a response completely prevented by K252a. **Conclusions & Inferences** These results suggest that NGF-TrkA-dependent pathways are implicated in colonic contractile alterations observed during OVA exposure in rats. NGF-TrkA system might represent a potential target for treatment of gastrointestinal disorders characterized by colonic motor alterations.

Introduction

Colonic dysmotility is a common finding in functional gastrointestinal disorders (FGDs). For instance, irritable bowel syndrome (IBS), the main FGD, is characterized by abdominal pain-discomfort associated with dysmotility and altered bowel habits (1). Moreover, in a large number of IBS patients, food ingestion has been associated with symptomatology exacerbation, suggesting a role for food allergy in its pathogenesis (2). Intestinal food allergy-related mechanisms in IBS seem to involve local mucosal responses to dietary antigens rather than classical type-1 hypersensitivity reactions (3). We have shown that chronic exposure to oral ovalbumin (OVA) in Sprague Dawley rats induces a non-IgE mediated alteration of smooth muscle colonic contractility resembling that observed by others and us in IBS and animal models of the disease (4-8).

Several observations support an involvement of mast cells in the pathophysiology of IBS (9). Morphological and functional studies with colonic biopsies from IBS patients point towards an important role of mast cell-derived mediators and the interaction mast cells-nerve fibers on the disturbed secretomotor and sensory functions characterizing IBS (10,11). In this line, we have demonstrated that in the rat model of chronic exposure to oral OVA, mucosal mast cells (MMCs) are implicated in the altered colonic contractile activity; thus suggesting that OVA-induced colonic motor alterations in rats are somehow MMC-dependent (4). Therefore, exposure to oral OVA in rats reproduces some pathophysiological components of IBS; at least the MMC hyperactivity and the changes in colonic contractility, thus representing a valid model for studying IBS-related altered colonic contractile responses and their potential relationship with MMCs.

Recent data suggest that, within the gut, neurotrophins, mainly nerve growth factor (NGF), interact with MMCs generating a neuroimmune circuit likely to play a potential role in the pathophysiology of FGDs. For instance, evidences obtained in animal models of IBS have implicated NGF in the neuronal remodeling of enteric circuitries and MMCs recruitment, as basis for the functional changes observed. In particular, anti-NGF treatment completely blocked intestinal hypermotility in *Trichinella spiralis*-infected rats, an accepted model of post-infectious-IBS (12), and reduced the interaction MMCs-nerve fibers in the rat maternal separation model (13). A recent study in colonic biopsies from IBS patients supports these observations, showing an increased neuronal sprouting

within the mucosa, an effect associated to NGF increased levels, possibly of mast cell origin (14). However, the exact origin of colonic NGF and the potential role for MMCs as the peptide source are still unclear.

NGF interacts with two classes of cell surface receptors: the TrkA high-affinity receptor, a selective NGF receptor; and the p75 low-affinity receptor, which presumably binds to all neurotrophins (15). To further understand the role of NGF in colonic motor alterations we have investigated the effects of the pharmacological blockade of TrkA on OVA-induced changes in colonic contractility in the rat by using K252a, which has been widely used in the rat as a TrkA antagonist (16-20). In addition, we evaluated the expression levels of NGF and TrkA in the rat colon during exposure to oral OVA, complementing this data by determining the colonic expression of the related neurotrophin brain-derived neurotrophic factor (BDNF) and its preferential receptor TrkB. Finally, we evaluated the interplay between MMCs-NGF/TrkA system, characterizing, in particular, if MMCs represent a cellular source of the neurotrophin and/or express TrkA receptors.

Materials and Methods

Animals

Adult (5 week-old at arrival), specific pathogen free (SPF), Sprague-Dawley (SD) male rats were used (Charles River, Les Oncins, France). Animals had free access to water and a standard pellet diet, free of traces of OVA or any other egg derivative (A04; Safe, Augy, France). Rats were maintained under conventional conditions in a light (12h/12h light-dark cycle) and temperature controlled (20-22ºC) room, in groups of two per cage. Animals were acclimatized to the new environment for 1 week before starting any experimental procedure. All the experimental protocols were approved by the Ethics Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya (protocols 1010 and 5351, respectively).

Experimental design

Rats received OVA by oral gavage (1mg mL^{-1}, 1 mL/rat, n=24), on a daily basis during a 6-week period (21). A group of rats receiving vehicle (1 mL/rat, n=21) was used as control. After the third week, 11 of the animals receiving OVA and 10 of the animals receiving vehicle were treated subcutaneously with K252a (50 µg kg^{-1}). Treatment with K252a was performed daily and lasted until the day before animals were euthanized, outlasting for 10 days OVA exposure. This antagonist, at the dose and pattern of administration followed here, has already been used, showing effective blockade of TrkA and biological effects *in vivo* (18-20). The rest of the animals (13 OVA- and 11 vehicle-exposed) were used as control groups in which the treatment protocol was the same but K252a was replaced by the corresponding vehicle (1 mL kg^{-1}, sc). Except for the *in vivo* experiments, at the time of euthanasia, tissue samples from the colon were obtained and either used for organ bath studies, fixed in 4 % paraformaldehyde for immunohistochemical studies or frozen in liquid nitrogen and stored at -80ºC until analysis.

Organ bath

Full thickness preparations, obtained from the mid portion of the colon, were cut 1 cm long and 0.3 cm wide and hung for organ bath study oriented to record circular muscle activity. Strips were mounted under 1 g tension in a 10-mL muscle bath containing carbogenated Krebs solution (95% O_2 – 5% CO_2) maintained at 37 ± 1 ºC. The composition of Krebs solution was (in mmol L^{-1}): 10.10 glucose, 115.48 NaCl, 21.90 $NaHCO_3$, 4.61 KCl, 1.14 NaH_2PO_4, 2.50 $CaCl_2$, and 1.16 $MgSO_4$ (pH 7.3–7.4). One strip edge was tied to the bottom of the muscle bath using suture silk and the other one to an isometric force transducer (Harvard VF-1 Harvard Apparatus Inc., Holliston, MA, USA). Output from the transducer was fed to a PC through an amplifier. Data were digitalized (25 Hz) using Data 2001 software (Panlab, Barcelona, Spain). Strips were allowed to equilibrate for about 1h. After this period, contractile responses to carbachol (CCh; 0.1–10 µM) and the NO inhibitor N^G-nitro-L-Arginine (L-NNA; 1 mM) were assessed. For CCh, cumulative concentration-response curves, with a 5-min interval between consecutive concentrations, were constructed. For L-NNA, spontaneous activity was recorded during a 10-min period after the addition of the drug.

To determine the spontaneous contractile activity, the preparation tone was measured for 15 min and the mean value (in g) determined. To test the effects of CCh, the maximum peak, from the basal tone, was measured after each concentration tested. To measure the response to L-NNA, the 10-min mean of the strip tone before the drug administration was determined and compared with the 10-min mean of the strip tone after L-NNA addition.

Strain-gauge recordings

After a fasting period of 6 h, animals were placed in an induction camera and anesthetized by inhalation of 4 % isofluorane (Isoflo®; Esteve, Barcelona) in 2 L min^{-1} oxygen to allow cannulation of a lateral vein of the tail. Thereafter, rats were maintained in level III of anesthesia by intravenous thiopental sodium, as required, and exposed to mask delivery of 1 L min^{-1} oxygen during all the procedure. A laparatomy was performed, the colon localized and a strain-gauge (F-04IS, Star Medical, Tokyo, Japan) was sutured to its wall (2 cm from de cecum) to record circular muscle activity. The strain gauge was connected to a high-gain amplifier (MT8P; Lectromed, Herts, UK), and signals were sent to a recording unit (PowerLab/800; ADInstruments) connected to a computer. Finally, an electrode holder with two platinum electrodes (WPI, Sarasota, FL, USA) was inserted into the colonic lumen at 1 cm distally to the strain-gauge to induce ascending excitation of the peristaltic reflex by electrical mucosal stimulation (EMS). EMS was applied by duplicate at 30 V, 0.6 ms and 4 Hz during 30 s each time, and the polarity of the stimulating electrodes was reversed at 15 s.

To analyze *in vivo* colonic motility, contractions of the colon were classified into low-frequency and high-amplitude (LFHA) contractions and high-frequency and low-amplitude (HFLA) contractions, as previously described (22). HFLA were identified by having a frequency within the range of 10–15 contractions min^{-1}, while LFHA were defined as contractions of an amplitude >300% of that of HFLA contractions at the same recording site (22). HFLA and LFHA were assessed over a 15-min period and the frequency and amplitude expressed as the mean. When assessing the responses to EMS, the recording analyzed corresponded to the 30 s of stimulation and the expressed value was the mean of the duplicates. All analysis was performed using Chart 5 software for Windows (both from AD Instruments, Castle Hill, NSW, Australia).

Immunohistochemistry (IHC)

Immunodetection of rat mast cell protease II (RMCPII) and NGF was carried out on paraformaldehyde-fixed colonic samples using a monoclonal antibody anti-RMCPII (Moredun, Edinburgh, UK) and a polyclonal rabbit anti-NGF (ab1526; CHEMICON International, Temecula, USA). Antigen retrieval for NGF was achieved by processing the slides in a pressure cooker, at full pressure, for 10 min in 10 mM citrate solution. The secondary antibodies included biotinylated horse antimouse IgG (BA-2000; Vector Laboratories, Burlingame, CA, USA) and biotinylated swine antirabbit Ig (E0353; DAKO, Carpinteria, CA, USA). Detection was performed with avidin/peroxidase kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) and counterstaining with haematoxylin. Specificity of the staining was confirmed by omission of the primary antibody. When performing IHC for NGF, mouse submaxillary glands were used as a positive control. Slides were viewed with an Olympus BH-2 microscope. For MMC quantification, at least 20 non-adjacent X400 fields of colonic mucosa were randomly selected and the number of RMCPII-immunopositive cells counted. All procedures were carried out using coded slides to avoid bias.

Immunofluorescence staining

For analyses of co-localization of NGF, proNGF or TrkA with RMCPII, double immunofluorescence was used. After 1 h of blocking with 10 % normal goat serum at room temperature, colonic sections were incubated with a mixture of anti-RMCPII and anti-NGF or anti-ProNGF (ab5583; CHEMICON International, Temecula, USA) or anti-TrkA (sc-118; Santa Cruz Biotechnology, CA, USA) overnight. Thereafter, sections were incubated with a secondary antibody cocktail consisting of fluorescence-conjugated Alexa Fluor 488 goat anti-mouse IgG (A11029; Molecular Probes, Eugene, OR, USA) and Cy3 goat anti-rabbit IgG (PA-43004; Amersham-Pharmacia, Buckinghamshire, UK). After washing, the slides were coverslipped with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA) and examined under an Axioskop 40 fluorescence microscope (Carl Zeiss, Jena, Germany). Merging of the images was analyzed with ImageJ Software (U. S. National Institutes of Health, Bethesda, Maryland, USA). To assess the percentage of cells with RMCPII and TrkA co-localization, Alexa Fluor 488- and Alexa Fluor 488-Cy3-stained cells were counted randomly using a 100x

objective. Specificity of the staining was confirmed by omission of the primary antibody and/or the secondary antibodies. The absence of cross-reactivity was confirmed in control single-labeled preparations.

ELISA

Protein was extracted from colonic tissue samples using lysis buffer (50 mM HEPES, 0.05 % Triton X-100, 0.0625 mM PMSF and the Mini Complete protease inhibitor Roche) and RMCPII concentration was determined by ELISA using a commercial kit (Moredun). Total protein was determined using the Bradford assay kit (BIO-RAD, Hercules, CA, USA).

RNA extraction and quantitative real-time PCR

Total RNA was extracted from colonic samples using Ribopure RNA Isolation Kit (Applied Biosystems, CA, USA) and quantified by Nanodrop (Nanodrop Technologies, Rockland, DE, USA). For cDNA synthesis, 1 µg of RNA was reverse-transcribed in a 20 µl reaction volume using a high capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA). Expression of NGF, BDNF, TrkA and TrkB was determined by quantitative real-time PCR performed with specific Taqman probes (Applied Biosystems; NGF: Rn01533872_m1, BDNF: Rn00560868, TrkA: Rn00572130_m1, TrkB: Rn01441749_m1, B-Actin: Rn00667869_m1) mixed with Taqman Universal Master Mix II for 40 cycles (95ºC for 15 s, 60ºC for 1 min) on a 7900 real-time PCR system (Applied Biosystems). Rat submaxillary gland and neocortex were used as positive controls for the gene expression of TrkA, TrkB and NGF and BDNF, respectively. B-Actin expression served as an endogenous control for normalizing the mRNA levels of the target genes. Expression levels were analyzed by the $2^{-\Delta\Delta CT}$ method.

Chemicals

Ovalbumin (Grade V; A5503) was purchased from Sigma-Aldrich (St.Louis, MO, USA) and was dissolved in saline solution. K252a [(9S,10R,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid methyl ester; Tocris Bioscience, Ellisville,

MO, USA] was reconstituted in 8.75 % ethanol in milli-q water. CCh (Sigma-Aldrich) stock solution and further dilutions were prepared in distilled water. L-NNA (Sigma-Aldrich) was prepared directly in carbogenated Krebs solution.

Statistics

All data are expressed as mean ± SEM. Motility results are presented as raw data (g of force) or frequency of contractions (number min^{-1}; *in vivo* recordings). EC_{50} for CCh was calculated by non-linear regression to a sigmoidal equation (GraphPad Prism 4.01, San Diego, California, USA). Comparisons between multiple groups were performed with two-factor ANOVA. When the two way ANOVA revealed significant effects of treatments, data were reanalyzed with one-way ANOVA followed, when necessary, by a Student-Newman-Keuls multiple comparison test to detect differences between experimental groups. P values < 0.05 were considered statistically significant.

Results

Colonic mucosal mast cell count and RMCPII content

The number of RMCPII-positive cells in the colon showed no significant differences between experimental groups despite the treatment received (Fig. 1A). Nevertheless, a two-way ANOVA analysis revealed an effect of OVA treatment increasing RMCPII content (P=0.022), although only the OVA-K252a group achieved statistical significance (P<0.05 vs. vehicle-vehicle; Fig. 1B). K252a, per se, showed a tendency to increase the levels of RMCPII, although statistical significance was not achieved (P=0.13).

Localization of NGF by immunohistochemistry

Within the colon, immunoreactivity for NGF was detected mainly in the submucosal and myenteric plexuses (Fig. 2C-D). A diffuse staining was observed in the epithelium, both on the villi and, occasionally, in the crypts. Within the villi, there were scarce cells, of undetermined type, showing NGF-like immunoreactivity (Fig. 2A-B). No labeling was detected in the muscle layers. No differences in the staining pattern or intensity were observed between OVA- and vehicle-treated animals or associated to the treatment with

K252a. Immunoreactivity was absent in sections in which the primary antibody was omitted, thus confirming the specificity of the staining. Staining was intense and well localized in positive controls from mouse submaxillary glands.

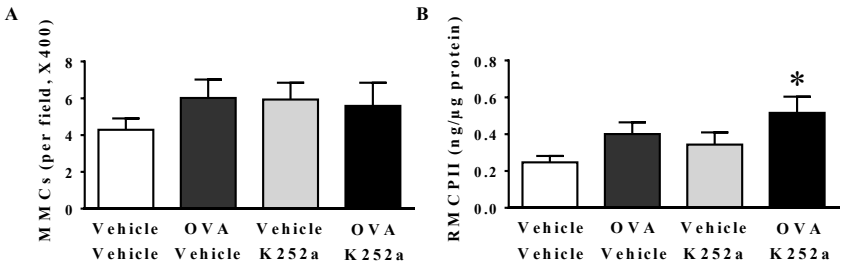

Figure 1. Colonic density of MMCs (A) and RMCPII content (B) in the different experimental groups. Data are mean ± SEM; n =5-10 per group. * : P<0.05 vs. vehicle-vehicle.

Co-localization of RMCPII and NGF, proNGF or TrkA

In vehicle-treated animals, double immunofluorescence studies demonstrated that the vast majority (63.6±10.4%) of RMCPII-positive cells (identified as MMCs) co-expressed TrkA-like immunoreactivity (Fig. 2G). Similar ratio of RMCPII-TrkA co-localization was observed after OVA exposure (75.0±5.7%). Apart from RMCPII-positive cells, other scarce cells within the lamina propria showed TrkA-labeling, indicating that not only MMCs express the receptor in the rat colonic mucosa.

No RMCPII-positive cells (MMCs) showed co-staining for NGF or ProNGF.

NGF, BDNF, TrkA and TrkB expression in the colon

Overall, colonic expression levels of NGF, TrKA and TrkB were relatively low, with no significant differences in expression levels among the different experimental groups (Fig. 3). However, it was noticeable that K252a and OVA , per se, increased TrkA expression levels by 32% and 26% respectively when compared to the expression levels in the control group although these effects were not evident in OVA-K252a-treated animals (Fig. 3B).

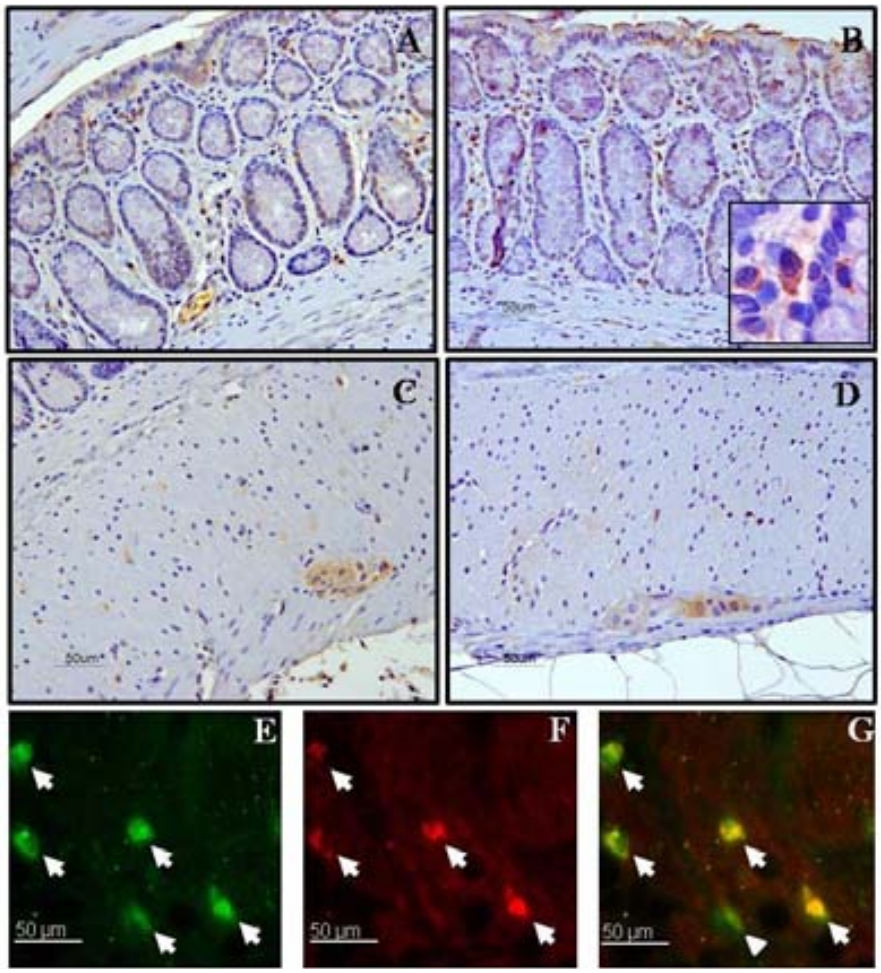

Figure 2. Immunohistochemistry for NGF and double immunofluorescence for TrkA and RM CPII (M MCs). Photomicrographs showing NGF-like immunoreactivity in colonic tissues of a vehicle-vehicle-treated (A, C) and OVA-vehicle-treated (B, D) rats. Labeling for NGF was detected in the submucosal and myenteric plexuses, surface of the villi and crypts, and in scarce, unidentified cells within the villi. Insert in (B) shows a higher magnification of these NGF-immunoreactive positive cells within the crypts. Note that no differences in the staining pattern or intensity are observed between OVA- and vehicle-treated animals. E-G: Dual label immunofluorescence showing the presence of TrkA in MMCs of the rat colon. E: Representative image of anti-RMCPII labeling (green) of cells (identified as MMCs) in the colonic mucosa. The arrows indicate positively labeled cells. F: Same field as in E showing labeling for TrkA (red). The arrows indicate positively labeled cells. G: Merged image of E and F showing extensive colocalization (yellow) of RMCPII (MMCs) and TrkA immunoreactivities. The arrows indicate double labeled MMCs, whereas the arrowhead indicates a MMC negative for TrkA.

In control tissues (rat submaxillary gland) expression levels of NGF, TrkA and TrkB were, respectively, 6-, 14- and 100-fold higher than those observed in the colon in control conditions.

BDNF was not detectable in the colon (ct values higher than 40), although high expression levels were found in the positive control (rat neocortex).

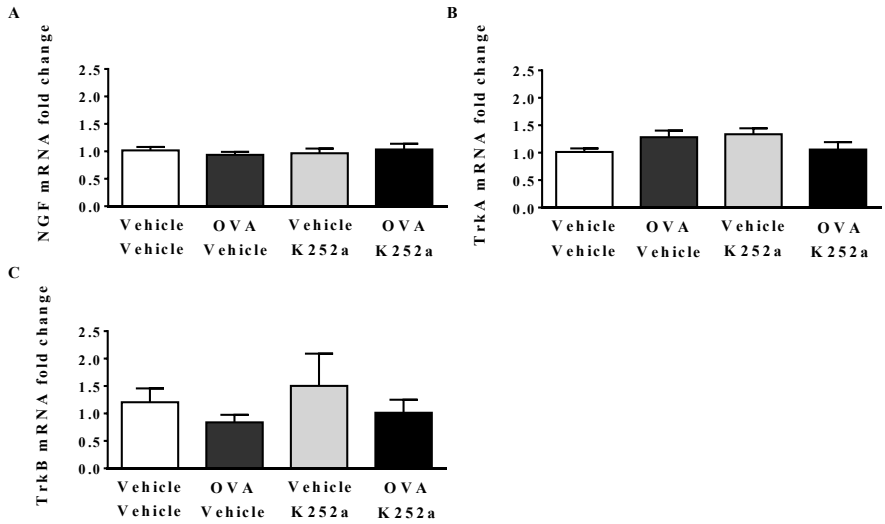

Figure 3. Real-time PCR analysis of mRNA for NGF (A), TrkA (B) and TrkB (C).
Data are mean ± SEM; n=5-9 per group.

Colonic contractility *in vitro*

Spontaneous colonic contractile activity, as assessed *in vitro*, was similar in vehicle- and OVA-exposed animals (vehicle: 0.53±0.06 g; OVA: 0.51±0.03 g; P>0.05; Fig. 4 A). K252a decreased spontaneous activity in similar proportion in vehicle- (0.40±0.05 g; P=0.07 vs. vehicle-vehicle; Fig.4A) or OVA-exposed animals (0.35±0.05 g, P<0.05 vs. OVA-vehicle; Fig. 4A).

In control conditions, CCh elicited a concentration-dependent contractile response with an estimated EC_{50} of 1.8±1.3 mM. Overall, a two-way ANOVA analysis revealed an OVA effect (P=0.031) enhancing the contractile responses to CCh, leading to a left-shift of the concentration-response curve and a 5-fold reduction in the estimated EC_{50}

(0.39±0.1 mM; Fig. 4B). Treatment with K252a did not affect the responses to CCh, neither in vehicle- nor in OVA-exposed animals (EC_{50}; vehicle-K252a: 1.7±1.0 mM; OVA-K252a: 0.16±0.04 mM; Fig. 4B).

In colonic strips obtained from vehicle-vehicle animals, blockade of NO synthesis by the addition of L-NNA to the organ bath increased spontaneous activity over pre-treatment values (P=0.036; Fig. 4C). Similar effects were observed in tissues from OVA-vehicle- or vehicle-K252a-treated rats, although motor effects of L-NNA were enhanced in OVA-vehicle-treated animals (Fig. 4C). However, in animals treated with OVA plus K252a, L-NNA-induced increased spontaneous activity was no longer observed (Fig. 4C).

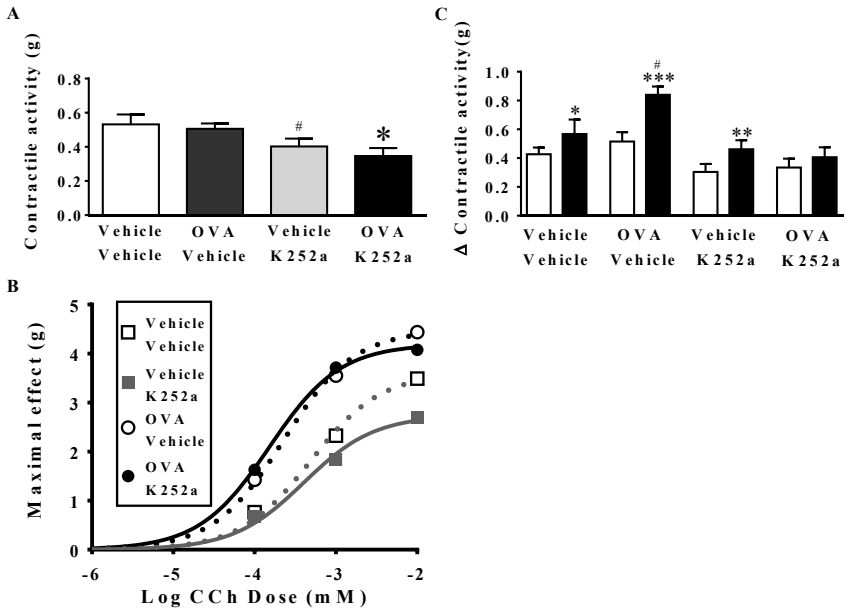

Figure 4. Effects of oral OVA and K252a on colonic contractility *in vitro*. A: Colonic spontaneous contractile activity in the different experimental groups. Data are mean ± SEM; n=5-10 per group. *: P<0.05 vs. OVA-vehicle; #: P=0.074 vs. vehicle-vehicle. B: Concentration-response curves for carbachol. Note that oral exposure to OVA leads to a left-shift of the concentration-response curve, an effect not modified by treatment with K252a. Data represent mean values (symbols) and non-linear regression curves. n=5-10 per group. C: Effect of NO blockade with L-NNA on spontaneous contractility in the different experimental groups. Data represent spontaneous contractility before (open bars) and after the addition of L-NNA (closed bars). Data are mean ± SEM; n=5-10 per group. *: P<0.05, **: P<0.01, ***: P<0.001 vs. respective contractile activity before the addition of L-NNA (Paired t-test); #: P<0.01 vs. other L-NNA-treated groups (ANOVA).

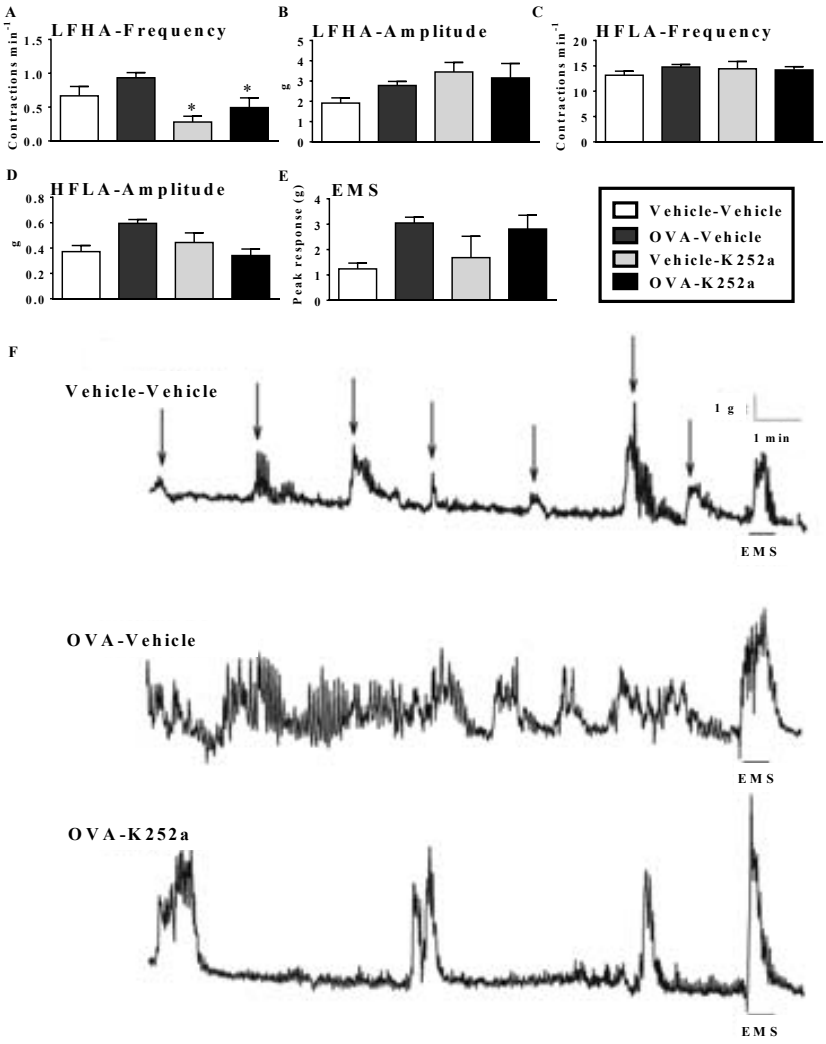

Figure 5. A-D: Effects of oral OVA and K252a treatment on colonic motility *in vivo*. A-B: Frequency (A) and amplitude (B) of LFHA colonic contractions in the different experimental groups. Note that oral exposure to OVA tends to increase the frequency of LFHA colonic contractions and treatment with K252a reduces it in both vehicle- and OVA-treated rats. *: P<0.05 vs. respective vehicle. C-D: Frequency (C) and amplitude (D) of HFLA colonic contractions in the different experimental groups. Note that oral exposure to OVA leads to an increase of the amplitude of HFLA colonic contractions, an effect prevented by treatment with K252a. Data are mean ± SEM; n=3-5 per group. E: Colonic response to EMS (30 V, 4 Hz, 30 s) showing that exposure to OVA increases EMS-elicited motor responses in a K252a-independent manner. Data are mean ± SEM; n=3-5 per group. F: Representative tracings showing spontaneous colonic motility and response to EMS in a vehicle-vehicle-, OVA-vehicle- and OVA-K252a-treated animals. The arrows indicate LFHA contractions. Note how OVA exposure increases the frequency of LFHA contractions, an effect prevented by K252a treatment.

Colonic motility *in vivo*

As previously described (22), strain-gauge recordings of spontaneous colonic motility in vehicle-vehicle-treated rats exhibited two different types of contractions; LFHA contractions with superimposed HFLA contractions (Fig. 5F). Overall, exposure to OVA altered spontaneous colonic motility by affecting both types of contractions (Fig. 5). Treatment with OVA tended to increase the frequency of LFHA contractions (vehicle: 0.67±0.14 contractions min^{-1}; OVA: 0.93±0.08 contractions min^{-1}; P=0.08; Fig. 5A) and also enhanced the amplitude (vehicle: 1.92±0.25 g; OVA: 2.78±0.20 g; Fig. 5B). As it refers to HFLA contractions, exposure to OVA did not alter the frequency but increased the amplitude (vehicle: 0.37±0.05 g; OVA: 0.59±0.03 g; Fig. 5D). However, in animals treated with K252, part of these effects of OVA exposure on spontaneous colonic motility was no longer observed. K252a inhibited the OVA-increased frequency of LFHA contractions (0.49±0.14 contractions min^{-1}; P<0.05 vs. OVA-vehicle; Fig. 5A) although it did not affect the amplitude. A similar effect of K252a on the frequency but not the amplitude of LFHA contractions was observed in vehicle-exposed animals (0.28±0.09 contractions min^{-1}; P<0.05 vs. vehicle-vehicle; Fig. 5A). Concerning to HFLA contractions, treatment with K252a showed a tendency to reverse the increase in the amplitude of HFLA contractions after OVA exposure, as suggested by a significant interaction between treatments in a two-way ANOVA (0.34±0.05 g; P<0.05; Fig. 5D).

In control conditions, EMS elicited a LFHA-type response that coincided with the stimulation time (Fig. 5F). The contractile response to EMS was increased by exposure to OVA (vehicle: 1.24±0.23 g; OVA: 3.05±0.23 g; P=0.05; Fig. 5E) in a K252a-independent manner (2.80±0.54 g; Fig. 5E).

Discussion

This study shows that, in the rat model of chronic exposure to oral OVA, changes in colonic motility might be related to an altered activity of the NGF-TrkA pathway. Although NGF expression levels were not changed, motor responses following the treatment with K252a suggest that NGF-dependent signaling pathways are involved in colonic spontaneous motor activity and mediate OVA-induced enhancement of NO-dependent inhibitory tone *in vitro*. Moreover, our results suggest that colonic NGF is not

MMC-derived, although these cells express TrkA receptors and, therefore, represent a target for NGF within the colonic mucosa.

The results of this study confirm that oral OVA activates MMCs in the colon, as indicated by the increase in RMCPII levels within the colonic wall, similarly to that described previously in the same model (4). Data derived from animal models of IBS have demonstrated the importance of MMCs as effector cells mediating the array of pathophysiological changes that characterize IBS in humans. For instance, degranulation of MMCs seems to be a key step in the onset of visceral hyperalgesia and the alterations of epithelial barrier function observed both in animal models and the human disease (23-26). In the model of chronic exposure to oral OVA in rats, although these salient features of IBS have not been characterized, observations reveal that MMCs also play a role on the changes within colonic smooth muscle contractility, thus supporting the validity of the model as an appropriate approach to IBS-like altered colonic motor responses.

Results obtained show that OVA-exposed rats have colonic contractility dysfunction, including increased responses to carbachol and L-NNA *in vitro* and enhanced spontaneous contractility and EMS-elicited responses *in vivo*. These results confirm previous observations in this model (4), resembling that observed in IBS patients and other animal models of the disease (5-8). Tissue histological examination excluded muscle hypertrophy as a potential cause for this carbachol and EMS enhanced responses (data not shown), thus suggesting an increased excitability of the smooth muscle to cholinergic stimulation. A possible explanation for these OVA-induced colonic motor alterations could be related to an excited-activated state of MMCs. This is suggested by the higher tissue concentration of RMCPII observed in OVA-treated animals and supported by evidences *in vivo* implicating mast cell degranulation in the onset of cecocolonic motor alterations in rats (27). MMC mediators act on nerve endings of intrinsic and extrinsic primary afferent neurons forming neural networks within both the submucosal and myenteric plexuses, leading to a local amplification of effector responses (28). Therefore, OVA-induced colonic motor alterations might arise as a result of altered afferent nerve input into myenteric motor circuits due to a tonic activation of MMCs.

In animal models of IBS, both mast cells and NGF have been implicated in colonic epithelial barrier function, propulsive motor activity and sensitivity to colorectal distension (12,24). However, the exact source(s) of colonic NGF remains elusive and the potential role of mast cells as the main source of intestinal NGF is controversial (24,29). In order to elucidate these points, immunohistochemistry for the neurotrophin was performed in colonic tissues. Although we were able to see specific NGF staining, with similar distribution patterns as those previously reported (17), we did not find any obvious difference in staining, intensity or distribution, among the different experimental groups. This was further confirmed by real time PCR, showing similar expression levels of NGF among experimental groups. Moreover, during double labeling studies, we were unable to detect NGF immunoreactivity in MMCs, identified as RMCPII-positive cells within the colonic mucosa. This contrasts with animal data suggesting that NGF is released by mast cells upon degranulation (24,30) and with data from colonic biopsies of patients with functional and inflammatory gastrointestinal disorders localizing NGF in MMCs (14,31). However, our data agree with a recent study in a rat colitis model in which NGF immunoreactivity was not associated to MMCs (29). Whether or not this represents a species-related difference (human vs. rat) and/or experimental model-dependent variations in the colonic source of NGF warrants further studies. Interestingly, Stanzel *et al.* (2008) evidenced that NGF was synthesized mainly by epithelial cells and hypothesized that MMCs could represent a source of pro-NGF, in agreement to that suggested also by studies on cultured rat peritoneal mast cells (32). Based on these data, we also attempted, although unsuccessfully, to localize proNGF in colonic MMCs. Overall, our observations indicate that MMCs are not a cellular source of NGF in the rat colon. Nevertheless, results obtained suggest a functional link between MMCs and NGF and, in particular, indicate that MMCs are a target for NGF. First, we were able to demonstrate the presence of TrkA receptors on a high proportion of colonic MMCs (by 60%). Second, K252a treatment tended to increase colonic RMCPII levels, thus suggesting that these receptors are functional and might mediate MMC degranulation upon stimulation with NGF. Indeed, the NGF ability to degranulate mast cells has been previously demonstrated, both *in vivo* (33) and *in vitro* (34).

In this study, we aimed also to elucidate the functional implication of NGF in the alterations of colonic smooth muscle contractility that characterize oral OVA exposure in rats. A role for NGF on IBS-like gastrointestinal motor alterations in animal models

has been previously suggested (12). In order to further assess this involvement of NGF we used a pharmacological approach based on the blockade of the NGF high-affinity receptor, TrkA, with K252a (35). In our conditions, treatment with K252a resulted in a decrease of spontaneous colonic motor activity both *in vivo* and *in vitro* and prevented the enhancement of the nitrergic inhibitory tone secondary to OVA exposure *in vitro*. Interestingly, direct addition of K252a to the organ bath also decreased spontaneous colonic contractility (data not shown), thus reinforcing the results obtained with the treatment with K252a and suggesting and effective blockade of TrkA in *in vivo* conditions. From these observations, it is feasible to speculate that a tonic NGF-dependent stimulation might be necessary to maintain basal spontaneous contractility at optimal conditions. Taking into consideration that rat enteric neurons express the high-affinity receptor TrkA, as previously described and also confirmed in this study by immunohistochemistry (data not shown; 36), we can hypothesize that K252a is likely to bind to TrkA receptors on myenteric neurons preventing NGF-mediated effects within the ENS and thus, affecting motor activity.

The dose and pattern of administration of K252a followed here has already been used, showing biological effects *in vivo* indicative of an effective blockade of TrkA (18-20). Therefore, it is feasible to assume that the responses observed here are related to an effective blockade of TrkA receptors. However, K252a not only binds to TrkA but also to other neurotrophins receptors, mainly TrkB and TrkC, and other kinases, such as the Ca^{2+}/calmodulin kinase II (37) or the myosin light chain kinase (38), which are implicated in the contractile activity of intestinal smooth muscle (39). From our observations, it cannot be ruled out that the effects observed might be associated, at least partially, to K252a effects on these targets. Nevertheless, several observations suggest that the responses to K252a are likely to be associated to the blockade of TrkA receptors. First, a recent study demonstrated that the pharmacological blockade of TrkA with K252a, the treatment with TrkA antisense oligonucleotides and the *in vivo* immunoneutralization of NGF were equally effective preventing chronic stress-induced visceral hypersensitivity to colorectal distension in rats (17). Second, *in vivo* immunoneutralization of NGF normalized post-infectious gut dysmotility in *T. spiralis*-infected rats (a model of post-infectious-IBS) (12), as observed here in the OVA model with K252a. Overall, these observations suggest that K252a-mediated effects within the gastrointestinal tract are related to the modulation of NGF-TrkA-dependent mechanisms.

Although up-regulation of NGF and its high-affinity receptor TrkA has been demonstrated during colonic acute inflammation (29,31,40), expression results in animal models of IBS are discordant (24,26,41). In the present study, only marginal changes in TrkA, and no changes in NGF expression levels were observed among experimental groups. This could seem surprising as we demonstrated that NGF/TrkA-dependent pathways are implicated in the OVA-induced colonic dysmotility in the rat. However, an interesting study in mice followed the increase in mRNA neurotrophins levels during the inflammation of the colon and revealed that these are back to control conditions one-week after the induction, suggesting a rapid and short-term secretion type of these molecules during pathological conditions (42). Taking into consideration that neurotrophins have both acute and long-term biological effects (43), it is feasible to speculate that NGF/TrkA mRNA colonic levels in the OVA-exposed rats at the time of euthanasia may be not be representative of those during the chronic-treatment, even when NGF-dependent colonic dysmotility is still present. In addition, there is also the possibility that the OVA-induced increase in MMC mediators release exerts its effects on the ENS through pathways involving NGF/TrkA-dependent mechanisms although these are not up-regulated by OVA exposure. In any case, NGF/TrkA expression results should be interpreted cautiously since the interaction between TrkA and other neurotrophin receptors (namely the p75 and the neurotrophin receptor homolog) leads to an enhanced activity of the signaling pathways, without increasing the amounts of NGF and/or TrkA receptors per se (44). It is feasible to assume that the marginal changes observed in TrkA expression after OVA or K252a treatment (32% and 26% increase, respectively) might have consequences at the protein level yet to be demonstrated. On the one hand, K252a-induced changes in TrkA expression could represent a compensatory mechanism to the receptor blockade. On the other hand, OVA-induced changes might be secondary to the OVA-mediated stimulation of MMCs and/or other cell types, including enteric neurons, as discussed above. Interestingly, these changes in TrkA expression were no longer observed in animals receiving OVA and K252a. Although we cannot explain the mechanisms behind this effect, this observation further supports an interplay between OVA effects and the NGF-TrkA pathway.

In summary, the present study suggests that NGF-TrkA-dependent mechanisms are implicated in basal colonic contractility and also in OVA-induced colonic motor alterations in rats. In addition, our results show that MMCs express TrkA receptors and,

therefore, represent a target for NGF, rather than being a source of the peptide, in the rat colon. Overall, this study highlights a potentially important role for NGF-TrkA-dependent signaling pathways on colonic motor alterations, as observed for instance in FGDs. NGF receptors antagonists could represent a therapeutic target for the treatment of gastrointestinal disorders characterized by altered colonic motility.

Acknowledgments

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Disclosures

F. J. designed and performed experiments, analyzed data and wrote the paper. V. M. designed and performed experiments and wrote the paper. P. V. designed experiments and wrote the paper.

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CHAPTER 2

CHAPTER 2

COLONIC MOTOR ALTERATIONS ASSOCIATED TO THE SENSITIZATION TO A LUMINAL ANTIGEN ARE ENHANCED DURING A DYSBIOTIC-LIKE STATE IN RATS

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Abstract

Background: Enteric dysbiosis is suggested as a risk factor for dietary proteins-associated intestinal alterations, contributing to the development of food allergies and the symptomatology of functional gastrointestinal disorders, mainly irritable bowel syndrome (IBS). We explored if a dysbiotic-like state, simulated by administration of bacterial lipopolysaccharides (LPS), facilitates the sensitization to the luminal antigen ovalbumin (OVA), in rats. **Methods:** Rats were exposed to oral OVA for 1 week, alone or with LPS. Thereafter, colonic histology, goblet cell density, mucosal eosinophils and mucosal (MMCs) and connective tissue mast cells (CTMCs) were evaluated. Colonic expression (RT-qPCR) of interleukins, interferon alpha-1 and integrins was assessed to determine local immune responses. Luminal and wall adhered microbiota were characterized by fluorescence *in situ* hybridization. Colonic contractility (*in vitro*) served to assess functional changes associated to OVA and/or LPS. **Results:** Neither OVA nor LPS, alone or combined, lead to structural alterations, except for a reduced goblet cell density in OVA-LPS-treated rats. MMC density was unaffected, while CTMC counts increased within the submucosa of OVA-LPS-treated animals. Marginal immune activation, with up-regulation of IL-6 and interferon-alpha-1, was observed in OVA-LPS-treated rats. LPS induced a dysbiotic-like state characterized by decreased luminal bacterial counts, with a specific loss of clostridia. LPS facilitated *Clostridium* spp. wall adherence, an effect prevented by OVA. Colonic contractility was altered in OVA-LPS-treated animals, showing increased basal activity and enhanced motor responses to OVA. **Conclusions:** Changes in gut microbiota might enhance/facilitate local neuroimmune responses to food antigens leading to motor alterations similar to those observed in IBS.

Introduction

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder and is characterized by abdominal pain-discomfort associated with dysmotility and altered bowel habits (1). Although its underlying pathophysiology remains unclear, recent evidences suggest that IBS is due to a dysregulation of the brain-gut axis, with various peripheral alterations contributing to the exacerbation of the symptomatology (2). For instance, both changes in gut microbiota and adverse reactions to food allergens have been implicated in the pathogenesis of the disease (2-5).

Intestinal food allergy-related mechanisms in IBS seem to involve local mucosal responses to dietary antigens with activation of resident mast cells (MCs), rather than classical type-1 hypersensitivity reactions (4,6). In this line, we have previously demonstrated that long-term exposure to oral ovalbumin (OVA), without adjuvants, results in a non-IgE mediated alteration of colonic motility in rats, an effect related to an excited-activated state of the tissue mucosal mast cells (MMCs) (7). These OVA-mediated changes are reminiscent of those observed in IBS patients and in animal models of the disease (7-11).

Gut commensal microbiota (GCM) has been implicated in the maintenance of the normal gastrointestinal hyporesponsive state to food antigens. For instance, results obtained in studies in infants with food allergy show a disturbed balance between beneficial and potentially harmful bacteria in the large intestine and that supplementation with probiotics appears to alleviate the allergic inflammation (12,13). Supporting these observations, results from animal models demonstrate a cause-effect relationship between dysbiotic states with reduced GCM and the development of allergic responses to oral antigens (14,15). Overall, these data suggest that a disruption of the GCM might cause an impairment of the intestinal tolerogenic mechanisms, increasing the risk of food protein-induced immune activation and the development of food allergy and/or IBS-like alterations.

The aim of the present study was to further explore the impact of dysbiotic states of the colon in the generation of abnormal responses to dietary antigens. For this purpose, we treated rats with low doses of bacterial lipopolysaccharides (LPS), simulating a

dysbiotic-like state (16). Simultaneously, animals were exposed orally to the allergic protein OVA. The potential facilitatory effects of LPS towards OVA sensitization were studied *in vitro* by assessing spontaneous colonic contractility and the contractile responses elicited by the presence of the antigen (OVA). To further understand the role of GCM in the functional alterations observed, changes in the colonic microbiome and bacterial wall adherence were determined by fluorescence *in situ* hybridization (FISH). Finally, to gain insight into the immune nature of the IBS-like responses related to dietary antigens, we assessed local changes in cytokines expression, the potential involvement of eosinophils and the dynamics of colonic MC populations.

Materials and methods

Animals

Adult (9 week-old), specific pathogen free (SPF), Sprague-Dawley (SD) male rats were used (Charles River, Les Oncins, France). Animals had free access to water and were fed with a standard diet (145 g/kg protein, 40 g/kg fat, 45 g/kg fiber and 13.4 kJ/g; free of traces of ovalbumin or any other egg derivative; Teklad Global 14% Protein Rodent Maintenance Diet 2014, Harlan Interfauna Iberica S.A.). During all the experiment, rats were maintained under conventional conditions in a light (12h/12h light-dark cycle) and temperature controlled (20-22 ºC) room, in groups of two per cage. Animals were acclimatized to the new environment for 1 week before starting any experimental procedure. All the experimental protocols were approved by the Ethics Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya (protocols 1010 and 5351, respectively).

Exposure to oral ovalbumin and treatment with LPS

Rats were exposed to OVA by a daily oral gavage (10 mg/mL, 1 mL/rat, n=15) during a one week period. A group of rats were used as controls, in which the same exposure protocol was followed but OVA solution was replaced by saline (1 mL/rat, n=15). In parallel, a subgroup of vehicle- and OVA-exposed rats (n=8 for each) was treated intraperitoneally with LPS (100 µg/kg/day for 7 days). The rest of the rats (7 vehicle-

and 7 OVA- exposed animals) received the vehicle for LPS (1 mL/kg/day for 7 days). Animals were examined for clinical signs and body weight changes on a daily basis (at the time of treatment) and were euthanized by decapitation 24 h after the last treatment. At the time of euthanasia, tissue samples from the colon were collected for functional studies (organ bath studies) or either fixed in 4 % paraformaldehyde or Carnoy's solution, for immunohistochemical and fluorescent *in situ* hybridization (FISH) studies respectively, or frozen in liquid nitrogen and stored at -80ºC until analysis.

Organ bath studies

Full thickness preparations were obtained from the mid portion of the colon, cut 1 cm long and 0.3 cm wide and hung, oriented to record circular muscle activity, for organ bath studies. Strips were mounted under 1 g tension in a 10-mL organ bath containing carbogenated Krebs solution (95% O_2 – 5% CO_2) maintained at 37 ± 1 ºC. The composition of Krebs solution was (in mmol/L): 10.10 glucose, 115.48 NaCl, 21.90 $NaHCO_3$, 4.61 KCl, 1.14 NaH_2PO_4, 2.50 $CaCl_2$, and 1.16 $MgSO_4$ (pH 7.3–7.4). One strip edge was tied to the bottom of the bath and the other one to an isometric force transducer (Harvard VF-1 Harvard Apparatus Inc., Holliston, MA, USA). Output from the transducer was fed to a PC through an amplifier. Data were digitalized (25 Hz) using Data 2001 software (Panlab, Barcelona, Spain). Strips were allowed to equilibrate for about 1h. After this period, contractile responses to carbachol (CCh; 0.1-10 µM), bovine serum albumin (BSA; 0.1 %) and OVA (0.1 %) were assessed. For CCh, cumulative concentration-response curves, with a 5 min interval between consecutive doses, were constructed. For BSA or OVA, spontaneous activity was recorded during a 15-min period after the addition of the protein.

To determine the spontaneous contractile activity, the area under the curve (AUC) was measured (in g) over a 15 min period. To assess the effects of CCh, the peak response, from the basal tone, was measured after each concentration tested. For assessing the response to BSA or OVA exposure, the AUC was measured for a 10 min period before the addition of the peptide and compared with the 10 min AUC measured during the 5 min to 15 min period post-addition.

Histology, immunohistochemistry and cell counting

Paraformaldehyde-fixed tissue samples were processed routinely for paraffin embedding, and 5-µm thick sections were obtained for haematoxylin and eosin (H&E), toluidine or alcian blue staining or for immunohistochemistry. All the preparations were viewed with an Olympus CH30RF200 microscope.

H&E-stained colonic sections were used for histological examination of the tissue and also for assessing eosinophil infiltration, as determined by counting the number of eosinophils in 20 non-adjacent fields of colonic mucosa (X600).

MMCs were identified by immunodetection of rat mast cell protease II (RMCPII) using a monoclonal antibody anti-RMCPII (Moredun Animal Health, Edinburgh, UK). The secondary antibody was a biotinylated horse antimouse IgG (BA-2000; Vector Laboratories, Burlingame, CA, USA). Detection was performed with avidin/peroxidase kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA). Specificity of the staining was confirmed by omission of the primary antibody. Sections were counterstained with 1% toluidine blue solution (pH 0.5) for 20 min, which served to identify CTMCs. For MMC quantification, at least 20 non-adjacent fields (X400) of colonic mucosa were randomly selected and the number of RMCPII-immunopositive cells determined manually. CTMCs were identified by the presence of toluidine blue-stained metachromatic granules in their cytoplasm. Total number of toluidine blue-stained cells in the preparation was determined and then normalized for the surface area of the tissue section. To identify goblet cells, colonic tissue sections were stained with Alcian Blue pH 2.5/Periodic Acid Schiff (AB 2.5/PAS kit; Bio-Optica, Milano, Italy) in order to specifically stain neutral (pink) and acidic (blue) mucins. Colonic goblet cells were counted in 20 longitudinally-oriented villus-crypt units. Length of the villus-crypt unit was determined to obtain goblet cell density (number of cells/mm). In all cases, cell counting was carried out on coded slides to avoid bias.

RMCPII quantification

Protein was extracted from colonic tissue samples using lysis buffer (50 mM HEPES, 0.05 % Triton X-100, 0.0625 mM PMSF and the Mini Complete protease inhibitor

Roche) and RMCPII concentration was determined by ELISA using a commercial kit (Moredun). Total protein was determined using the Bradford assay kit (BIO-RAD, Hercules, CA, USA).

Bacterial identification by fluorescence *in situ* hybridization

For FISH, oligonucleotide probes (Biomers, Ulm/Donau, Germany and Tib Molbiol, Mannheim, Germany) with a 5'-Cy3 (carbocyanine 3) dye were used (5 ng/µL). The bacterial groups characterized and the specific probes used are specified in Table 1.

In situ hybridization of bacteria in the luminal content was performed on glass slides, as previously described by us (17-19). Samples were hybridized for 16 h by addition of the hybridization buffer (20 mM Tris-HCl, 0.9 M NaCl, 0.1% SDS at pH 7.2) and thereafter washed with the washing buffer (20 mM Tris-HCl, 0.9 M NaCl at pH 7.2) for 30 min. 4',6-diamidino-2-phenylindole (DAPI) served as a control signal in all samples. Hybridized slides were viewed under oil immersion, using a Carl Zeiss Axioskop 40 FL epifluorescence microscope (filter for Cy3) equipped with a digital camera (Zeiss AxioCam MRm) for obtaining digital images (Zeiss AxioVision Release 4.8.1; Carl Zeiss, Jena, Germany). For quantification of bacteria, 20 randomly selected fields were photographed, the number of hybridized cells counted using the CellC software (20) and the mean value obtained.

To assess bacterial adherence to the colonic wall, sections from Carnoy-fixed tissues were hybridized in the same conditions. Slides were viewed under oil immersion and 20 randomly selected fields were photographed. Analysis of the images was performed manually by three independent researchers that observed the pictures and localized hybridized bacteria within the mucus layer or attached to the epithelial surface. A coincidence between two out of the three observers in bacterial location in at least three out of the 20 pictures observed was required to decide that there was bacterial attachment to the epithelium (18). All procedures were performed on coded slides, to avoid bias.

Table 1. Probes used for fluorescent *in situ* hybridization (FISH) and hybridization conditions

Probe	Sequence (5'→3')	Target	Hybridization Temp (ºC)
EUB 338	GCTGCCTCCCGTAGGAGT	Bacteria	50
NON 338	ACATCCTACGGGAGGC	Non bacteria (negative control)	50
EREC 482	GCTTCTTAGTCAGGTACCG	*Clostridium coccoides* cluster XIVa	50
ENT-D	TGCTCTCGCGAGGTCGCTT-CTCTT	Enterobacteria	50
BIF 164	CATCCGGCATTACCACCC	*Bifidobacterium* spp	50

RNA extraction and quantitative real-time PCR

Total RNA was extracted from frozen colonic samples by homogenization in Trizol reagent (Ambion, Austin, Texas, USA) followed by isopropanol precipitation and quantification by Nanodrop (Nanodrop Technologies, Rockland, DE, USA). For cDNA synthesis, 1 µg of RNA was reverse-transcribed in a 20 µl reaction volume using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR (RT-qPCR) was performed to determine mRNA levels of inflammatory markers, integrins and resistin-like molecule-beta (RELMb) in the colon. The TaqMan® probes listed in Table 2 were used (Applied Biosystems). The PCR reaction mixture was incubated on a 7900 real-time PCR system (Applied Biosystems) for 40 cycles (95ºC for 15 s, 60 ºC for 1 min). B-Actin expression served as an endogenous control for normalizing the mRNA levels of the target genes. Expression levels were analyzed by the comparative Ct method (2-ΔΔCT) using the vehicle-vehicle group as the calibrator.

Table 2. TaqMan® gene expression assays

Protein	Gene symbol	Assay reference
Inflammatory markers		
Interleukin 6 (IL-6)	*Il6*	Rn01410330_m1
Interleukin 13 (IL-13)	*Il13*	Rn00587615_m1
Interleukin 10 (IL-10)	*Il10*	Rn00563409_m1
Interleukin 12 (subunit beta) (IL-12)	*Il12b*	Rn00575112_m1
Interferon alpha-1 (IFN-alpha-1)	*Ifna1*	Rn02395770_g1
Markers of host-microbial interactions		
Integrin beta-1	*Itgb1*	Rn00566727_m1
Integrin alpha-2	*Itga2*	Rn01489315_m1
Resistin-like molecule beta (RELM-beta)	*Retnlb*	Rn01439306_m1
Reference gene		
B-Actin	*Actb*	Rn00667869_m1

Chemicals

OVA (Grade V; A5503), BSA (A9085) and LPS (LPS from *Escherichia Coli* 055:B5; 62326) were purchased from Sigma-Aldrich (St.Louis, MO, USA) and were dissolved in saline solution. Carbachol (Sigma-Aldrich) was dissolved in distilled water at a 10^{-1} M, further dilutions were prepared in distilled water.

Statistics

All data are expressed as mean ± SEM; except for bacterial counts that are expressed as media (interquartile range) ± SD. A robust analysis (one iteration) was used to obtain mean ± SEM for RT-qPCR data. Comparisons between multiple groups were performed by a one-way or a two-way ANOVA, as appropriate, followed when necessary by a Fisher's least significant difference (LSD) post hoc test to detect differences between experimental groups. P values < 0.05 were considered statistically significant.

Results

Animal monitoring

Exposure to oral OVA alone during one week did not affect body weight compared to animals receiving saline. Addition of LPS produced a transitory loss of body weight, evident only during the first day of treatment. LPS-induced body weight loss was similar in vehicle-LPS (5.24 ± 1.01 %; $P < 0.05$ vs. vehicle-vehicle group) and OVA-LPS-treated groups (4.05±0.77%; $P < 0.05$ vs. OVA-vehicle group). Thereafter, the repeated administration of LPS did not longer affect body weight and similar weight gain was observed across experimental groups. No other clinical signs were observed.

Colonic histology

Histological examination of the colon revealed no substantial effects associated to treatments, without evidences of tissue damage or inflammatory-like changes. The number of eosinophils infiltrating the colonic mucosa was similar across experimental groups (Fig. 1 A). Quantification of goblet cells in AB-PAS-stained sections revealed that LPS treatment was associated to a diminished cell density, as assessed by a two-way ANOVA, reaching statistical significance in OVA-LPS-treated rats (122.7±3.8 cells/mm; $P<0.05$ vs. vehicle-vehicle: 153.8±4.0 cells/mm; Fig. 1B). Animals exposed to OVA alone also showed a tendency for a decrease in the relative abundance of goblet cells (133.8±6.75 cells/mm; $P=0.05$ vs. vehicle-vehicle; Fig. 1 B). In vehicle-vehicle-treated animals, combined AB-PAS staining revealed that nearly all the goblet cells of the colonic mucosa contained acidic mucins (blue staining), with very few goblet cells showing mixed or neutral mucins (purple and pink staining, respectively). Relative abundance of acidic, mixed and neutral mucins was not affected by OVA or LPS or their combination.

Mast cells counts and RMCPII content

Colonic MMC counts remained unaltered after one week exposure to oral OVA (OVA-vehicle: 5.26±0.90 cells/field; $P>0.05$ vs. vehicle-vehicle: 5.80±2.18 cells/field; Fig. 1C).

Addition of LPS had no effect on the colonic density of MMCs (Fig. 1C). Similarly, colonic content of RMCPII was unaffected by OVA (OVA-vehicle; 0.55±0.21 ng/µg protein; vehicle-vehicle: 0.56±0.23 ng/µg protein; P>0.05) and remained unaltered after LPS treatment (Fig. 1D).

CTMCs, identified by its methacromatic granules, were mainly localized in the submucosa of the rat colon, with a relatively low density in control conditions (2.73±0.29 cells/field; Figs. 1E and 2). Neither OVA nor LPS, per se, affected CTMCs counts. However, in OVA-LPS-treated animals the counts of CTMCs were increased by 2-fold when compared with the separate treatments or the vehicle-vehicle group (P<0.05; Figs. 1E and 2).

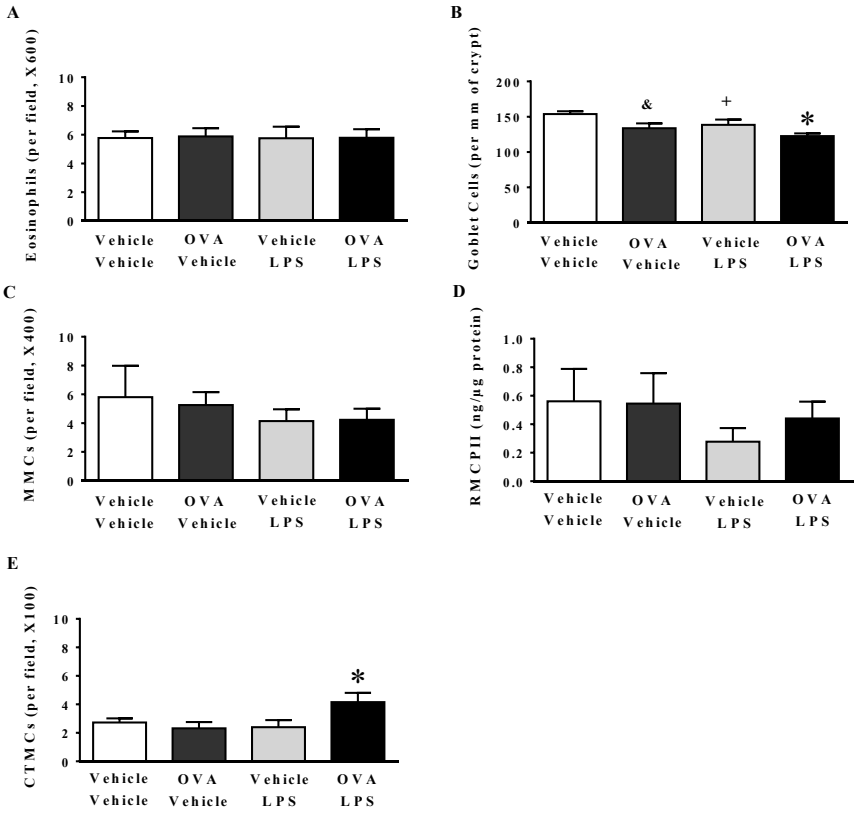

Figure 1. Colonic density of eosinophils (A), goblet cells (B), mucosal mast cells (C), RMCPII content (D) and connective tissue mast cells (E) in the different experimental groups. Data are mean±SEM of 4-8 animals per group. *: P<0.05 vs. vehicle-vehicle group. &: P=0.05 vs. vehicle-vehicle group. +: P=0.09 vs. vehicle-vehicle group.

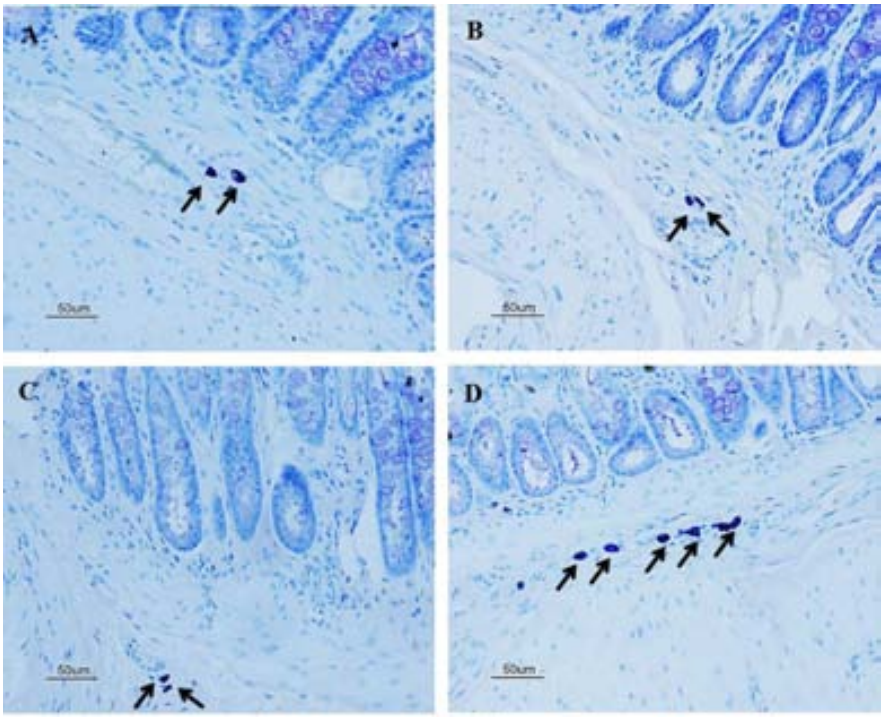

Figure 2 . Co nnective tiss ue mast cells. Representative microphotographs sh owing connective ti ssue m ast c ells (CTMCs) in to luidine blue-stained colonic slice s f rom vehicle-vehicle- (A), OVA-vehicle- (B), LPS-vehicle- (C) and LPS-OVA-treated (D) rats. The arrows indicate CTMCs in the submucosa, as identified by the presence of toluidine blue-stained m etachromatic g ranules in th eir c ytoplasm. No tice th e in creased density o f the CTMC population in the colonic submucosa of LPS-OVA-treated animals (D).

Characterization of luminal and wall-adhered microbiota

In ve hicle-vehicle-treated an imals, to tal b acterial co unts i n th e luminal content of t he colon, determined by FISH as EUB338-positive cells and confirmed by DAPI staining, were within t he margins p reviously d escribed b y us (17,19) ($3.74\times10^{10}\pm1.16\times10^{10}$ cells/mL; Fig. 3). Total bacterial counts were not altered by the administration of OVA alone ($2.90\times10^{10}\pm6.10\times10^{9}$ cells/mL; P>0.05 v s. v ehicle-vehicle; Fi g. 3). Ho wever, treatment with LPS diminished by 50% total bacterial counts vs. vehicle-vehicle values, irrespective of the coadmnistration of OVA (LPS-vehicle: $1.75\times10^{10}\pm3.10\times10^{9}$ cells/mL; LPS-OVA: $1.62\times10^{10}\pm1.84\times10^{9}$ cells/mL; both P<0.05 vs. vehicle-vehicle; Fig. 3). This effect was further confirmed by similar results obtained in DAPI-positive nuclei counts (Fig. 3).

Independently of the changes in total bacterial counts, LPS and OVA resulted in states of dysbiosis, affecting selectively the Clostridium cluster XIV or *coccoides* group (EREC482 probe). Overall, *Clostridium* spp. was the most abundant strain, irrespective of the treatment considered. Exposure to oral OVA alone or LPS alone decreased *Clostridium* spp. counts in similar proportion (OVA-vehicle: $7.83\times10^{9}\pm1.41\times10^{9}$ cells/mL; vehicle-LPS: $7.53\times10^{9}\pm1.19\times10^{9}$ cells/mL; P=0.07 and P=0.05 vs. vehicle-vehicle, respectively; Fig. 3). The combination of both treatments resulted in a further reduction in *Clostridium* spp. counts to $4.26\times10^{9}\pm4.03\times10^{8}$ cells/mL (P<0.05 vs. vehicle-vehicle; Fig. 3). Other bacterial groups assessed, namely *Bifidobacterium* spp. (BIF164 probe) and Enterobacteria (ENT-D probe) were not affected by either OVA, LPS or their combination.

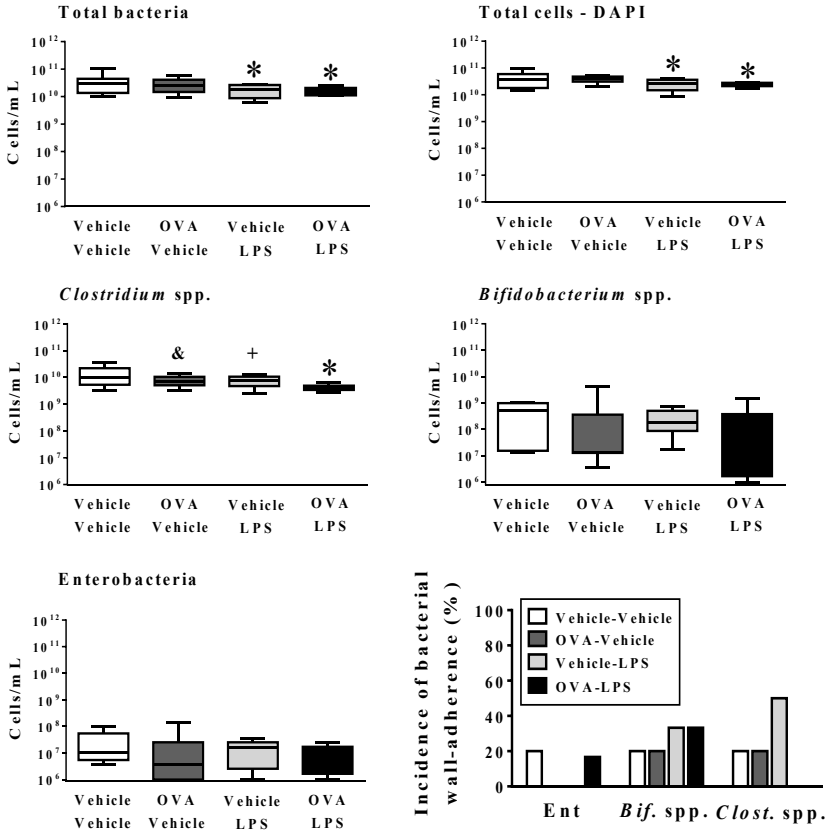

Figure 3. Composition of the luminal microbiota and incidence of bacterial wall adherence, as assessed by FISH. Bacterial counts are medias (interquartile range) ± SD, 7-8 animals per group. *: P<0.05 vs.vehicle-vehicle group. +: P=0.05 vs. vehicle-vehicle group. &: P=0.07 vs. vehicle-vehicle group. Incidence of bacterial wall adherence (bottom right corner): data represent the percentage of animals showing bacterial wall adherence for the different bacterial groups assessed. Ent: Enterobacteria; *Bif.* spp: *Bifidobacterium* spp; *Clost.* spp: *Clostridium* spp.

In vehicle-vehicle-treated animals, bacterial adherence to the colonic epithelium was relatively low and similar for all the bacterial groups assessed (by 20% incidence; Fig. 3). Treatment with OVA, did not affect the incidence of bacterial wall adherence. However, LPS showed a tendency to favour the adherence of *Bifidobacterium* spp. (incidence: 35%) and *Clostridium* spp. (incidence: 50%) (Fig. 4). The combination of OVA and LPS completely prevented the adherence of *Clostridium* spp. (0% incidence) without affecting the adherence of *Bifidobacterium* spp. (incidence: 35%).

Expression of inflammatory markers and markers of host-bacterial interactions

In control conditions (vehicle-vehicle-treated animals), colonic mRNA expression of pro-inflammatory (IFN-alpha-1, IL-6, IL-12 and IL-13) and anti-inflammatory markers (IL-10) was relatively low, but within detectable ranges in all samples. Overall, relative expression of cytokines was: IFN-alpha-1>IL-10>IL-12-beta>IL-6~IL-13.

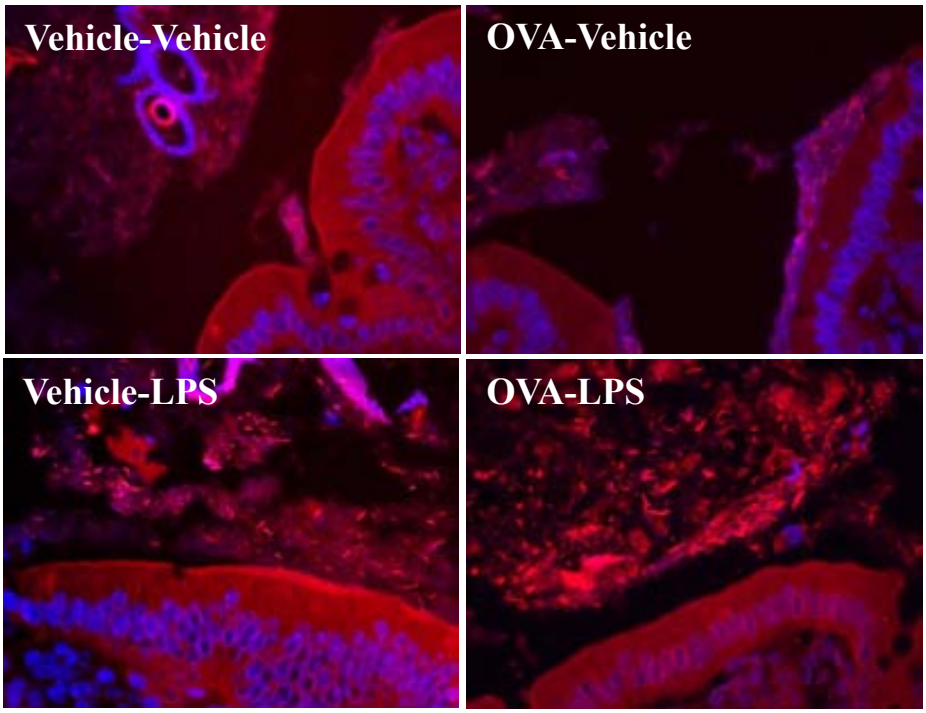

Figure 4. Representative colonic tissue images showing wall adherence of *Clostridium* spp (EREC 482 probe) in the different experimental groups.

Exposure to oral OVA alone did not affect cytokines expression; with the exception of IL-12-beta, which was slightly up-regulated although statistical significance was not reached. Similarly, LPS alone had minor effects on cytokines expression, with only a tendency (P=0.06) to increase colonic IL-6 levels (Fig. 5). However, combined treatment with OVA and LPS resulted in a two-fold increase in the expression of IFN-alpha-1 (Fig. 5).

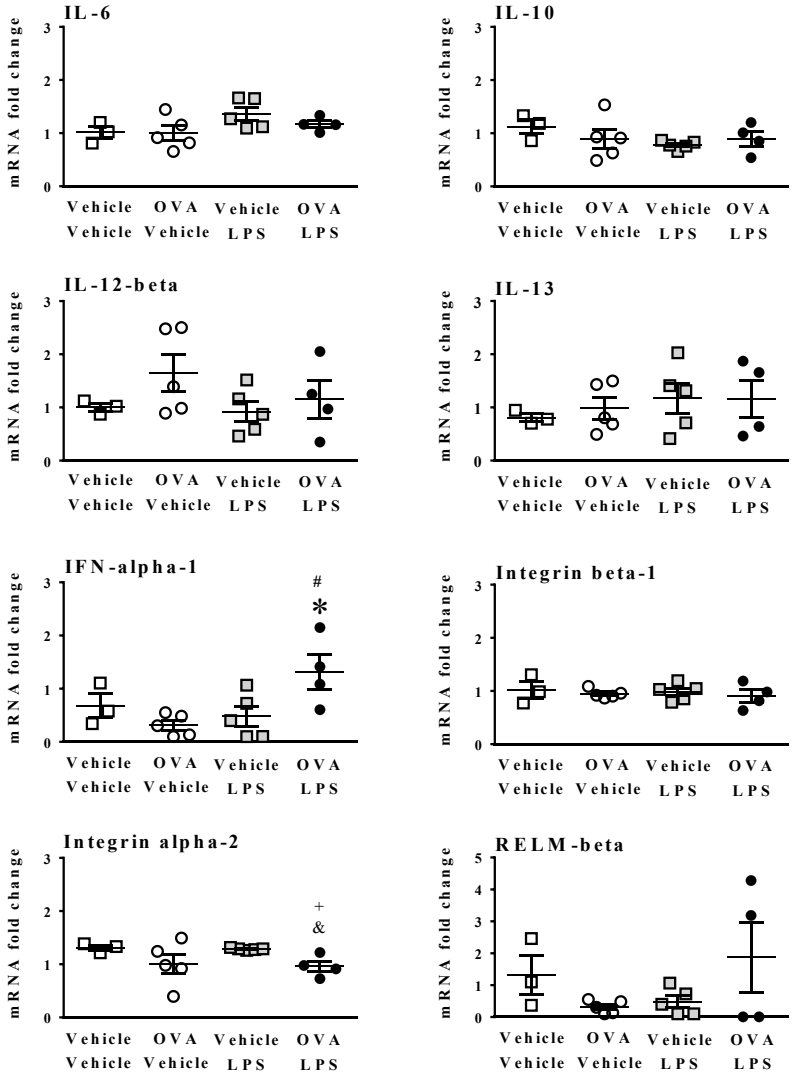

Figure 5. Colonic expression of inflammatory markers and markers of host-bacterial interactions. Each symbol represents an individual animal, the horizontal lines with errors represent the mean±SEM. *: P<0.05 vs. vehicle-LPS and OVA-vehicle group. # : P=0.08 vs. vehicle-vehicle group. + : P=0.09 vs. vehicle-vehicle group. &: P=0.07 vs. vehicle-LPS group.

In control conditions, markers of host-bacterial interactions, namely integrin beta-1, integrin alpha-2 and RELM-beta, were moderately expressed. Relative expression levels were: integrin beta-1>integrin alpha-2>RELM-beta. OVA tended to down-regulate integrin alpha-2, particularly in animals co-treated with LPS (Fig. 5).

Colonic contractility *in vitro*

Spontaneous colonic contractile activity was similar in vehicle-vehicle- and OVA-vehicle-treated animals (OVA-vehicle: 39.81±4.88 g ; vehicle-vehicle: 34.19±2.39 g ; $P>0.05$; Fig. 6A). A two way ANOVA analysis revealed that the challenge with LPS had a significant effect ($P=0.04$) enhancing contractile activity both in vehicle-LPS- and OVA-LPS-treated rats (Fig. 6A). However, further post hoc test found no differences between specific groups.

In vehicle-vehicle-treated animals, CCh elicited contractile responses in a concentration-dependent manner. Neither OVA nor LPS, alone or in combination, affected the contractile responses to CCh (Fig. 6B).

The spontaneous contractile activity of tissue samples obtained from vehicle-vehicle-treated animals was not affected by the direct addition of OVA to the organ bath. Similarly, in tissues from animals exposed to oral OVA during one week, direct exposure to OVA was without effect. However, OVA challenge on tissues obtained from OVA-LPS-treated rats resulted in an increase in the frequency and magnitude of spontaneous contractile activity ($P<0.05$). Nevertheless, some variability was observed in the responses to direct OVA exposure, with 60% of the animals being clearly "responders", as determined by a response to OVA challenge above two standard errors from the mean response in the vehicle-vehicle group (Fig. 6C). The frequency of "responders" in the other experimental groups oscillated between 0% and 25% (Fig. 6C). Regardless the experimental group considered, direct addition of a BSA solution to the organ bath did not affect colonic spontaneous contractile activity.

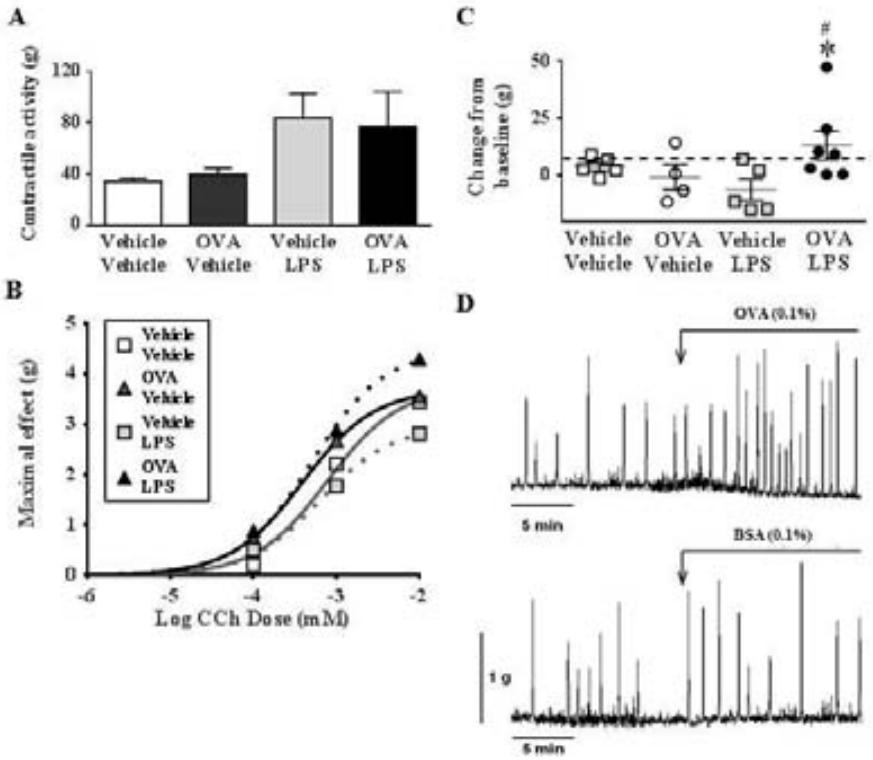

Figure 6. Colonic contractility *in vitro*. (A) Colonic spontaneous contractile activity in the different experimental groups. Data are mean ± SEM; n = 4 -6 per group. (B) Concentration-response curves for carbachol. Data represent mean values (symbols) and non-linear regression curves. n = 4 –6 per group. (C) Colonic contractile responses to OVA challenge. Each symbol represents an individual animal, the horizontal lines with errors represent the mean±SEM. The broken horizontal line corresponds to the mean+2x SEM.*: P <0.05 vs. vehicle-LPS group. #: P=0.09 vs. OVA-vehicle group. (D) Representative tracings showing the effects of OVA and BSA challenge on spontaneous colonic contractility in an OVA-LPS-treated animal. Notice the increase in the magnitude and frequency of colonic contractions after addition of OVA to the organ bath, an effected non-observed after the addition of BSA.

Discussion

Dietary antigens and gut microbiota are potential factors contributing to the pathophysiology of IBS. In this study, we show an interaction between a dietary antigen (OVA) and microbial components that leads to a situation of dysbiosis together with an altered colonic contractility, mimicking some of the features described in IBS patients. Our results indicate that LPS favors the colonic sensitization to OVA, thus suggesting that alterations of the commensal microbiota and the gut host-microbiotal interactions

might be factors facilitating the intestinal (colonic) sensitization against dietary antigens and the development of IBS-like states.

We previously showed that rats exposed to oral OVA, without adjuvants, during a 6-week period, develop a colonic contractility dysfunction, resembling that observed in IBS patients and other animal models of the disease (7-11,21). However, repeated exposure to low doses of a dietary antigen might lead to a process of oral tolerance (22,23). Therefore, in the present work, we assessed the effectiveness of a shorter exposure period to high doses of oral OVA in the induction of IBS-like colonic motor alterations. Results obtained show that colonic contractility remained unaltered after 1-week exposure to 10 mg of OVA, thus suggesting that, in contrast to long-term exposure, sensitization to oral antigens induced by few administrations might require the presence of facilitatory elements, such as adjuvants (24). We also explored if GCM might represent a potential factor favoring the induction of adverse reactions to food antigens. Administration of low-doses of LPS, which mimics a gram-negative bacterial overload, resulted in a specific dysbiosis of the colon. In addition, when combined with oral OVA, LPS induced an enhanced colonic contractility to the direct addition of the antigen to the organ bath, thus indicating a sensitization of the tissue to OVA. These altered responses of the colon are reminiscent of the exacerbated symptomatology reported in a subgroup of IBS patients after the ingestion of certain foods, reinforcing the hypothesis that adverse reactions to dietary components might be a contributing factor to the pathophysiology of the disease (25). Moreover, results shown here support the view that gut microbiota might facilitate the sensitization process to luminal antigens, thereby representing a factor leading to the development or the exacerbation of symptoms in IBS.

Dietary-related IBS-like responses have been associated, both in patients and animal models, to local mechanisms of the colonic mucosa rather than a systemic reaction, more characteristic of food allergies (4,6,7). In agreement with these observations, in this study, OVA-induced altered contractility in OVA-LPS-treated rats was neither related to the presence of circulating specific IgEs (unpublished results) nor to the altered expression of pro- (IL-6 and IL-13) or anti-allergic (IL-12 and IL-10) cytokines (26,27). However, these animals showed a specific up-regulation of IFN-alpha-1, similar to that observed in IBS-like states (28;29).

Colonic motor changes after long term exposure to oral OVA are related to an excited-activated state of MMCs (21). Moreover, MMCs have been directly implicated in the pathophysiology of IBS, although their exact role remains unclear (30-32). Here, no changes in MMC density or their excited-activated state was observed after a 1-week OVA exposure, with or without LPS. However, CTMC counts were increased in the submucosa of OVA-LPS-treated animals, while unaffected in animals treated only with OVA or LPS. Although the potential involvement of CTMCs in IBS has been less studied, several observations suggest that they could act also as effector cells leading to functional alterations within the gut. For instance, we have described a similar increase in CTMCs in the jejunum of *Trichinella spiralis*-infected rats, an accepted model of post-infectious-IBS that also courses with dysmotility (33). Moreover, CTMC counts are increased in mice with food allergy (34,35) and the degranulation of CTMCs excites the intestinal smooth muscle *in vitro* (36). Although further in deep studies are required, these observations suggest that submucosal CTMCs could be taking part in the altered contractile responses observed in OVA-LPS-treated rats.

Gut microbiota is a dynamic component of gastrointestinal homeostasis. Epidemiological and clinical data support the hypothesis that significant perturbations of the GCM can disrupt the mechanisms of oral tolerance leading to allergic responses (37). In agreement, in the current study, low doses of LPS facilitated the sensitization to oral OVA at the same time that induced changes in GCM (an overall decrease in luminal bacterial counts with a specific dysbiotic state characterized by a loss of clostridia). At the same time, LPS favored the adherence of *Clostridium* spp. to the colonic wall. A cause-effect relationship between adherence and luminal microbiota can be suggested from these changes. It is feasible to speculate that increased host-bacterial interactions, driven by the adherence of clostridia, might trigger a local response that results in the dysbiotic-like state observed when assessing the luminal microbiota. The apparent mismatch between adherence and luminal bacterial counts is similar to that observed previously in other models of dysbiosis and suggests that, besides the absolute number of bacteria, other factors modulate host-bacterial interactions (18). Nevertheless, we did not observe changes in the overall expression of adhesion molecules or RELM-beta, important components facilitating host-bacterial interactions within the gut. From the present studies, we cannot state that the observed changes in the microbiome are relevant in the facilitatory effects of LPS towards OVA sensitization. However, this hypothesis is

supported by previous results that demonstrate a direct relationship between altered states of GCM and increased susceptibility to sensitization to oral dietary antigens (14).

Abnormal intestinal responses to dietary antigens have been related in part to an altered epithelial barrier function (38). Indeed, altered gut microbiota is able to favor sensitization towards dietary antigens throughout changes in epithelial permeability (38). Here, although not directly assessed, it is feasible to assume the presence of an altered barrier function as the doses and pattern of administration of LPS were similar to other studies demonstrating an increased epithelial permeability (39,40). In addition, adherence of *Clostridium* spp. to the colonic epithelium, favored by LPS, could be a contributing factor in the alteration of the barrier function and the subsequent sensitization to OVA. Surprisingly, in OVA-LPS-treated rats no adherence of *Clostridium* spp. was observed (0% incidence). This might be associated to the presence of colonic hypercontractility generated by the presence of OVA in a state of LPS-induced enhanced sensitization. In these conditions, increased colonic motility might difficult the interaction between luminal bacteria and the epithelium, preventing bacterial adherence. In agreement with this hypothesis, we observed that direct addition of OVA to the organ bath only increased colonic contractile activity in tissues from OVA-LPS-treated animals. Moreover, this effect on bacterial dynamics might be potentiated by the enhanced mucus release present in OVA-LPS-treated rats, as suggested by the reduced density of goblet cells (41). Thus, in OVA-LPS-treated animals, the mucus discharge could facilitate the trapping of bacteria and prevent their attachment to the epithelial surface, while the altered motor responses would assist in their subsequent expulsion (42).

In summary, this study shows an interaction between luminal dietary antigens and components of the gut microbiota leading to potential alterations in colonic motor activity. In particular, we show that a dysbiotic situation favors the sensitization against a luminal dietary antigen, namely OVA, and the generation of abnormal motor responses against that antigen. Similar mechanisms might contribute to the pathophysiology of IBS, where a significant proportion of patients show an altered microbiota and sensitivity to certain food components. Alterations of the microbiota might enhance/facilitate the local neuroimmune responses to specific food antigens leading to motor alterations reminiscent of those observed in IBS.

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Disclosures

F. J. designed and performed experiments, analyzed data and wrote the paper. M. A. designed and performed experiments. P. V. designed experiments and wrote the paper. V. M. designed and performed experiments, and wrote the paper.

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CHAPTER 3

CHAPTER 3

PLASTICITY OF DORSAL ROOT GANGLION NEURONS IN A RAT MODEL OF POST-INFECTIOUS GUT DYSFUNCTION: POTENTIAL IMPLICATION OF NERVE GROWTH FACTOR

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Abstract

Background Intestinal infections are suggested as a risk factor for the development of irritable bowel syndrome (IBS)-like visceral hypersensitivity. The mechanisms implicated might involve long-term changes in visceral afferents, with implication of nerve growth factor (NGF). We explored plastic changes in dorsal root ganglion (DRG) neurons innervating the gut, and the potential implication of NGF, in a rat model of IBS-like post-infectious gut dysfunction. **Methods** Rats were infected with *T. spiralis* larvae. 30 days post-infection, inflammatory markers, including interleukins (ILs) and mucosal mast cell (MMC) infiltration [rat mast cell protease II (RMCPII)], and NGF and TrkA expression was determined in the jejunum and colon (RT-qPCR). In the same animals, morphometry (neuronal body size) and NGF content (immunofluorescence) were assessed in thoracolumbar DRG neurons. **Results** In infected animals, a low grade inflammatory-like response, characterized by up-regulated levels of RMCPII and IL-6, was observed in the jejunum and colon. TrkA expression was increased in the jejunum while in the colon showed a slight reduction. NGF levels remained unaltered regardless the gut region. Overall, the mean cross-sectional area of DRG neurons was increased in *T. spiralis*-infected animals, with a reduction in both TrkA and NGF staining. **Conclusions & Inferences** Results suggest that during *T. spiralis* infection in rats, there is a remodeling of sensory afferents that might imply a NGF-mediated mechanism. Plastic changes in sensory afferents might mediate the long-lasting functional alterations that characterize this model of IBS. Similar mechanisms might be operating in patients with post-infectious-IBS.

Introduction

Alterations in epithelial, neural and immune functions are likely to contribute to the initiation and perpetuation of functional gastrointestinal disorders (FGDs). For instance, remodeling of enteric sensory afferents is suggested to be implicated in the development of the visceral hypersensitivity observed in irritable bowel syndrome (IBS) (1). In this line, it has been shown that intestinal infections evoke hyperexcitability of colonic dorsal root ganglion (DRG) neurons and increase afferent nerve activity of the jejunum, suggesting a potential role for these processes on the modulation of afferents' properties (2-4).

Data suggest that neurotrophins, mainly nerve growth factor (NGF), are implicated in the pathophysiology of IBS-like sensorial alterations. For instance, NGF content is increased in rectal mucosa of diarrhea-predominant IBS children (5). In addition, the ability of NGF to induce long-lasting changes in the morphological and electrophysiological properties of DRG neurons has been widely reported *in vitro* (6,7) and *in vivo* (8). Thus, it is feasible to hypothesize that NGF might contribute to the remodeling of enteric afferents and the onset and/or maintenance of the visceral hypersensitivity states that characterize IBS.

In the model of experimental infection with *Trichinella spiralis* in rats, a validated model of post-infectious-IBS (PI-IBS), we have demonstrated the presence of a long-lasting jejunal epithelial barrier dysfunction associated to a persistent state of local immune activation and mucosal mast cell (MMC) infiltration (9,10). In the present study, we used the same model with the aim of characterizing changes in enteric sensory afferents and the potential implication of NGF. At 30 days post-infection (PI), we assessed the presence of a persistent inflammatory-like state in the jejunum and colon. In the same animals, morphometric changes in thoracolumbar DRGs (innervating the small intestine and colon) were used as a surrogate marker for alterations in sensory afferents. Finally, to assess the potential implication of NGF we determined the expression of the peptide and its high-affinity receptor TrkA within the gut and in DRGs.

Materials and Methods

Animals

Adult (6 week-old at arrival), specific pathogen free (SPF), Sprague-Dawley male rats were used (Charles River, Les Oncins, France). Rats were maintained under conventional conditions in a light (12h/12h light-dark cycle) and temperature controlled (20-22ºC) room, in groups of two to three per cage, with access to tap water and laboratory rat chow (A04; Safe, Augy, France) *ad libitum*. Animals were acclimatized to the new environment for one week before starting any experimental procedure. All experimental procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya (protocol numbers 1013 and 5352, respectively).

Trichinella spiralis infection

Muscle-stage larvae of *T. spiralis* were obtained from infected CD1 mice as previously described (9,10). Rats were infected at 7–8 weeks of age by administration of 7.500 *T. spiralis* larvae, suspended in 1 mL of saline, by oral gavage. Studies were performed on days 30±2 post-infection (PI). Age-matched rats dosed orally with 1 mL of saline were used as controls. After the infection, animals were regularly monitored for clinical signs and body weight changes. Normal course of the infection was confirmed by a significant decrease of body weight in infected animals compared with controls, with a peak reduction on days 8-to-10 PI and a subsequent linear increase over time, as previously described by us (9,10).

Experimental procedures and tissue sampling

Non-infected controls or animals at 30 days PI were euthanatized by decapitation. A laparotomy was performed and jejunal and middle colon samples were obtained, frozen in liquid nitrogen and stored at -80ºC until analysis. At the same time, the spinal thoracolumbar segment was removed and fixed for 24 h in cold 4 % paraformaldehyde. Thereafter, dorsal root ganglia (DRGs) from T10 to L2 (T10-L2) were dissected, pooled together and processed for morphological and immunohistochemical studies. T10-L2

DRGs contain afferent fibers innervating both the small intestine and the colon of the rat (11-13).

NGF and TrkA immunofluorescence staining in DRGs

Immunodetection of NGF and TrkA was carried out separately on paraformaldehyde-fixed DRG sections (5 µm) by overnight incubation at 4ºC with a rabbit polyclonal antibody against NGF (ab1526; Chemicon International, Temecula, CA, USA) or TrkA (sc-118; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antigen retrieval was achieved by microwave processing of the slides (10mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0; 2 cycles of 5 min, 800W). Expression was visualized using a Cy3 goat anti-rabbit IgG (1 h, room temperature; PA-43004; Amersham-Pharmacia, Buckinghamshire, UK). The slides were coverslipped and observed with a Zeiss Axioskop 40 microscope (Carl Zeiss, Jena, Germany) equipped with a digital camera (Zeiss AxioCam MRm). At least 25 photographs (original magnification X400) were taken per animal. All procedures were carried out using coded slides to avoid bias.

Image analysis of DRG neurons: Morphometry and quantification of NGF and TrkA expression

Intensity of staining for NGF and TrkA and cell body area of DRG neurons were determined in 100 randomly selected neurons from each animal, using the ImageJ software. The neuronal cross-sectional area together with the mean pixel density of NGF and TrkA labeling were determined for each neuron and a mean value obtained per animal.

Neuronal cross-sectional areas were determined by outlining the perimeter of clearly defined neuronal cell bodies. According to their size, cells were classified as: small (cross-sectional area $\leq$500 μm^2), medium (500-1000 μm^2), large (1000-1500 μm^2) and very large (>1500 μm^2) (14,15).

When measuring NGF- and TrkA-signal intensity, fluorescent intensity in negative controls (omission of the primary antibody) served to establish a mean background value. Immunofluorescence intensity in *T. spiralis*-infected animals was expressed as the

relative change compared to the mean immunofluorescence intensity in non-infected controls, taken as 100%. Comparisons were performed between DRG neurons with a similar cross-sectional area. All measurements were carried out in a blinded manner to avoid bias.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from frozen jejunal and colonic samples by homogenization in Trizol reagent (Ambion, Austin, Texas, USA) followed by isopropanol precipitation and quantification by Nanodrop (Nanodrop Technologies, Rockland, DE, USA). For cDNA synthesis, 1 µg of RNA was reverse-transcribed in a 20 µl reaction volume using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed to determine mRNA levels of inflammatory markers, NGF and TrkA in the jejunum and colon. For this purpose, the Taqman® probes listed in Table 1 were used (Applied Biosystems). The PCR reaction mixture was incubated on a 7900 real-time PCR system (Applied Biosystems) for 40 cycles (95ºC for 15 s, 60ºC for 1 min). B-Actin expression served as an endogenous control for normalizing the mRNA levels of the target genes. Expression levels were analyzed by the $2^{-\Delta\Delta CT}$ method.

Table 1. TaqMan® gene expression assays

Protein	Gene symbol	Assay reference
Inflammatory markers		
Interleukin 6 (IL-6)	*Il6*	Rn01410330_m1
Interleukin 13 (IL-13)	*Il13*	Rn00587615_m1
Interleukin 10 (IL-10)	*Il10*	Rn00563409_m1
Rat mast cell proteinase II (RMCPII)	*Mcpt2*	Rn00756479_g1
Inducible nitric oxide synthase (iNOS)	*Nos2*	Rn00561646_m1
Neurotrophins and receptors		
Nerve growth factor (NGF)	*Ngf*	Rn01533872_m1
High affinity nerve growth factor receptor (TrkA)	*Ntrk1*	Rn00572130_m1
Reference gene		
B-Actin	*Actb*	Rn00667869_m1

Statistical analysis

All data are expressed as mean ± SEM. A robust analysis (one iteration) was used to obtain mean ± SEM for RT-qPCR data. Comparisons between groups were performed using Student's unpaired t test or nonparametric Mann–Whitney test, as appropriate. P values <0.05 were considered statistically significant.

Results

Jejunal and colonic expression of inflammatory markers

In vehicle-treated rats, expression of inflammatory (IL-6, IL-13 and iNOS) and anti-inflammatory markers (IL-10) was detected at similar, relatively low, levels both in jejunum and colon. IL-13 content in the jejunum was particularly low compared to the other markers, with 2, out of the 10 samples analyzed, below levels of detection. Overall, relative expression for the jejunum was IL-10>iNOS>IL-6>IL-13; while for the colon was IL-10>IL-6~IL-13>iNOS.

In the jejunum of infected animals, IL-6 and IL-13 expression was up-regulated by 2.5-fold over controls ($P<0.05$; Fig. 1). In the colon, changes were restricted to IL-6 expression, which was up-regulated by 2-fold ($P<0.05$; Fig. 1). Other markers assessed (iNOS, IL-10) were not affected in infected animals.

T. spiralis infection induced a 7-fold and 4-fold increase in RMCPII expression in the jejunum and colon, respectively (both $P<0.05$ vs. non-infected controls; Fig. 1).

Jejunal and colonic expression of NGF and TrkA

In non-infected animals, NGF and TrkA expression was detected at moderate-to-low levels in both jejunum and colon. In the jejunum of *T. spiralis*-infected animals, TrkA expression showed a 2-fold increase, without changes in NGF expression ($P<0.05$; Fig. 2). However, in the same animals, colonic expression of TrkA was reduced by 30% ($P<0.05$; Fig. 2) while NGF expression was unaffected.

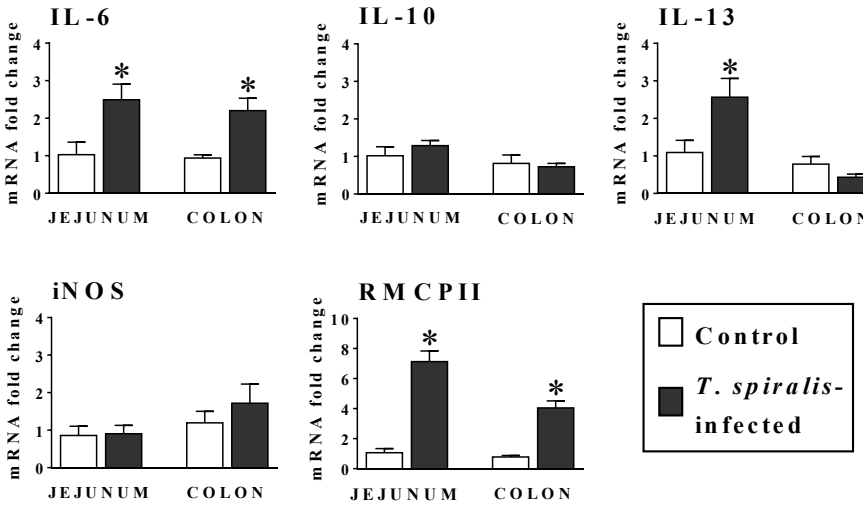

Figure 1. Jejunal and colonic expression of inflammation-related markers in non-infected controls and *T. spiralis*-infected animals at day 30 PI. Data are mean±SEM of 4-6 animals per group. *: P<0.05 vs. non-infected controls.

Morphometric characterization of DRG neurons

In non-infected animals, the majority of DRG neurons were of small (<500 μm^2, 51%) or medium size (500-1000 μm^2, 33%) (Fig. 3C). In *T. spiralis*-infected animals this pattern of distribution was altered, with a reduction in the relative abundance of small-sized neurons (11%) and an increase in the number of very large-sized neurons (>1500 μm^2, 8%) (Fig. 3). Overall, infected-animals showed a 27% increase in the mean cross-sectional area of DRG neurons (P<0.05; Fig. 3D).

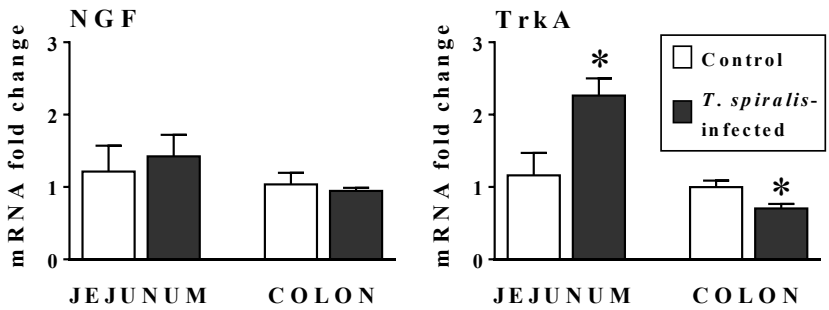

Figure 2. Jejunal and colonic expression of NGF and TrkA in non-infected controls and *T. spiralis*-infected animals at day 30 PI. Data are mean±SEM of 4-6 animals per group. *: P<0.05 vs. non-infected controls.

NGF- and TrkA-immunoreactivity in DRG neurons

In DRGs from non-infected animals, neurons showing NGF- or TrkA-labeling were distributed throughout the ganglia (Fig. 4). Both NGF and TrkA-signal were particularly abundant in the cytoplasm of small/medium-sized DRG neurons (area ≤1000 μm^2) while large neurons (area >1000 μm^2) showed immunoreactivity at background levels (Fig. 4). Labeling for TrkA was clearly visualized in glial satellite cells surrounding DRG neurons (Fig. 4).

In *T. spiralis*-infected animals, the pattern of expression of NGF or TrkA was maintained. However, signal intensity for both markers was reduced, particularly within small/medium-sized neurons (Fig.4).

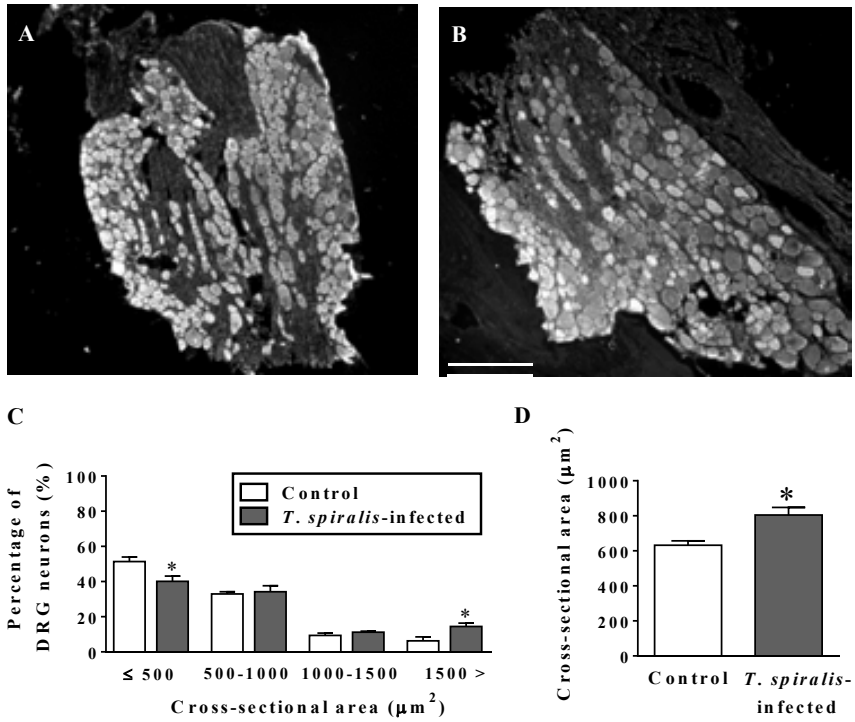

Figure 3. Morphometric changes in DRG neurons. A-B: Representative photomicrographs of thoracolumbar DRGs from a non-infected (A) and a *T. spiralis*-infected rat at day 30 PI (B). Scale bar: 100 μm. C: Size-distribution of thoracolumbar DRG neurons in non-infected and *T. spiralis*-infected animals. D: Mean cross-sectional area of thoracolumbar DRG neurons. Data are mean±SEM, 4-5 animals per group and 100 neurons per animal. *: P<0.05 vs. non-infected controls.

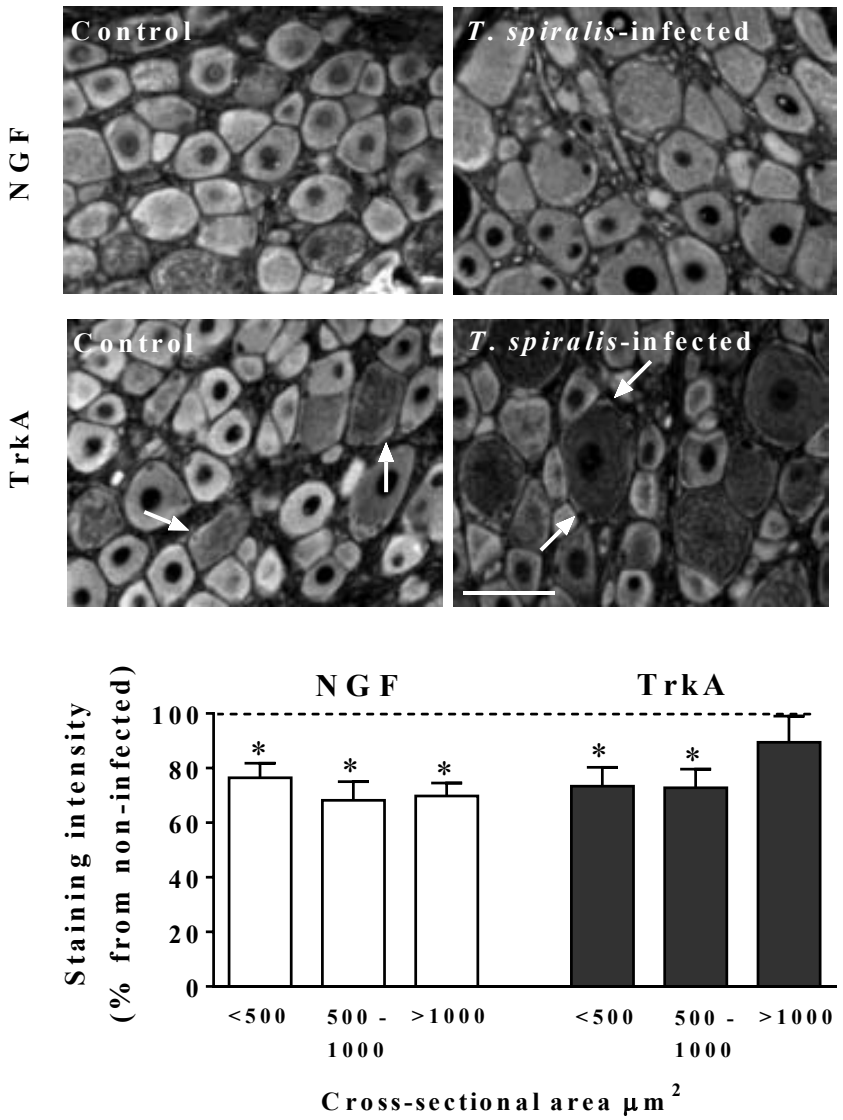

Figure 4. NGF- and TrkA-immunoreactivity in DRG neurons. Photomicrographs of NGF and TrkA immunofluorescence in thoracolumbar DRG neurons from a non-infected and a *T. spiralis*-infected rat at day 30 PI. Note that labeling intensity within neurons was clearly decreased after *T. spiralis* infection. Arrows point glial satellite cells with TrkA immunoreactivity, surrounding DRG neurons. Scale bar: 50 µm. The bar graph shows the quantification of NGF and TrkA immunofluorescence intensity (according to the cross-sectional area of DRG neurons) expressed as the relative change compared to the mean immunofluorescence intensity in non-infected animals (taken as 100, broken horizontal line). Data are mean±SEM, 4-5 animals per group and 100 neurons per animal. *: $P<0.05$ vs. staining intensity in non-infected controls (100%).

Discussion

The present study evidences morphological changes in the intestinal afferent innervation in a model of post-infectious gut dysfunction in rats. The altered expression of NGF and TrkA in DRGs suggests an implication of neurotrophins in the plastic remodeling of sensory neurons. Similar changes in gut afferents might be taking place in PI-IBS patients, as basis for the sensorial alterations observed.

Persistent inflammatory states of the gut have been associated to the remodeling of sensory neurons innervating the affected region (16,17). In the present report, a chronic inflammatory-like response of the intestine (up to day 30 PI) was observed, as determined by an up-regulation of inflammatory cytokines (primarily IL-6) and RMCPII (a MMC marker). This is in agreement with previous observations in *T. spiralis*-infected animals revealing a long-term increase in the tissue expression of inflammatory mediators and a persistent infiltrate of MMCs (9,10,18). RMCPII and IL-6 levels showed a positive correlation (unpublished data), suggesting that the increased cytokine transcript content might result from the activated state of MMCs, as supported by *in vitro* observations (19,20). Interestingly, we show that this inflammatory state extends to intestinal areas not directly affected by the parasite. Indeed, a similar inflammatory-like reaction was demonstrated in the small intestine (jejunum) and in the colon. Since the colon is regarded as the main affected organ in IBS, these observations further support the validity of the *T. spiralis* infection model as a good approach to IBS in general, and PI-IBS in particular. Further in deep studies are required to assess if the colon of *T. spiralis*-infected animals also presents functional alterations reminiscent of those observed in IBS patients.

Plasticity of afferent sensory pathways during intestinal inflammation might imply both functional and morphological adaptations (2,16,17,21,22). Here, the cross-sectional area of thoracolumbar DRG neurons of *T. spiralis*-infected animals was increased by 27% when compared with non-infected controls. Similar neuronal changes were observed during TNBS-induced enteritis in guinea pigs or after partial obstruction of the intestine in rats (16,22). In these studies, hypertrophy of DRG neurons was accompanied with an active inflammation of the gut, leading the authors to propose that the retrograde transport of inflammatory mediators from the intestine into DRGs contributed to the

observed changes. Similar mechanisms might be operating during *T. spiralis* infection, since morphological alterations of DRGs were associated to an extended enteric inflammatory-like response, as discussed above.

NGF is regarded as an active mediator in inflammation-related neuronal remodeling of afferent innervation (7,23). For instance, hypertrophy of DRG neurons has been related to increased levels of the target-tissue NGF and is prevented by immunoneutralization of the peptide (24-26). In the present studies, although DRG neurons showed an increase in size during intestinal inflammation, NGF levels, both in the jejunum and colon, remained unchanged. This apparent discrepancy might be explained by the rapid and short-term up-regulation that neurotrophic factors exhibit during pathological conditions (7). For instance, in *T. spiralis*-infected animals, an early peak in jejunal NGF levels has been described by day 3 PI, followed by normalization at later stages (27). Taking into account the long-term biological effects of neurotrophins (28), it is feasible to hypothesize that the hypertrophy of sensory neurons observed here is a persisting phenotypic effect of an early up-regulation of enteric NGF expression. Supporting this hypothesis, changes in the afferent innervation of the bladder of streptozotocin-diabetic rats were associated to an increase in DRG neuronal size that was two weeks delayed relative to the peak levels of NGF (26). Alternatively, up-regulation of jejunal levels of TrkA receptors, as observed in the jejunum, can also be interpreted as an enhanced NGF-dependent afferent signaling arising from inflamed tissues, without changes in NGF expression, thus contributing to the induction and maintenance of morphological changes within DRG neurons. However, TrkA immunoreactivity in DRG neurons was reduced during infection. This might suggest a compensatory mechanism constraining the neuroplastic effects associated to the enhanced NGF-TrkA-dependent signaling. In fact, since NGF is uptaken by TrkA receptors in axon terminals, DRG neurons could limit NGF-signaling by down-regulating their TrkA expression (29).

It is feasible to speculate that the morphological changes described here in DRG neurons, together with the expression changes in the NGF-TrkA axis, are associated to an altered signaling of intestinal afferents. In fact, compelling evidences reveal that, during intestinal inflammation, both electrophysiological properties of DRG neurons and intestinal afferent nerve activity are altered, showing states of sensitization (2-4,16,22). Moreover, NGF has been directly implicated in the induction of long-term phenotypical

changes in sensory neurons leading to states of central and peripheral sensitization (30). Therefore, and even though the descriptive characteristic of our study, we can hypothesize that the changes observed might be associated to alterations in visceral sensitivity, similar to that observed in other animals models of IBS or in IBS patients. Further functional studies are required to confirm such a hypothesis.

In summary, we show the presence of a long-lasting extended low grade inflammation in the gut of *T. spiralis*-infected rats. In these conditions, there are persisting morphological changes in the intestinal afferent innervation, namely a hypertrophy of DRG neuronal cell bodies. Alterations in NGF-TrkA-depending signaling might participate in these plastic changes. Overall, these observations suggest that long-term activation of the enteric immune system during intestinal infections might account for changes in gut afferents properties, thereby providing a neuroimmune basis for the states of chronic visceral hypersensitivity.

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Disclosures

F. J. designed and performed experiments, analyzed data and wrote the paper. J.A. F-B designed and performed experiments. V. M. designed and performed experiments, and wrote the paper. P. V. designed experiments and wrote the paper.

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CHAPTER 4

CHAPTER 4

PERSISTENT ALTERATIONS IN COLONIC AFFERENT INNERVATION IN A RAT MODEL OF POST-INFECTIOUS GUT DYSFUNCTION: ROLE FOR CHANGES IN PERIPHERAL NEUROTROPHIC FACTORS

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Abstract

Background Visceral hypersensitivity in the inflamed gut is related partly to the effects of peripheral neurotrophic factors (NTFs) on local afferent neurons. However, alterations in sensory afferents of distant areas remain unexplored. Using the *Trichinella spiralis* infection model, which courses primarily with a jejunitis, we investigated the remodeling of colonic afferents and the potential role of NTFs. **Methods** Rats were infected with *T. spiralis*. Inflammatory-like changes, mucosal mast cells (MMCs) dynamics, and expression of nerve growth factor and glial cell line-derived NTFs (GDNF, artemin and neurturin) were determined in the colon up to day 30 post-infection. Functionality of colonic afferents was determined assessing changes in the expression of sensory-related markers in thoracolumbar/lumbosacral DRGs (TL, LS) following intracolonic capsacin. **Results** *T. spiralis* induced an inflammatory-like response within the colon, partly resolved at day 30 post-infection, except for a persistent MMC infiltrate. While the jejunum of infected animals showed an up-regulation in the expression of NTFs, a transitory down-regulation was observed in the colon. Overall, *T. spiralis* effects on DRGs gene expression were restricted to a transient down-regulation of TRPV1. Stimulation with intracolonic capsaicin induced a down-regulation of TRPV1 levels in TL and LS DRGs, an effect enhanced in LS DRGs of infected animals, regardless the post-infection time considered. **Conclusions & Inferences** During intestinal inflammation, spread morphological and functional alterations, including remodeling of visceral afferents, are observed outside the primary region affected by the insult. Similar mechanisms might be operating in states of widespread alterations of visceral sensitivity.

Introduction

Inflammatory processes of the intestine have been associated to the remodeling of sensory afferents innervating the inflamed area, leading to the development of functional alterations, frequently manifested as visceral hypersensitivity (1,2). For instance, a persistent state of low grade inflammation has been suggested as a contributing factor for the development of visceral hypersensitivity in irritable bowel syndrome (IBS) patients (3). Inflammation-related neuroplastic changes also occur at intestinal regions not directly affected by the primary inflammatory insult (4). The exact pathophysiological mechanisms involved in this phenomenon remain largely unknown. Different hypothesis have been suggested, such as the release to the blood stream of inflammation-related mediators from the primary inflamed area, the activation of pro-inflammatory cascades in the distant regions or the implication of neurally-mediated responses (4,5).

Data accumulated during the last years suggest that neurotrophic factors (NTFs), including nerve growth factor (NGF) and the family of glial cell line-derived NTFs (GDNF, artemin and neurturin), are implicated in the sensorial alterations observed in inflammatory and functional gastrointestinal disorders (FGDs). For instance, evidences obtained from animal models of IBS and gut inflammation show an up-regulation in NTFs expression and suggest their implication in the sensitization of enteric afferent neurons, as basis for the functional changes observed (6,7). In particular, knockout mice for the glial cell line-derived neurotrophic factor family receptor α3 (GFRα3), a component of the receptor of the growth factor artemin, showed reduced visceral pain-related responses during TNBS-induced colitis, in agreement with observations *in vitro* showing that colorectal afferents of GFRα3 knockout mice also failed to sensitize when exposed to inflammatory mediators (8). Moreover, a recent study in IBS patients showed a positive correlation between abdominal pain scores and content of brain-derived neurotrophic factor (BDNF) in the colonic mucosa, thus further supporting a role for NTFs in the sensitization of visceral afferents (9).

Special attention has received the dynamic interplay between NGF and intestinal mucosal mast cells (MMCs). In particular, observations derived from animals models suggest that, within the intestine, MMCs are a target for NGF and that NGF is involved

in the recruitment and activation of MMCs and mediates the interaction between mast cells and nerve fibers (10-12).

In the present study, we explored the potential implication of NTFs in the afferent neuronal remodeling associated to gut inflammation. We used the model of *Trichinella spiralis* infection in rats, a validated model of MMC-associated post-inflammatory gut dysfunction resembling post-infectious-IBS (PI-IBS) (13,14). In this model, we assessed, up to 30 days post-infection (PI), inflammatory-like changes and mast cell (mucosal and connective type) dynamics in the colon, a site not directly affected during *T. spiralis* infection, which courses primarily with a jejunitis. To study the potential implication of NTFs we determined the expression of NGF, GDNF, artemin and neurturin and their receptors (TrkA and GFRα3) in colonic tissues as well as in thoracolumbar (TL) and lumbosacral (LS) dorsal root ganglion (DRG) neurons, site of origin of the afferent innervation of the colon. Functionality of colonic afferents was determined assessing changes in the expression of sensory-related markers and NTFs' receptors in DRG neurons following stimulation of the ion channel transient receptor potential vanilloid 1 (TRPV1) with intracolonic capsaicin.

Materials and Methods

Animals

Adult (6 week-old at arrival), specific pathogen free (SPF), Sprague-Dawley (SD) male rats were used (Charles River, Les Oncins, France). Rats were maintained under conventional conditions in a light (12h/12h light-dark cycle) and temperature controlled (20-22ºC) room, in groups of two to three per cage, with access to tap water and laboratory rat chow (A04; Safe, Augy, France) *ad libitum*. Animals were acclimatized to the new environment for one week before starting any experimental procedure. All experimental procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya (protocol numbers 1013 and 5352, respectively).

Trichinella spiralis infection

Muscle-stage larvae of *T. spiralis* were obtained from infected CD1 mice as previously described (13,14). Rats were infected at 7–8 weeks of age by administration of 7.500 *T. spiralis* larvae, suspended in 1 mL of saline, by oral gavage. Studies were performed on days 2, 6, 10, 14, 23 and 30 post-infection (PI). Age-matched rats dosed orally with 1 mL of saline were used as controls. After the infection, animals were regularly monitored for clinical signs and body weight changes. Normal course of the infection was confirmed by a significant decrease of body weight in infected animals compared with controls, with a peak reduction on days 8-to-10 PI and a subsequent linear increase over time, as previously described by us (13,14).

Experimental procedures and tissue sampling

Non-infected controls or animals at 2, 6, 10, 14, 23 and 30 days PI were deeply anesthetized with isoflurane followed by euthanasia by decapitation. A laparotomy was performed and jejunal and middle colon samples were obtained and either fixed in ice-cold 4 % paraformaldehyde or Lana's fixative (4% paraformaldehyde, 14% picric acid in 0.4 M phosphate buffer) for histological and immunohistochemical studies, or frozen in liquid nitrogen and stored at -80ºC until analysis. In addition, paired thoracolumbar (TL, T12-L2) and lumbosacral (LS, L6-S2) dorsal root ganglia (DRGs) were removed and processed immediately for gene expression studies (see below). TL and LS DRGs were selected in order to distinguish the influence of the gut region (small intestine vs. colon) in the changes observed. In the rat, TL DRGs contain both small intestine and colonic afferents fibers while LS DRGs are mostly devoided of neurons innervating the upper gastrointestinal tract (15-17).

In some cases, before tissue sampling, colonic sensory afferents were stimulated with intracolonic capsaicin. For this, non-infected controls or animals at 14 and 30 days PI were used. Animals were anesthetized with isoflurane and capsaicin (0.1 mL/rat, 0.1% in ethanol:Tween 80:saline; 1:1:8, v:v:v; Sigma Aldrich, St. Louis, MO, USA) was administered in tracolonically (2 cm from the anus) using a 18 g auge catheter. Thereafter, rats were maintained under anesthesia for 25 min and subsequently

euthanized by decapitation. Colonic tissue samples and TL and LS DRGs were obtained as described above.

Histopathological studies

Paraformaldehyde-fixed colonic tissue samples were processed routinely for paraffin embedding, and 5-µm sections were obtained for haematoxylin and eosin (H&E) staining. H&E slides were evaluated and a histopathological score (ranging from 0, normal, to 9, maximal alterations) was assigned to each animal. Parameters scored included: epithelial structure (0: normal; 1: mild alterations of the epithelium; 2: local epithelium destruction; 3: generalized epithelium destruction); presence of inflammatory infiltrate (0: normal; 1: mild localized infiltrate; 2: mild generalized infiltrate; 3: severe generalized infiltrate) and relative density of goblet cells (0: normal; 1: mild increase in cell density; 2: moderate/zonal increase in cell density; 3: severe/generalized increase in cell density). The same samples were also used for morphometric studies in which the thickness of mucosa, submucosa and muscularis externa layers was measured. For this, digital images were acquired at X200 magnification (Nikon Eclipse 90i, equipped with a digital camera, DXM 1200F; Nikon corporation, Japan) and were analyzed with the NIH ImageJ software. At least 10 random measurements per sample (from 2 slices) were used to obtain a mean thickness value. All procedures were performed in a blinded manner on coded slides to avoid bias.

Immunohistochemistry for Rat Mast Cell Proteinases II and VI and MC counts

Immunodetection of rat mast cell proteinases II (RMCPII) and VI (RMCPVI) was carried out on paraformaldehyde-fixed colonic samples using a monoclonal antibody anti-RMCPII (Moredun, Edinburgh, UK) or a goat polyclonal anti-mast cell tryptase (RMCPVI) antibody (sc-32473; Santa Cruz Biotechnology, Dallas, Texas, USA), respectively. Antigen retrieval for RMCPVI was achieved by processing the slides in a microwave (10mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH 9.0; 2 cycles of 5 min, 800W). The secondary antibodies included biotinylated horse antimouse IgG (BA-2000; Vector Laboratories, Burlingame, CA, USA) and biotinylated rabbit anti-goat IgG (sc-2774; Santa Cruz Biotechnology). Detection was performed with avidin/peroxidase kit (Vectastain ABC kit; Vector Laboratories) and counterstaining

with haematoxylin. Specificity of the staining was confirmed by omission of the primary antibody. Slides were viewed with a Zeiss Axioskop 40 microscope (Carl Zeiss, Jena, Germany). Mucosal mast cells (MMCs), identified as RMCPII-immunopositive cells, were quantified in, at least, 20 non-adjacent, randomly selected, fields of colonic mucosa (X400), covering the whole mucosal thickness. Density of connective tissue mast cells (CTMCs), identified as RMCPVI-immunopositive cells, was determined by counting the total number of cells in the submucosa, external smooth muscle and serosa areas in two complete tissue sections of the colon for each animal. All procedures were carried out using coded slides to avoid bias.

Immunofluorescence for RMCPII

Following fixation for 4 h in Lana's fixative, colonic samples were cryoprotected by sequential overnight incubation with 10% and 20% sucrose, embedded in OCT (Sakura Finetek, Japan), frozen, and sectioned at 20 µm thickness. Tissue sections were incubated with the monoclonal antibody anti-RMCPII overnight at 4 °C and then with Alexa Fluor 488 goat anti-mouse IgG (A11029; Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. Images of the immunostained tissue were acquired at X400 magnification using a scanning confocal microscope (Zeiss LSM 700; Carl Zeiss, Jena, Germany) and subsequently analyzed using the ImageJ software. Microphotographs of at least 50 randomly selected RMCPII-immunopositive cells were obtained, the immunofluorescence intensity quantified (ImageJ) and a mean intensity obtained for each animal. Immunofluorescence intensity in *T. spiralis*-infected animals is expressed as the relative change compared to the mean immunofluorescence intensity in non-infected animals, taken as 100%. In all cases, sections from control and *T. spiralis*-infected animals were processed simultaneously. Specificity of the staining was confirmed by omission of the primary antibody. Analysis of the data was carried out in a blinded manner to avoid bias.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from frozen colonic samples and fresh DRGs by homogenization in Trizol reagent (Ambion, Austin, Texas, USA) followed by isopropanol precipitation and quantification by Nanodrop (Nanodrop Technologies,

Rockland, DE, USA). For cDNA synthesis, 1 µg of RNA was reverse-transcribed in a 20 µl reaction volume using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed to determine mRNA levels of inflammatory markers and NTFs in the colon and expression of proteinase-activated receptors, NTFs' receptors, neuropeptides and cation channels in TL and LS DRGs. For this purpose, the Taqman® probes listed in Table 1 were used (Applied Biosystems). The PCR reaction mixture was incubated on a 7900 real-time PCR system (Applied Biosystems) for 40 cycles (95ºC for 15 s, 60ºC for 1 min). B-Actin expression served as an endogenous control for normalizing the mRNA levels of the target genes. Expression levels were analyzed by the $2^{-\Delta\Delta CT}$ method.

Table 1. Taqman® gene expression assays

Protein	Gene symbol	Assay reference
Inflammatory markers		
Rat mast cell proteinase II (RMCPII)	*Mcpt2*	Rn00756479_g1
Interleukin 13 (IL-13)	*Il13*	Rn00587615_m1
Inducible nitric oxide synthase (iNOS)	*Nos2*	Rn00561646_m1
Proteinase-activated receptors		
Protease-activated receptor 2 (PAR-2)	*F2rl1*	Rn00588089_m1
Neurotrophic factors		
Nerve growth factor (NGF)	*Ngf*	Rn01533872_m1
Artemin	*Artn*	Rn01761472_g1
Glial cell line-derived neurotrophic factor (GDNF)	*Gdnf*	Rn01402432_m1
Neurturin	*Nrtn*	Rn01527513_g1
Neurotrophic factors' receptors		
High affinity nerve growth factor receptor (TrkA)	*Ntrk1*	Rn00572130_m1
GDNF family receptor alpha 3 (GFRα3)	*Gfra3*	Rn01760829_m1
Neuropeptides and Cation channels		
Calcitonin gene-related peptide (CGRP)	*Calca*	Rn01511354_m1
Transient receptor potential vanilloid channel-1 (TRPV1)	*Trpv1*	Rn00583117_m1
Reference gene		
B-Actin	*Actb*	Rn00667869_m1

Statistical analysis

All data are expressed as mean ± SEM. Comparisons between multiple groups were performed by a one-way or a two-way ANOVA, as appropriate, followed when necessary by a Fisher's least significant difference (LSD) post hoc test to detect differences between experimental groups. P values < 0.05 were considered statistically significant.

Results

Histopathology and colonic expression of inflammatory markers

Compared with non-infected controls, colonic microscopic histological score was significantly increased at 14 days PI, due to the presence of an inflammatory infiltrate and an increased goblet cell density (Table 2; Fig. 1 A-B). By day 30 PI, no signs of histological alterations were observed (Table 2; Fig. 1 C). Thickness of the mucosa, submucosa or muscularis externa was not altered by the infection, regardless the day considered (Table 2). RT-qPCR analysis revealed a rapid up-regulation of colonic IL-13 expression after *T. spiralis* infection (by 9-fold increase over control values at day 2 PI; P<0.05; Fig. 2). IL-13 up-regulation persisted up to day 14 PI (by 6-fold increase over control values; P<0.05), declining towards the expression observed in non-infected animals by day 30 PI (Fig. 2). Although iNOS mRNA levels showed at day 2 and 6 PI, respectively, a 3 and 4 -fold increase over baseline, no statistical significance was achieved (Fig. 2).

Characterization of colonic mast cell populations

T. spiralis infection was associated to a hyperplasia of MMCs (identified as RMCPII-positive cells) in the mucosa of the colon (Fig. 3A-C). MMC counts started to increase on day 10 PI, reaching a peak value on day 14 PI (by 6.5-fold increase over control values, P<0.05) and persisted at similar levels up to day 30 PI (Fig. 3D). Similarly, gene expression for RMCPII was up-regulated in *T. spiralis*-infected animals, with a peak of expression on day 14 PI (by 32-fold increase over control values, P<0.05; Fig. 2). At day 30 PI, RMCPII expression levels were still increased (by 10-fold) over control values,

although statistical significance was not reached. RMCPII immunofluorescence intensity, as assessed by confocal microscopy, was similar in control and *T. spiralis*-infected animals, regardless the time PI considered (Fig. 4).

Table 2. Histological changes in the colon after *T. spiralis* infection

			T. spiralis-infected	
		Control	Day 14 PI	Day 30 PI
Histological score	Epithelial structure (0-3)	0	0	0
	Inflammatory infiltrate (0-3)	0.25±0.07	1.02±0.15*	0.35±0.16
	Goblet cell density (0-3)	0.36±0.12	0.88±0.07*	0.30±0.10
	Total (0-9)	0.6±0.12	1.91±0.19*	0.65±0.23
Thickness (μm)	Mucosa	280.2±10.1	280.2±12.8	262.4±0.5
	Submucosa	58.5±6.0	49.6±7.0	68.7±6.1
	Muscularis externa	259.9±25.8	253.0±20.6	262.6±24.7

Data are mean±SEM of 6-8 animals per group. *: P<0.05 vs. respective control group.

In non-infected animals, CTMCs (identified as RMCPVI-positive cells) were rarely found and, when present, were located mainly in the submucosa of the colon. *T. spiralis* infection did not affect the density or the pattern of distribution of CTMCs (control: 8.6±1.7 cells/tissue section; day 30 PI: 10.4±2.8 cells/tissue section)

Colonic and jejunal expression of neurotrophic factors

Expression of NGF, GDNF, artemin and neurturin was detected, at similar levels, in colonic and jejunal samples from non-infected controls. Relative expression levels were similar in both areas (colon: artemin~GDNF>NGF>neurturin; jejunum: artemin~GDNF>neurturin>NGF). In *T. spiralis*-infected animals, specific site- (jejunum vs. colon) and time-related changes in expression were observed depending on the NTF considered.

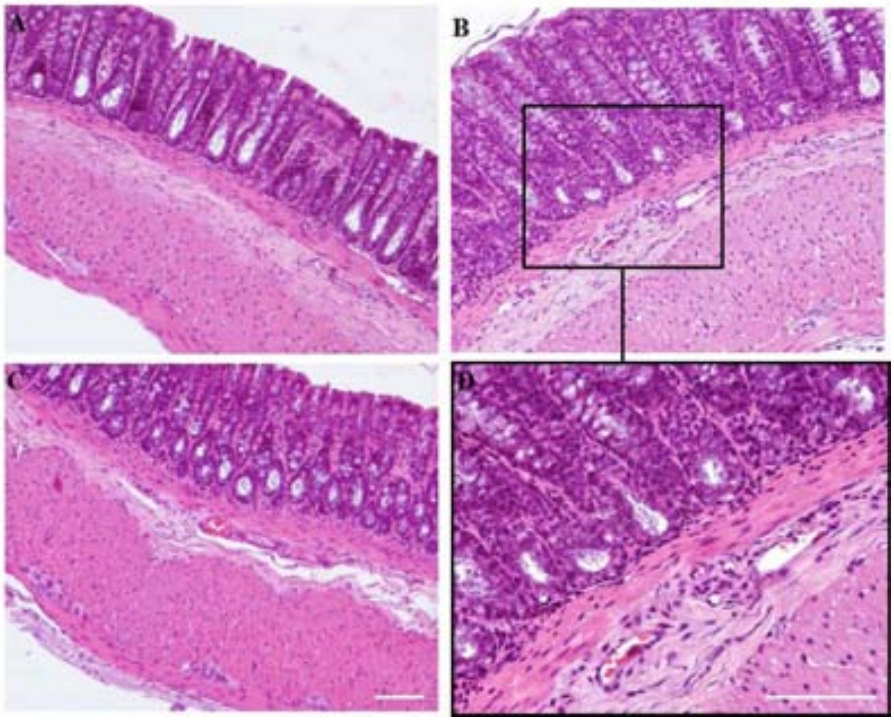

Figure 1. Colonic histology. Representative microphotographs showing haematoxylin and eosin-stained colonic slices from a control (A) and previously infected rats at days 14 (B) and 30 PI (C). Notice the presence of an inflammatory infiltrate without evidence of epithelium damage at 14 days PI. Scale bar: 100 µm. D: Magnification of the area indicated in B. Scale bar: 100 µm.

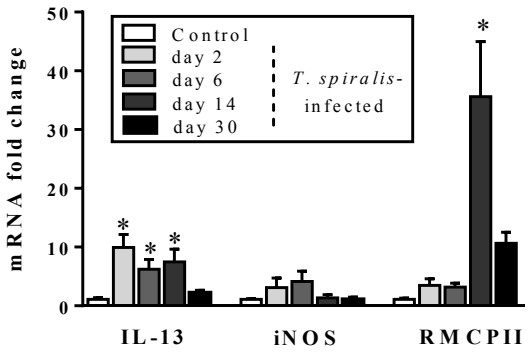

Figure 2. Effects of *T. spiralis* infection on colonic expression of inflammation-related markers. Relative expression of IL-13, iNOS and RMCPII mRNA in the colon of non-infected controls and previously infected rats at days 2, 6, 14 and 30 PI. Data are mean±SEM of 4-5 animals per group. *: P<0.05 vs. respective control group.

In the colon of infected animals, NGF showed a time-related down-regulation from days 2 to 14 PI, with minimum expression levels reached at day 14 PI (by 50% reduction, $p<0.05$ vs. control) and a recovery to normal levels by day 30 PI (Fig. 5). Other NTFs showed only a transient reduction in expression by day 14 PI (25% - 40% reduction) with the exception of artemin, which showed a biphasic response with an up-regulation (by 30%) at day 30 PI (Fig. 5).

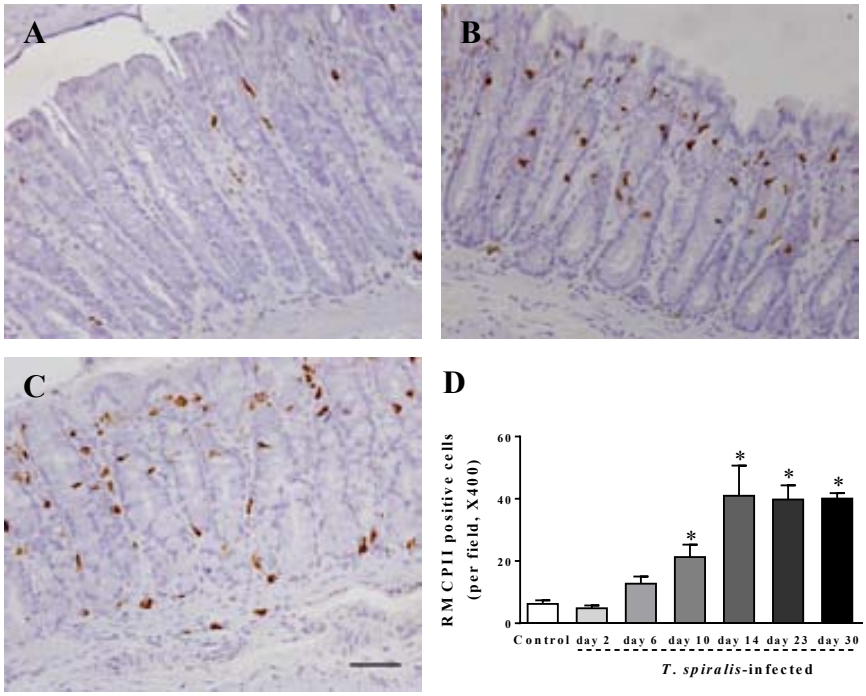

Figure 3. Immunohistochemistry for colonic mucosal mast cells. A-C: Representative microphotographs showing RMCPII immunopositive cells (corresponding to MMCs) in the colonic mucosa of a control (A) and previously infected rats at days 14 (B) and 30 PI (C). Scale bar: 50 µm. D: Quantification of colonic MMCs (number per field, X400). Data are mean±SEM of 4-6 animals per group. *: $P<0.05$ vs. control group.

In the jejunum, changes in NTFs expression in response to *T. spiralis* infection were more variable, depending upon the NTF considered. NGF showed a sustained increase from day 2 to 30 PI, although no statistical significance was achieved, probably because of the relatively large variability observed (Fig. 5). Artemin and GDNF showed a similar pattern, with an early, transitory, up-regulation (75%-100% increase) by day 2 PI followed by a normalization in expression (Fig. 5). On the other hand, neurturin showed

a progressive down-regulation between days 6 and 14 PI and a recovery by day 30 PI (Fig. 5).

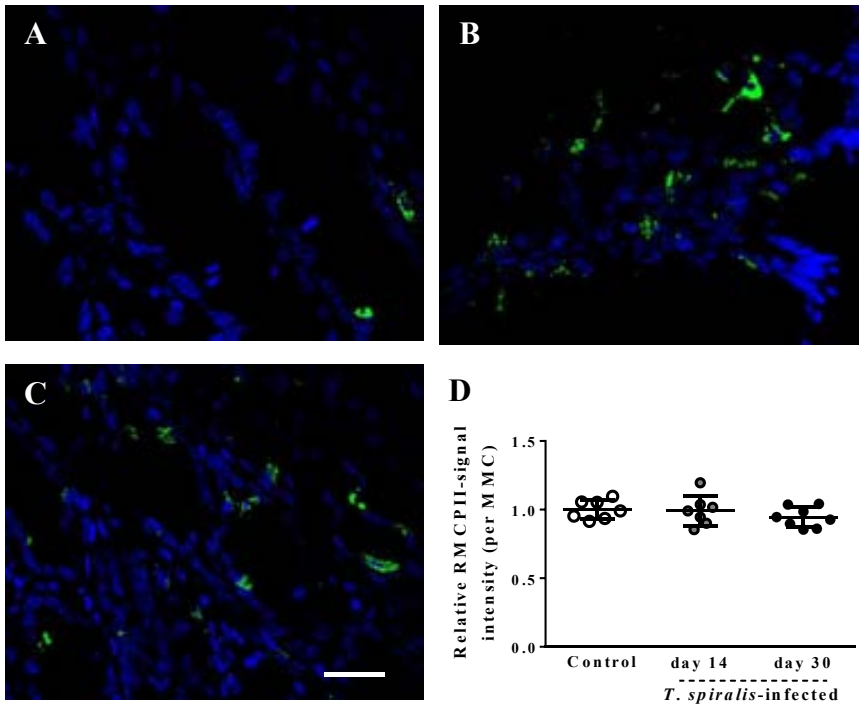

Figure 4. Immunofluorescence for colonic mucosal mast cells. A-C: Representative confocal microphotographs showing RMCPII immunopositive cells (corresponding to MMCs, green-stained cells) in the colonic mucosa of a control (A) and previously infected rats at days 14 (B) and 30 PI (C). Scale bar: 50 µm. D: Quantification of RMCPII immunofluorescence intensity (per cell) expressed as the relative change compared to the mean immunofluorescence intensity in non-infected animals (taken as 1.0) (see methods for details of the quantification procedure). Each point represents an individual animal, the horizontal line with error represents the mean±SEM.

Effects of intracolonic capsaicin on colonic neurotrophic factors

In non-infected controls, intracolonic capsaicin induced a specific up-regulation of artemin, without changes in the expression of the other NTFs assessed (Fig. 6). In *T. spiralis*-infected animals, a similar significant increase in the expression levels of artemin was observed at day 14 PI; however, this effect was totally absent at day 30 PI

(Fig. 6). Expression of other NTFs in *T. spiralis*-infected animals was not affected by intracolonic capsaicin, regardless the PI time considered.

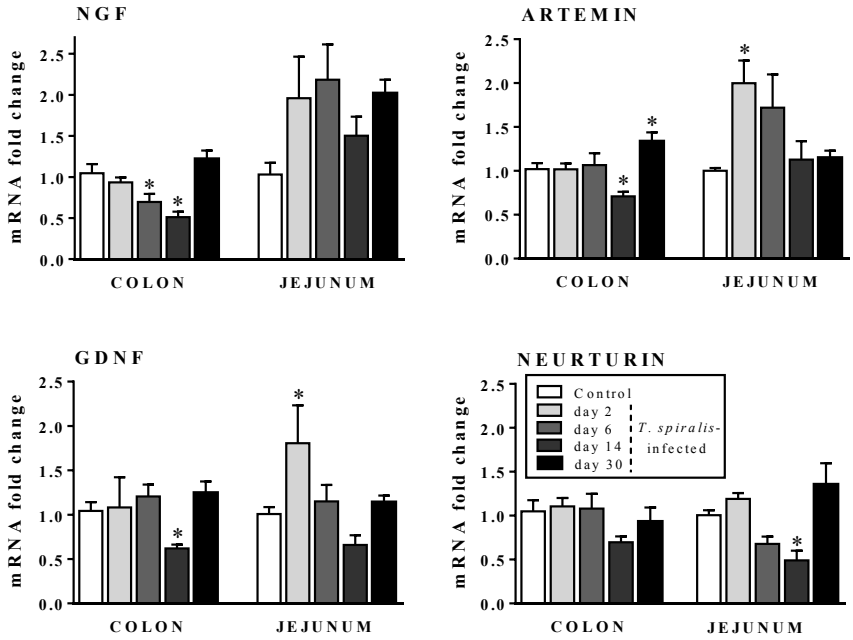

Figure 5. Effects of *T. spiralis* infection on colonic and jejunal expression of neurotrophic factors. Relative expression of NGF, artemin, GDNF and neurturin mRNA in the colon of control and previously infected rats at days 2, 6, 14 and 30 PI. Data are mean±SEM of 4-10 animals per group. *: P <0.05 vs. respective control group.

Expression of neurotrophic factors' receptors and sensory-related markers in TL and LS DRGs: effects of intracolonic capsaicin

mRNA for TRPV1, CGRP, PAR-2, TrkA and GFRα3 was detectable in all samples assessed. Expression levels of these genes in control conditions were similar in LS and TL DRGs, being the expression levels of TRPV1, TrkA and GFRα3 relatively high compared to those of PAR-2 and CGRP.

Overall, *T. spiralis* infection did not affect the expression of the different genes assessed, neither in TL nor LS ganglia; with the exception of TPRV1 in TL DRGs that showed a

down-regulation that reached statistical significance at day 14 PI (30 % decrease over control values; P<0.05; Fig.7).

Intracolonic capsaicin down-regulated TRPV1 expression in both TL and LS DRGs. Relative down-regulation of TRPV1 expression in TL DRGs was similar across groups (30% - 40% reduction; Fig. 7). However, responses in LS DRGs were enhanced in *T. spiralis*-infected animals, particularly at 30 days PI (change in expression: control, -16±9%; day 30 PI, -52±4.15%; P<0.05; Fig.7). Regardless of the experimental group considered, there was a negative correlation between the relative capsaicin-induced down-regulation of TRPV1 in LS DRGs and the changes in colonic expression of artemin (R^2=0.37; P=0.04; Fig. 7).

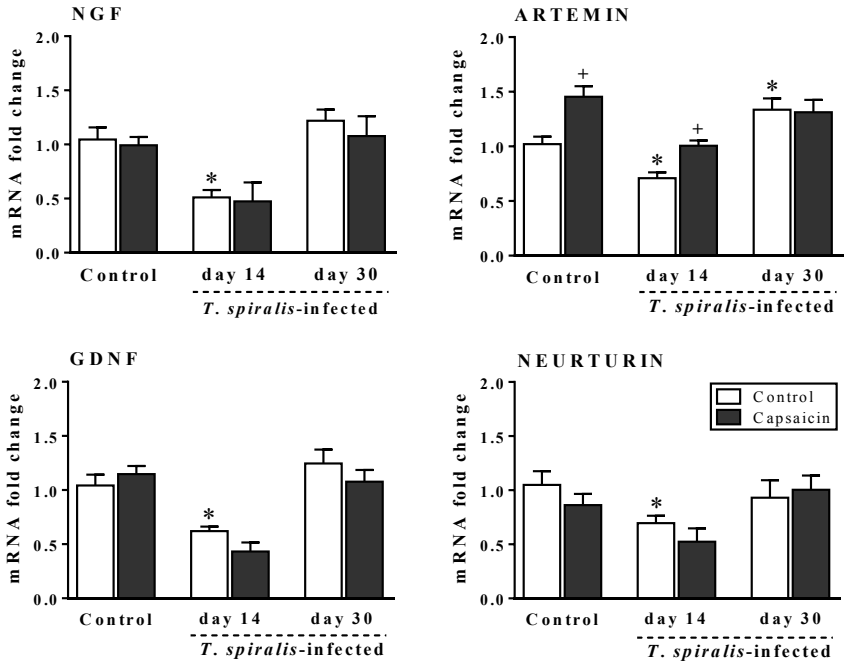

Figure 6. Effects of intracolonic capsaicin on neurotrophic factors expression in the colon. Relative expression of NGF, artemin, GDNF and neurturin mRNA in the colon from control and previously infected rats at days 14 and 30 PI. Data are mean±SEM of 3-10 animals per group. *: P<0.05 vs. control-control group +: P<0.05 vs. respective control group.

Regardless the experimental group considered, intracolonic capsaicin had a clear tendency to down-regulate the expression of TrkA and GFRα3. These effects were

particularly evident in TL DRGs at day 30 PI (Fig. 7). Similar effects were observed for PAR-2, which was down-regulated by capsaicin particularly in TL DRGs in *T. spiralis*-infected animals (Fig. 7). Expression of CGRP was similar across groups, independently of the region considered, and was not affected by intracolonic capsaicin (Fig. 7).

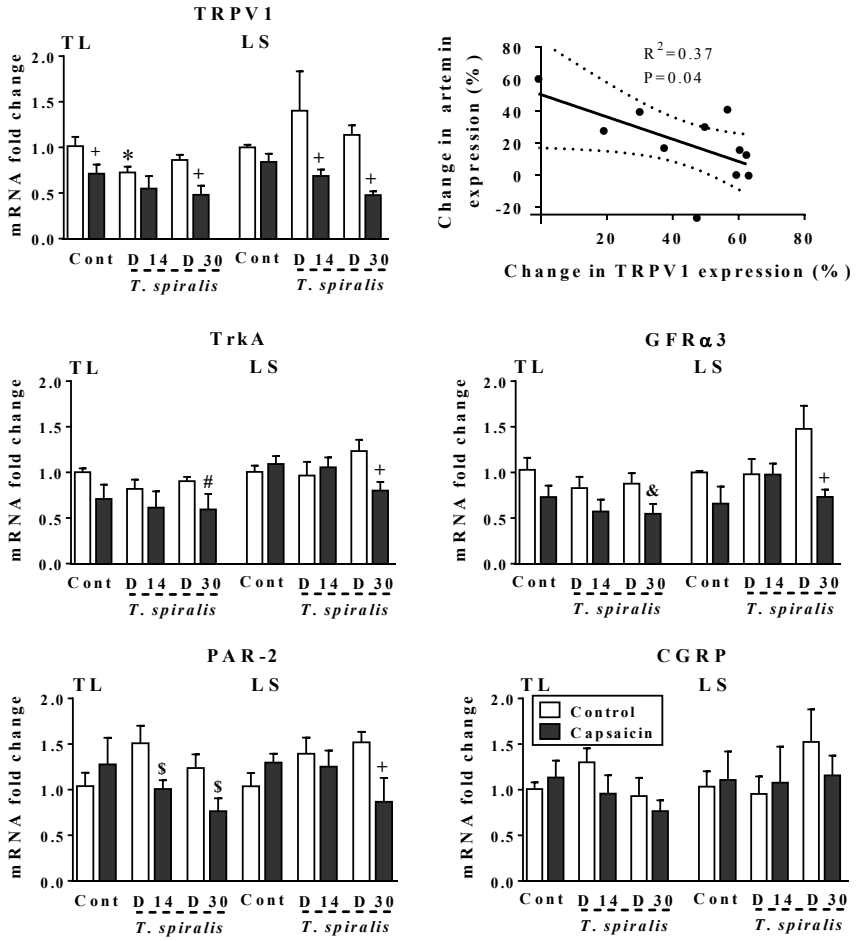

Figure 7 . Effe cts of *T. spiralis* infection a nd in tracolonic capsaicin on neurotrophic fa ctors' receptors and sensory-related markers in TL and LS DRGs. Relative expression of TRPV1, TrkA, GFRα3, PAR-2 and CGRP in TL and LS DRGs from control and previously infected rats (*T. spiralis*) at days 14 (D 14) and 30 (D 30) PI. Data are mean±SEM of 3-5 animals per group. *: P<0.05 vs. control-control group +, #, & and $: P<0.05, P=0.07, P=0.06 and P=0.09 vs. respective control group. The rig ht panel in th e u pper ro w shows the correlation b etween c hanges in c olonic expression of artemin and TRPV1 expression in LS DRGs after intracolonic capsaicin. Each p oint represents a n i ndividual animal; th e br oken l ines r epresent the 9 5% confidence interval.

Discussion

The present study evidences a remodeling of the colonic afferent innervation in a model of post-infectious gut dysfunction in rats. Results obtained show the presence of an inflammatory reaction within the colon characterized by a persistent MMC infiltrate, although this organ was not directly affected by *T. spiralis* larvae. In parallel, long-lasting changes in colonic sensory afferents properties were revealed by intracolonic capsaicin administration, suggesting that both inflammation and neuronal remodeling can be spread widely after localized injury of the intestine.

The infection with *T. spiralis* in rodents is an accepted model of post-infectious gut dysfunction, which reproduces some of the alterations observed in patients with PI-IBS (13,14). Most of the studies using this model focus on the jejunum, the main area affected by the larvae invasion, which shows structural and functional alterations directly related to the inflammatory response induced by the presence of the parasite (18). However, enteric dysfunction has also been reported in worm-free regions of the gastrointestinal tract (19). Here, we focused on the rat colon, observing the development of an inflammatory response resembling that previously described by us in the jejunum (14). The main characteristic of the jejunal response was the presence of a long-lasting mast cell infiltrate, with increases in the counts of both MMCs and CTMCs (13,14). In the colon, a similar long-lasting infiltration of MMCs was observed, but CTMC density was unaffected. Moreover, presence and migration of the parasite resulted in significant alterations of the epithelium and muscle layers of the jejunum, while no structural alterations were observed in the colon, consistent with the absence of larvae. However, despite this larvae absence, the colon developed a Th2-like cytokine pattern (up-regulation of IL-13), which has been related to the expulsion of the parasites (18). Overall, these observations suggest the existence of an extended inflammatory-like response to the infection, with some region-specific features likely associated to the presence and migration of the parasite. The affection of the colon, as demonstrated here, further increases the validity of *T. spiralis* infection in rats as a model of IBS, since the colon is regarded as the main affected organ in the disease.

Motor and sensorial alterations observed in the inflamed gut have been associated, at least partially, to an up-regulation in NTFs expression (10,20,21). Data obtained from animal models and intestinal biopsies of patients with intestinal inflammatory disorders

confirm an enhanced expression of NGF and GDNF family members during active enteric inflammation (20,22,23). In agreement with these data, here we show changes in the pattern of expression of NGF, GDNF, artemin and neurturin along the course of *T. spiralis* infection in rats. Within the jejunum, the early up-regulation of NGF, GDNF and artemin might be associated to the intestinal response to the presence of the larvae in the lumen and the mucosa. Indeed, early increases in NGF have been related to the hypermotility responses associated to the expulsion of the parasite (20). However, the most striking changes in NTFs expression during *T. spiralis* infection were observed in the colon. Conversely to that observed in the jejunum, in the colon, NTFs expression was reduced between days 6-14 PI. The reason for this down-regulation is not clear, since an inflammatory-like response was also observed in the colon. Indeed, during states of chemically-induced colitis, colonic contents of NTFs increase, following a pattern similar to the observed here for the jejunum (24). Thus, it is feasible to speculate that these differences might be related to the degree of inflammation observed. During *T. spiralis* infection, the primary site of inflammation is the jejunum, with the colon showing, comparatively, an attenuated inflammatory-like response. Therefore, inflammation-dependent modulation of NTFs might depend upon the intensity of the inflammatory response elicited. Since NTFs are active in flammatory components, mediating for instance neural sensitization, a local down-regulation might help to constrain the effects and extend of inflammation, particularly in areas not directly affected by the inflammatory insult.

Inflammatory states of the colon have been associated to the development of visceral hypersensitivity, subsequent to the sensitization of colonic afferents (1,2,25). In particular, observations in animal models reveal that, during inflammation, colonic afferents show altered electrophysiological properties together with changes in the expression profile of sensory-related markers, including NTFs' receptors (1,2,8,24,26). Here, effects of *T. spiralis* infection on DRGs gene expression were restricted to a transient TPRV1 down-regulation. Interestingly, this effect was observed in TL but not LS DRGs. Taking into account the distribution of the afferent innervations along the spinal ganglia, this suggests that, rather than related to the colon, the effect resulted from the *T. spiralis*–induced inflammation of the small intestine, which originates mainly in the TL region (16). Both up- and down-regulation of TRPV1 expression in DRGs have been described during intestinal inflammation, depending upon the experimental model

considered (24,27,28). Overall, levels of TRPV1 seem to reflect a balance between expression of the transcript and its transport from the soma of DRG neurons to central and peripheral axonal terminals (29). Therefore, a reduced content on transcript in DRGs might be indicative of an increased expression of the channel at the periphery and/or at spinal cord with subsequent changes in afferent sensitivity, thus contributing to the development of afferent sensitization.

TRPV1 channels have been related to the development and maintenance of colonic inflammatory hypersensitivity (30,31). Here, modulation of TRPV1 mRNA levels in DRGs was determined as a way to assess potential changes in colonic afferent excitability. Stimulation of afferents by intracolonic capsaicin caused down-regulation of TRPV1 mRNA levels in TL and LS DRGs, similarly to that observed in previous studies (32,33). Interestingly, capsaicin effects were enhanced in *T. spiralis*-infected animals, particularly in LS DRGs (main origin of colonic afferents), up to day 30 PI, thereby suggesting a long-lasting sensitization of colonic afferents by the infection. Many mediators released during inflammatory processes have been described to participate in the sensitization of TRPV1 channels, including MMC-derived proteases and growth factors (24,34). In this line, we had previously described that, during *T. spiralis* infection in rats, there is an increase in local (intestinal) and systemic (serum) levels of MMC-derived proteases (13). These observations, together with the results obtained here, showing a persistent MMC infiltrate and an up-regulation of RMCPII transcripts in the colon, support the view that MMC-derived proteases might participate in the sensitization of visceral afferents. However, in the present conditions, we were unable to demonstrate an activated state of MMCs, at least as it relates to changes in RMCPII content per cell.

The early down-regulation of NTFs observed in the colon of *T. spiralis*-infected animals might reflect a compensatory mechanism to the development of inflammation, thereby constraining afferent hyperexcitability. Similarly, late (by day 30 PI) increase in artemin might contribute to the long-lasting maintenance of afferent hypersensitivity, as suggested by observations from GFRα3 knockout mice (8). A role for artemin modulating afferent functionality is further supported by the correlation observed between the changes in artemin and TRPV1 expression under afferent stimulation, suggesting that TRPV1 expression in LS DRGs might act restraining artemin up-

regulation in the colon and, therefore, the excitability of afferents. Finally, we have observed that cultured rat MMCs express GFRα3 (data not shown), suggesting that the interaction artemin-MMCs might be a potential mechanism contributing to visceral afferent sensitization.

In summary, we show that changes consistent with an inflammatory-like response are present in the colon of *T. spiralis*-infected rats. In these conditions, there are long-lasting changes in afferent sensitivity, as suggested by the responses to intracolonic capsaicin. The neuroimmune bases underlying this remodeling of colonic afferents might be related to changes in NTFs levels and MMC mediators, derived from a persistent MMC infiltrate. Overall, these observations suggest that, during intestinal inflammation, a spread of morphological and functional alterations, including remodeling of visceral afferents leading to altered sensitivity, can be observed outside the primary site of action of the insult. Similar mechanisms might be operating in states of widespread alterations of visceral sensitivity.

Acknowledgments

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Disclosures

F. J. designed and performed experiments, analyzed data and wrote the paper. J.A. F-B designed and performed experiments. V. M. designed and performed experiments, and wrote the paper. P. V. designed experiments and wrote the paper.

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DISCUSSION

IBS, the main functional gastrointestinal disorder, affects primarily the colon and is characterized by recurrent abdominal pain/discomfort associated with changes in bowel habits and dysmotility (27). Although the pathogenesis behind this disorder is not clearly understood, symptoms generation has been related to certain luminal-derived stimuli, including gut commensal microbiota (GCM), intestinal infections and food antigens (31,37,40,41).

Several authors suggest that the presence of a continuous immune activation, leading to a state of persistent low grade inflammation, has to be considered as a characteristic feature of IBS (108). Accordingly, evidences suggest an activation of intestinal MCs as the common pathophysiological pathway through which luminal factors contribute to the initiation/perpetuation of IBS symptomatology (109,110). For instance, colonic biopsies of patients with IBS, either related to dietary components or to intestinal infections, show an excited-activated state of enteric MCs (36,109,110). Moreover, studies in animal models of the disease indicate that MCs are key effector cells mediating the changes in visceral sensitivity and the intestinal dysmotility that characterize IBS (55,111,112).

In addition, locally released NTFs are suggested to participate in the neuroimmune circuits that characterize the pathophysiology of the disorder. Indeed, the ability of NTFs, mainly NGF, to degranulate MCs has been widely described (113-116). Similarly, NGF is able to produce long-lasting phenotypical changes in afferent sensory neurons that might account for the sensory dysregulation observed in IBS patients (117). Evidences obtained from colonic biopsies of patients with IBS reveal an up-regulation in the intestinal expression of neurotrophins, namely NGF and BDNF; with BDNF showing a positive correlation with the symptomatology observed (103,106). Although these evidences, the intestinal sources of NTFs and its cause-effects relationship with IBS remain unknown. Thus, here, we hypothesized that NTFs, in conjunction with MCs, might be collaborating in the onset of IBS-like changes in colonic motility and sensitivity.

In the present work, we aimed to characterize the ability of luminal-derived stimuli (GCM, intestinal infections and food antigens) to trigger IBS-like colonic functional alterations and the potential implication of NTFs. For these purposes, two validated

animal models of MMC-dependent intestinal dysfunction in rats were used: chronic oral exposure to the food antigen OVA and experimental infection with *T. spiralis*, together with a new protocol of OVA exposure in rats that included the coadministration of the dietary antigen and bacterial components (LPS).

Mucosal mast cells of the colon are activated during the exposure to a food antigen or the infection with *T. spiralis*

Our results demonstrate that both oral exposure to the food antigen OVA and intestinal infection with *T. spiralis* stimulate MCs of the rat colon (Fig. 4). These effects are reminiscent of the excited-activated state of MCs observed in colonic biopsies of patients with dietary-related and PI-IBS (36,109,110). However, differences in MC responses can be observed depending upon the luminal stimuli considered. *T. spiralis* infection was characterized by an abundant, long-lasting, MMC infiltrate, while chronic exposure to oral OVA resulted in a state of excitement/activation of MMCs without changes in cell density. These responses might be related to the changes that the original stimuli (parasite vs. food antigen) induce in the tissue microenvironment (64). During *T. spiralis* infection, there is a strong immune response, mainly Th2-like, which drives a large recruitment of MMCs to the intestinal mucosa (118). On the other hand, chronic exposure to oral OVA is not associated to an overt immune response (55), leading to a limited recruitment of MMCs. Nevertheless, in both cases, MMC activation is observed, indicating that these cells are important in the functional changes observed in these models. Thus, depending on the degree of inflammation associated to the luminal-derived stimuli, the MMC response observed (activation vs. recruitment and activation) might differ. This phenomenon would explain, at least in part, the contradictory results in MMC counts reported for IBS patients (72,103,119), and suggests that the initial trigger of the disease might determine the relative MMC participation in its development.

Interestingly, during a short-term exposure to oral OVA (1-week), MC dynamics were unaffected. However, combination of OVA with a dysbiotic-like state for the same time period resulted in increased counts of CTMCs in the submucosa of the colon. Although the potential implication of CTMCs in IBS has received little attention, these observations, together with previous data (120), suggest that CTMCs could act also as

effector cells in IBS, contributing to the functional alterations that characterize the disease.

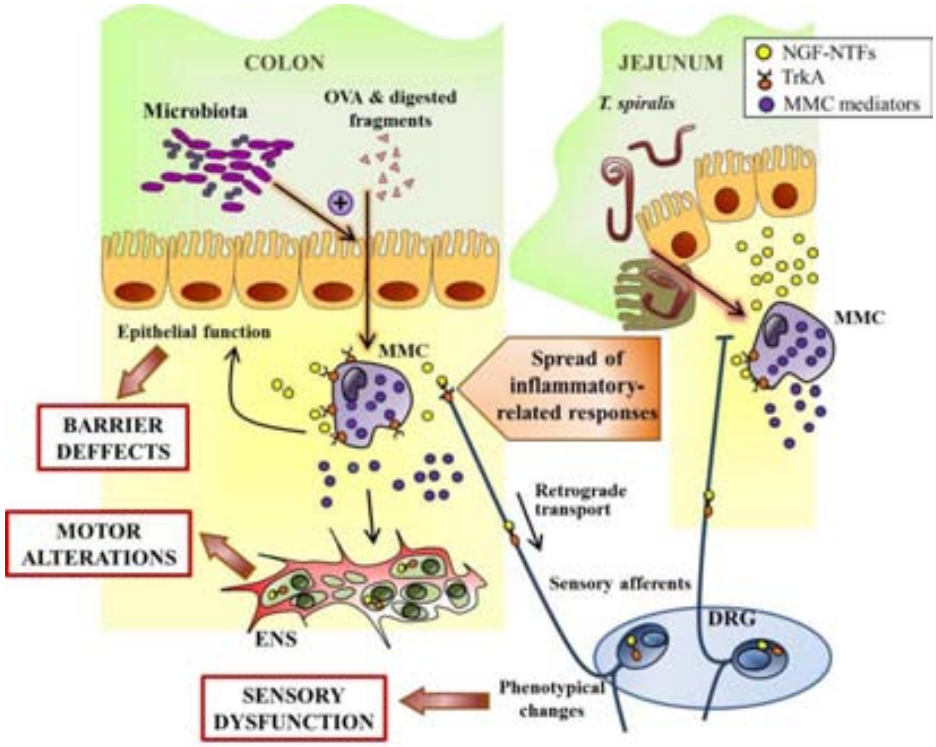

Figure 4. Schematic representation of the putative luminal stimuli-MMCs-NGF-dependent mechanisms implicated in OVA- and *T. spiralis*-induced colonic dysfunctions in rats. In the colon, OVA and/or OVA digested fragments and the extended inflammatory-like response derived from *T. spiralis* infection activate a neuroimmune circuitry that includes, at least, MMCs and peripheral NTFs. Local actions of MMC-derived mediators and NTFs are likely to alter the colonic motility and epithelial barrier function (not addressed in the present work). NGF, of a yet to be determined source, might bind to TrkA receptors located on MCs, maintaining an excited-activated state, and also to TrkA receptors located in enteric sensory afferents. Retrograde transport of NGF-TrkA complexes to DRG neurons is likely to induce long-term neuronal phenotypical changes that contribute to the sensory dysfunction that characterizes IBS. Luminal microbiota-derived factors (bacterial LPS) facilitate OVA effects.

Exposure to OVA-derived food antigens induces IBS-like motor alterations

Digestive motility disturbances are a common finding in alimentary allergies (121,122). Here, long-term (6-week) exposure to oral OVA was associated to a contractile dysfunction of the colon, both *in vivo* and *in vitro*. These motor changes resemble the alterations described in IBS patients and animal models of the disease, thereby

supporting a role for dietary antigens in the development of IBS-like changes in colonic motility (55,123-126). Moreover, as mentioned above, oral OVA induced an excited-activated state of colonic MMCs. Since MC degranulation has been related to the onset of colonic motor alterations (127), it is feasible to speculate that, during OVA exposure, excited-activated MMCs might be part of the underlying mechanisms mediating the observed motor changes (Fig. 4). In our conditions, the ability of OVA to develop colonic dysmotility was affected by the duration of the exposure period and the state of the colonic commensal microbiota. In contrast to that observed after a 6-week OVA exposure, colonic contractility was unaffected after a short-term (1-week) exposure. Therefore, these results suggest that the sensitization to oral allergens induced by few administrations might require the presence of facilitatory elements, such as adjuvants (128). Furthermore, in animals in which a dysbiotic-like state was generated (LPS co-administration), short-term administration of OVA was effective in inducing altered contractile responses to the antigen. Thus, overall, these observations support the hypothesis that adverse food reactions are implicated in the pathogenesis of IBS and suggest that concomitant alterations of the microbiota, commonly observed during IBS (41), might facilitate abnormal responses towards dietary antigens (Fig. 4).

NGF-TrkA-dependent mechanisms modulate OVA-induced colonic dysmotility

NTFs, mainly neurotrophins, have been suggested to play a role in the development of IBS-like motor alterations (58,129). Similarly, we show that the colonic dysmotility associated to long-term exposure to a dietary antigen is mediated, at least partially, by NGF-TrkA-dependent pathways. Indeed, treatment with K252a, an antagonist of TrkA (45,130-133), prevented part of the *in vivo* and *in vitro* colonic motor alterations associated to OVA exposure. These results agree with a previous study in which NGF *in vivo* immunoneutralization was able to block the motor alterations associated to *T. spiralis* infection in rats (58). Overall, these observations support a role for NGF-TrkA-dependent mechanisms on the onset/maintenance of IBS-like gastrointestinal motor alterations. Since enteric neurons express TrkA, as previously described and also confirmed by us (134, and data not shown), we can hypothesize that K252a binding to TrkA receptors on myenteric neurons prevents NGF-mediated effects within the ENS and, thus, modulates motor activity. In addition, K252a could affect the ability of NGF to interact with TrkA receptors expressed on MMCs, thus modulating their

excited/activated sate and, therefore, the release of MMC mediators (Fig. 4). However, despite the implication of NGF in the motor alterations observed, long-term OVA exposure was not associated to changes in NGF tissue expression. According to that reported, inflammation-dependent changes in NGF expression seem to be short-lasting and take place in early phases of the inflammatory course (104). In agreement, in the *T. spiralis* infection model in rats, we observed an early peak in jejunal NGF expression at day 2 PI. Since transitory increases in NGF availability have been associated to long-lasting phenotypical changes in the target tissue (43), the persistent colonic dysmotility observed after the 6-week exposure period to OVA could be related to changes in NGF content at early times, not assessed in the present studies. We cannot exclude the possibility that effects on the ENS associated to OVA-induced MMC mediators release are mediated through pathways involving NGF/TrkA-dependent mechanisms, although NGF is not directly up-regulated by OVA.

Infection with *T. spiralis* induces long-term changes in the morphological and functional properties of enteric afferents

Remodeling of visceral afferents has been suggested to be implicated in IBS-like visceral hypersensitivity states (135). Moreover, in some patients, first appearance of altered gut perception has been related to the resolution of a transient intestinal infection (the so-called PI-IBS) (136). Thus, we explored if morphological and functional changes in sensory neurons are also present in the *T. spiralis* infection model in rats.

At late phases of the infection (day 30 PI), sensory neurons of T10-L2 DRGs, which contain afferent fibers innervating both the small intestine and the colon of the rat (14,15,137), showed a significant increase in their cross-sectional area. These observations indicate a wide effect of the infection within the gastrointestinal tract, covering both regions primarily affected by the parasite (jejunum) and also worm-free areas (colon) (Fig. 4). To our knowledge, this is the first evidence of remodeling of sensory afferents outside the regions of the gut primarily affected by a harmful insult. This observation agrees with some opinions considering IBS an alteration affecting the whole intestine vs. a disease affecting solely the colon (138).

In order to elucidate if these morphological changes translate into an altered afferent function, we determined changes in TRPV1 levels in TL (T12-L2) and LS (L6-S2) DRGs following intracolonic stimulation of TRPV1 receptors with capsaicin. TL and LS DRGs were selected in order to distinguish the gut area potentially affected by these changes (small intestine vs. colon) (14,15,137). Stimulation of sensory afferents with intracolonic capsaicin induced a down-regulation of TRPV1 expression in TL and LS DRGs, similarly to that observed in previous studies (139,140). Interestingly, capsaicin effects were significantly enhanced in LS DRGs of *T. spiralis*-infected rats, regardless the PI time considered. These observations agree with data obtained in murine models of PI-IBS showing a persisting hyperexcitability of enteric DRG neurons after the resolution of the infectious process (141,142). Thus, taken together, the results support the view that structural and functional remodeling of enteric afferents are a significant component of IBS, at least in those cases in which the disease is triggered by an intestinal infection (PI-IBS). These changes might serve as a basis for the altered visceral sensitivity that appears as a characteristic feature in a significant proportion of IBS patients.

Neurotrophic factors are implicated in the remodeling of the enteric sensory afferent innervation associated to post-infectious states

As mentioned above, NTFs have been related to the remodeling of visceral afferents observed in states of inflammation in different organs, including the gastrointestinal tract (105,143). In our conditions, the infection with *T. spiralis* was associated to time- (early vs. late phases of the infection) and site-specific (jejunum vs. colon) changes in NTFs content. Within the jejunum, an early (day 2 PI) increase in the levels of NGF, GDNF and artemin was observed. However, in the colon, a general down-regulation of NTFs, including neurturin, was observed between days 6-14 PI. These regional differences in expression might be related to the local degree of inflammation; since, compared to the jejunum, the colon of *T. spiralis*-infected rats showed an attenuated inflammatory response.

Exposure to NTFs, particularly NGF, has been related to long-term neuroplastic changes in sensory neurons (117). Therefore, it is feasible to hypothesize that there is a cause-effect relationship between the early peak in jejunal NGF expression and the increase in

the mean cross-sectional area of T10-L2 DRG neurons observed at day 30 PI (Fig. 4). A similar role for NGF in the development of DRG neuronal hypertrophy has been suggested during urethral obstruction in rats (144,145). In addition, TrkA immunoreactivity in DRG neurons was also reduced along the infectious process, thus suggesting a compensatory mechanism to constraint the effects of an increased level of ligand. Since NTFs are uptaken in afferents axon terminals after binding to their receptors (146), their signaling in DRG neurons can be limited by diminishing neuronal receptor expression. Local down-regulation of NTFs at peripheral sites, as observed here in the colon of infected-rats in early phases, can also contribute to constraint these effects on visceral afferents. Supporting this hypothesis, the correlation observed between the changes in artemin and TRPV1 expression under afferent stimulation suggests that TRPV1 expression in LS DRGs might act restraining artemin up-regulation in the colon and, therefore, the excitability of afferents. Thus, overall, the results shown here support the view that locally released NTFs are modulating the changes in enteric sensory neurons observed during intestinal inflammation and suggest the presence of regulatory mechanisms, both within the gut and in its afferent innervation, to limit NTFs' effects on neuronal remodeling.

Mucosal mast cells of the rat colon are not a source of NGF

Effects of MCs on the development of IBS-like symptomatology have been suggested to be mediated, at least in part, by NGF (43). Indeed, studies in colonic biopsies of patients with IBS and in animal models reveal an up-regulation in the expression levels of the peptide (43,45,103). However, the exact source(s) of NGF in the colon, including the potential role of MCs, is controversial. In order to elucidate these points, we assessed the expression of NGF or its precursor, proNGF, in the colon and, in particular, in resident MMCs. Our results confirm previous observations showing specific NGF staining in the epithelium, ENS and scarce cells, of unidentified type, within the colonic crypts (45). However, we have been unable to detect NGF or its precursor in MMCs. This is in agreement with a study in a rat colitis model describing colonic NGF as an epithelial-derived, but not MC-derived, product (94) and supports the hypothesis that MMCs do not represent a source of NGF in the rat colon. It is worthy to mention that in colonic biopsies of patients with IBD, NGF has been localized in MMCs (92). Therefore, taking into consideration that MCs show a high interspecific variability (147), it is feasible to

speculate that these variations might reflect species-related differences (human vs. rat) and that the possibility that human MMCs synthesize NGF cannot be ruled out.

Mucosal mast cells of the rat colon are a target for NGF

Both *in vivo* and *in vitro* studies have demonstrated the ability of NGF to degranulate MCs (113-116). Thereby, it is feasible to hypothesize the implication of NGF in the excited-activated state of colonic MMCs that characterize the pathophysiology of IBS (43,148, present observations) (Fig. 4). Our results demonstrate the presence of TrkA receptors on colonic MMCs of the rat, in agreement with human data (92) (Fig. 4). Presence of TrkA on MMCs could explain, at least in part, the up-regulation in receptor levels during colonic states of MMC activation, as observed in long-term exposure to oral OVA. In addition, treatment with the TrkA antagonist K252a tended to increase colonic RMCPII levels, suggesting that these receptors are functional and might mediate MMC degranulation upon stimulation with NGF. Indeed, the functionality of TrkA receptors expressed by MCs has been previously demonstrated *in vitro* (98,113,149). Thus, overall, the present results suggest that, in the rat colon, MMCs represent a target for NGF, rather than being a source of the peptide. Therefore, the characteristic excited-activated state of these cells during IBS conditions could be modulated by increased levels of NGF binding to TrkA receptors (Fig. 4).

In summary, we show that the axis luminal-derived stimuli-NTFs is implicated in the alterations in sensory and motor functions observed in MMC-dependent models of IBS in rats. Our results highlight a potentially important role for NTF-related pathways in the onset/maintenance of the symptomatology in, at least, a subset of IBS patients. Thus, NTFs represent a therapeutic target for the treatment of gastrointestinal disorders characterized by altered colonic sensitivity and dysmotility.

CONCLUSIONS

1. Luminal-derived stimuli comprising intestinal infection with *Trichinella spiralis* and exposure to the food antigen ovalbumin activate colonic mast cells. Differences in mast cell responses are observed depending upon the inductor stimulus. *T. spiralis* infection results in a long-lasting mast cell infiltrate while oral exposure to ovalbumin stimulates mucosal mast cell activity without changes in cell density.

2. Oral exposure to the food antigen ovalbumin leads to a colonic motor dysfunction. The ability of ovalbumin to alter colonic contractility depends on the time of exposure and the gut commensal microbiota homeostasis.

3. Colonic motor alterations induced by oral exposure to ovalbumin are neurotrophin-mediated, as demonstrated by the pharmacological blockade of tropomyosin-receptor-kinase receptors with K252a.

4. The neurotrophins-tropomyosin-receptor-kinase receptors pathway participates in the maintenance of colonic basal contractility in rats.

5. Experimental infection with *T. spiralis* in rats, although primarily affecting the small intestine, induces long-term morphological and functional changes in the colon, the main affected organ in irritable bowel syndrome. This reinforces the validity of *T. spiralis* infection in rats as a post-infectious-irritable bowel syndrome model.

6. During *T. spiralis*-induced intestinal inflammation, there is a morphological and functional remodeling of intestinal sensory afferents, manifested as changes in morphology, neurotrophin content in dorsal root ganglia neurons and responses to peripheral stimulation.

7. During *T. spiralis* infection, a transient up-regulation of neurotrophic factors is observed at early times in the primary site affected by the parasite (jejunum) and is followed by an extended down-regulation affecting both jejunum and colon.

8. In rats, colonic mucosal mast cells are not a source of nerve growth factor, but express tropomyosin-receptor-kinase A receptors and are, therefore, a target for nerve growth factor.

9. The luminal-derived stimuli-mucosal mast cells-neurotrophic factors axis is implicated in the colonic functional alterations observed in food antigen and infection-related models of irritable bowel syndrome in rats.

1. Los estímulos de origen luminal, en concreto la infección intestinal por *Trichinella spiralis* y la exposición al antígeno de la dieta ovoalbúmina, activan los mastocitos del colon. Las diferencias en las respuestas mastocitarias dependen del estímulo inductor. La infección por *T. spiralis* produce un infiltrado mastocitario de larga duración mientras que la exposición oral a ovoalbúmina estimula los mastocitos de mucosa sin cambiar su densidad.

2. La exposición oral al antígeno de la dieta ovoalbúmina altera la actividad motora del colon. Los cambios en la contractilidad colónica dependen del tiempo de exposición al antígeno y de la homeostasis de la microbiota comensal.

3. Las alteraciones de la motilidad del colon debidas a la exposición a ovoalbúmina están mediadas por neurotrofinas, tal y como demuestra el bloqueo farmacológico de los receptores tropomiosina-receptor-quinasa con K252a.

4. La vía neurotrofinas-receptores tropomiosina-receptor-quinasa participa en el mantenimiento de la contractilidad basal del colon en la rata.

5. La infección experimental por *T. spiralis* en la rata, aunque afecta de manera primaria al yeyuno, induce cambios morfológicos y funcionales a largo plazo en el colon, el órgano que se considera principalmente afectado en el síndrome del intestino irritable. Tal observación refuerza el valor de la infección por *T. spiralis* en la rata como modelo experimental del síndrome de intestino irritable post-infeccioso.

6. En el curso de la inflamación intestinal asociada a la infección por *T. spiralis*, se produce una remodelación morfológica y funcional de las vías aferentes sensoriales del intestino. Esta remodelación se manifiesta en forma de cambios morfológicos y en el contenido en neurotrofinas en las neuronas de los ganglios de la raíz dorsal de la médula espinal, así como en la respuesta a la estimulación periférica.

7. Durante las fases tempranas de la infección por *T. spiralis*, se observa un incremento en el contenido de factores neurotróficos en la región intestinal primariamente afectada (yeyuno), seguida de una regulación a la baja que afecta tanto al yeyuno como al colon.

8. En la rata, los mastocitos de mucosa del colon no son una fuente celular de factor de crecimiento n ervioso. S in e mbargo, expresan r eceptores tropomiosina-receptor-quinasa A y, por tanto, representan una diana para factor de crecimiento nervioso.

9. El ej e estímulos luminales-mastocitos d e mucosa-factores n eurotróficos está implicado en las alteraciones f uncionales d el colon observadas en modelos d el síndrome del intestino irritable asociados a la exposición a antígenos de la dieta y a infecciones entéricas en la rata.

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APPENDIX

PUBLICATIONS DERIVED FROM THIS WORK

PAPERS

- F. Jardí, V. Martínez, P. Vergara. NGF is involved in oral ovalbumin-induced altered colonic contractility in rats: Evidence from the blockade of TrkA receptors with K252a. *Neurogastroenterol Motil* 2012, 24, e580–e590.

- F. Jardí, M. Aguilera, P. Vergara, V. Martínez. Colonic motor alterations associated to the sensitization to a luminal antigen are enhanced during a dysbiotic-like state in rats. Submitted to *Life Sciences*.

- F. Jardí, J.A. Fernández-Blanco, V. Martínez, P. Vergara. Plasticity of dorsal root ganglion neurons in a rat model of post-infectious gut dysfunction: potential implication of nerve growth factor. Submitted to *Neuroscience Letters*.

- F. Jardí, J.A. Fernández-Blanco, V. Martínez, P. Vergara. Persistent alterations in colonic afferent innervation in a rat model of post-infectious gut dysfunction: role for changes in peripheral neurotrophic factors. Submitted to *Neuroscience*.

ABSTRACTS

- F. Jardí, J.A. Fernández-Blanco, V. Martínez, P. Vergara. Remodeling of colonic sensory afferents in a rat model of post-infectious gut dysfunction: implication of neurotrophic factors. Poster.
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- F. Jardí, J.A. Fernández, E. Cepeda, V. Martínez, P. Vergara. Are mast cells a source of neurotrophins in the inflamed gut? Mismatch between neurotrophin expression and mast cells in a model of enteritis in rats. Poster.
  EMBRN-COST International Mast Cell and Basophil Meeting 2013. August 28-30, 2013. Udine, Italy.

- F. Jardí, J.A. Fernández-Blanco, V. Martínez, P. Vergara. Mucosal mast cells maintain normal contractility of the colon in a rat model of post-infectious gut dysfunction. Poster.
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- F. Jardí, J.A. Fernández-Blanco, V. Martínez, P. Vergara. Neuronal plasticity of lumbosacral DRG in a model of post-infectious gut dysfunction in rats. Poster.
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- F. Jardí, J.A. Fernández-Blanco, V. Martínez, P. Vergara. Mucosal mast cells maintain normal contractility of the colon through NGF-dependent mechanisms in a rat model of post-infectious IBS. Poster.
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NGF is involved in oral ovalbumin-induced altered colonic contractility in rats: evidence from the blockade of TrkA receptors with K252a

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Abstract

Background *Nerve growth factor (NGF)-mucosal mast cell (MMC) interaction has been implicated in the remodeling of enteric circuitries and associated functional changes. We investigated the involvement of NGF and its receptor TrkA in the altered colonic contractile activity observed in the model of oral ovalbumin (OVA)-induced MMC hyperactivity in rats. We also studied the role of colonic MMCs as a source of NGF.* ***Methods*** *Rats received oral OVA, alone or with the TrkA antagonist K252a. Colonic co-expression of NGF/TrkA and rat mast cell protease II (RMCPII) (double immunofluorescence), RMCPII content (ELISA) and expression of NGF, Brain-derived neurotrophic factor (BDNF) and TrkA/B (QT-PCR) were assessed. Colonic contractile activity was determined in vivo and in vitro.* ***Key Results*** *TrkA, but not NGF, was localized in colonic MMCs (RMCPII-positive). Oral ovalbumin exposure increased colonic RMCPII levels but did not change the percentage of TrkA-positive MMCs. Neither OVA nor K252a, alone or combined, altered NGF, BDNF or TrkA/B expression. Spontaneous colonic activity in vivo and in vitro was altered by OVA, an effect prevented by K252a. Electrical stimulation-induced contractile responses in vivo and carbachol responses in vitro were increased by OVA in a K252a-independent manner. In OVA-treated animals, inhibition of NO synthesis with L-NNA significantly enhanced spontaneous colonic activity in vitro, a response completely prevented by K252a.* ***Conclusions & Inferences*** *These results suggest that NGF-TrkA-dependent pathways are implicated in colonic contractile alterations observed during OVA exposure in rats. NGF-TrkA system might represent a potential target for treatment of gastrointestinal disorders characterized by colonic motor alterations.*

Keywords *colonic contractility, K252a, mucosal mast cells, nerve growth factor, ovalbumin, TrkA.*

Abbreviations: BDNF, brain-derived neurotrophic factor; CCh, carbachol; EMS, Electrical Mucosal Stimulation; ENS, enteric nervous system; FGD, functional gastrointestinal disorder; HFLA, high-frequency and low-amplitude (contractions); IBS, irritable bowel syndrome; IHC, immunohistochemistry; LFHA, low-frequency and high-amplitude (contractions); L-NNA, N^G-nitro-L-arginine; MMC, mucosal mast cell; NGF, nerve growth factor; OVA, ovalbumin; RMCPII, rat mast cell protease II.

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INTRODUCTION

Colonic dysmotility is a common finding in functional gastrointestinal disorders (FGDs). For instance, irritable bowel syndrome (IBS), the main FGD, is characterized by abdominal pain-discomfort associated with dysmotility and altered bowel habits.[1] Moreover, in a large number of IBS patients, food ingestion has been associated with symptomatology exacerbation, suggesting a role for food allergy in its pathogenesis.[2] Intestinal food allergy-related mechanisms in IBS seem to involve local mucosal responses to dietary antigens rather than classical type-1 hypersensitivity reactions.[3]

We have shown that chronic exposure to oral ovalbumin (OVA) in Sprague–Dawley (SD) rats induces a non-IgE-mediated alteration of smooth muscle colonic contractility resembling that observed by others and us in IBS and animal models of the disease.[4–8]

Several observations support an involvement of mast cells in the pathophysiology of IBS.[9] Morphological and functional studies with colonic biopsies from IBS patients point towards an important role of mast cell-derived mediators and the interaction mast cells-nerve fibers on the disturbed secretomotor and sensory functions characterizing IBS.[10,11] In this line, we have demonstrated that in the rat model of chronic exposure to oral OVA, mucosal mast cells (MMCs) are implicated in the altered colonic contractile activity; thus suggesting that OVA-induced colonic motor alterations in rats are somehow MMC-dependent.[4] Therefore, exposure to oral OVA in rats reproduces some pathophysiological components of IBS; at least the MMC hyperactivity and the changes in colonic contractility, thus representing a valid model for studying IBS-related altered colonic contractile responses and their potential relationship with MMCs.

Recent data suggest that, within the gut, neurotrophins, mainly nerve growth factor (NGF), interact with MMCs generating a neuroimmune circuit likely to play a potential role in the pathophysiology of FGDs. For instance, evidences obtained in animal models of IBS have implicated NGF in the neuronal remodeling of enteric circuitries and MMCs recruitment, as basis for the functional changes observed. In particular, anti-NGF treatment completely blocked intestinal hypermotility in *Trichinella spiralis*-infected rats, an accepted model of postinfectious IBS,[12] and reduced the interaction MMCs-nerve fibers in the rat maternal separation model.[13] A recent study in colonic biopsies from IBS patients supports these observations, showing an increased neuronal sprouting within the mucosa, an effect associated with NGF increased levels, possibly of mast cell origin.[14] However, the exact origin of colonic NGF and the potential role for MMCs as the peptide source are still unclear.

Nerve growth factor interacts with two classes of cell surface receptors: the TrkA high-affinity receptor, a selective NGF receptor; and the p75 low-affinity receptor, which presumably binds to all neurotrophins.[15] To further understand the role of NGF in colonic motor alterations we have investigated the effects of the pharmacological blockade of TrkA on OVA-induced changes in colonic contractility in the rat by using K252a, which has been widely used in the rat as a TrkA antagonist.[16–20] In addition, we evaluated the expression levels of NGF and TrkA in the rat colon during exposure to oral OVA, complementing this data by determining the colonic expression of the related neurotrophin brain-derived neurotrophic factor (BDNF) and its preferential receptor TrkB. Finally, we evaluated the interplay between MMCs-NGF/TrkA system, characterizing, in particular, if MMCs represent a cellular source of the neurotrophin and/or express TrkA receptors.

MATERIALS AND METHODS

Animals

Adult (5 weeks old at arrival), specific pathogen free (SPF), SD male rats were used (Charles River, Les Oncins, France). Animals had free access to water and a standard pellet diet, free of traces of OVA or any other egg derivative (A04; Safe, Augy, France). Rats were maintained under conventional conditions in a light (12 h/12 h light–dark cycle) and temperature controlled (20–22 °C) room, in groups of two per cage. Animals were acclimatized to the new environment for 1 week before starting any experimental procedure. All the experimental protocols were approved by the Ethical Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya (protocols 1010 and 5351, respectively).

Experimental design

Rats received OVA by oral gavage (1 mg mL^{-1}, 1 mL per rat, $n = 24$), on a daily basis during a 6-week period.[21] A group of rats receiving vehicle (1 mL per rat, $n = 21$) was used as control. After the third week, 11 of the animals receiving OVA and 10 of the animals receiving vehicle were treated subcutaneously with K252a (50 $\mu g\ kg^{-1}$). Treatment with K252a was performed daily and lasted until the day before animals were euthanized, outlasting for 10 days OVA exposure. This antagonist, at the dose and pattern of administration followed here, has already been used, showing effective blockade of TrkA and biological effects *in vivo*.[18–20] The rest of the animals (13 OVA- and 11 vehicle-exposed) were used as control groups in which the treatment protocol was the same but K252a was replaced by the corresponding vehicle (1 mL kg^{-1}, s.c.). Except for the *in vivo* experiments, at the time of euthanasia, tissue samples from the colon were obtained and either used for organ bath studies, fixed in 4% paraformaldehyde for immunohistochemical studies or frozen in liquid nitrogen and stored at −80 °C until analysis.

Organ bath

Full thickness preparations, obtained from the mid portion of the colon, were cut 1 cm long and 0.3 cm wide and hung for organ bath study oriented to record circular muscle activity. Strips were mounted under 1 g tension in a 10-mL muscle bath containing carbogenated Krebs solution (95% O_2 – 5% CO_2) maintained at 37 ± 1 °C. The composition of Krebs solution was (in mmol L^{-1}): 10.10 glucose, 115.48 NaCl, 21.90 $NaHCO_3$, 4.61 KCl, 1.14 NaH_2PO_4, 2.50 $CaCl_2$, and 1.16 $MgSO_4$ (pH 7.3–7.4). One strip edge was tied to the bottom of the muscle bath using suture silk and the other one to an isometric force transducer (Harvard VF-1 Harvard Apparatus Inc., Holliston, MA, USA). Output from the transducer was fed to a PC through an amplifier. Data were digitalized (25 Hz) using Data 2001 software (Panlab, Barcelona, Spain). Strips were allowed to

equilibrate for about 1 h. After this period, contractile responses to carbachol (CCh; 0.1–10 μmol L^{-1}) and the NO inhibitor N^G-nitro-L-arginine (L-NNA; 1 mmol L^{-1}) were assessed. For CCh, cumulative concentration-response curves, with a 5-min interval between consecutive concentrations, were constructed. For L-NNA, spontaneous activity was recorded during a 10-min period after the addition of the drug.

To determine the spontaneous contractile activity, the preparation tone was measured for 15 min and the mean value (in g) determined. To test the effects of CCh, the maximum peak, from the basal tone, was measured after each concentration tested. To measure the response to L-NNA, the 10-min mean of the strip tone before the drug administration was determined and compared with the 10-min mean of the strip tone after L-NNA addition.

Strain-gauge recordings

After a fasting period of 6 h, animals were placed in an induction camera and anesthetized by inhalation of 4% isofluorane (Isoflo®; Esteve, Barcelona, Spain) in 2 L min^{-1} oxygen to allow cannulation of a lateral vein of the tail. Thereafter, rats were maintained in level III of anesthesia by intravenous thiopental sodium, as required, and exposed to mask delivery of 1 L min^{-1} oxygen during all the procedure. A laparatomy was performed, the colon localized and a strain-gauge (F-04IS; Star Medical, Tokyo, Japan) was sutured to its wall (2 cm from the cecum) to record circular muscle activity. The strain gauge was connected to a high-gain amplifier (MT8P; Lectromed, Herts, UK), and signals were sent to a recording unit (PowerLab/800; ADInstruments, Castle Hill, NSW, Australia) connected to a computer. Finally, an electrode holder with two platinum electrodes (WPI, Sarasota, FL, USA) was inserted into the colonic lumen at 1 cm distally to the strain-gauge to induce ascending excitation of the peristaltic reflex by electrical mucosal stimulation (EMS). Electrical mucosal stimulation was applied by duplicate at 30 V, 0.6 ms and 4 Hz during 30 s each time, and the polarity of the stimulating electrodes was reversed at 15 s.

To analyze *in vivo* colonic motility, contractions of the colon were classified into low-frequency and high-amplitude (LFHA) contractions and high-frequency and low-amplitude (HFLA) contractions, as previously described.[22] High-frequency and low-amplitude were identified by having a frequency within the range of 10–15 contractions min^{-1}, while LFHA were defined as contractions of an amplitude >300% of that of HFLA contractions at the same recording site.[22] High-frequency and low-amplitude and LFHA were assessed over a 15-min period and the frequency and amplitude expressed as the mean. When assessing the responses to EMS, the recording analyzed corresponded to the 30 s of stimulation and the expressed value was the mean of the duplicates. All analysis was performed using Chart 5 software for Windows (both from ADInstruments).

Immunohistochemistry (IHC)

Immunodetection of RMCPII and NGF was carried out on paraformaldehyde-fixed colonic samples using a monoclonal antibody anti-RMCPII (Moredun, Edinburgh, UK) and a polyclonal rabbit anti-NGF (ab1526; Chemicon International, Temecula, CA, USA). Antigen retrieval for NGF was achieved by processing the slides in a pressure cooker, at full pressure, for 10 min in 10 mmol L^{-1} citrate solution. The secondary antibodies included biotinylated horse antimouse IgG (BA-2000; Vector Laboratories, Burlingame, CA, USA) and biotinylated swine antirabbit Ig (E0353; Dako, Carpinteria, CA, USA). Detection was performed with avidin/peroxidase kit (Vectastain ABC kit; Vector Laboratories) and counterstaining with hematoxylin. Specificity of the staining was confirmed by omission of the primary antibody. When performing IHC for NGF, mouse submaxillary glands were used as a positive control. Slides were viewed with an Olympus BH-2 microscope (Olympus, Hamburg, Germany). For MMC quantification, at least 20 non-adjacent ×40 fields of colonic mucosa were randomly selected and the number of RMCPII-immunopositive cells counted. All procedures were carried out using coded slides to avoid bias.

Immunofluorescence staining

For analyses of co-localization of NGF, proNGF or TrkA with RMCPII, double immunofluorescence was used. After 1 h of blocking with 10% normal goat serum at room temperature, colonic sections were incubated with a mixture of anti-RMCPII and anti-NGF or anti-ProNGF (ab5583; Chemicon International) or anti-TrkA (sc-118; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight. Thereafter, sections were incubated with a secondary antibody cocktail consisting of fluorescence-conjugated Alexa Fluor 488 goat anti-mouse IgG (A11029; Molecular Probes, Eugene, OR, USA) and Cy3 goat anti-rabbit IgG (PA-43004; Amersham-Pharmacia, Buckinghamshire, UK). After washing, the slides were coverslipped with Vectashield Mounting Medium (Vector Laboratories) and examined under an Axioskop 40 fluorescence microscope (Carl Zeiss, Jena, Germany). Merging of the images was analyzed with ImageJ Software (U. S. National Institutes of Health, Bethesda, MD, USA). To assess the percentage of cells with RMCPII and TrkA co-localization, Alexa Fluor 488- and Alexa Fluor 488-Cy3-stained cells were counted randomly using a 100× objective. Specificity of the staining was confirmed by omission of the primary antibody and/or the secondary antibodies. The absence of cross-reactivity was confirmed in control single-labeled preparations.

ELISA

Protein was extracted from colonic tissue samples using lysis buffer (50 mmol L^{-1} HEPES, 0.05% Triton X-100, 0.0625 mmol L^{-1} PMSF and the Mini Complete protease inhibitor Roche) and RMCPII concentration was determined by ELISA using a commercial kit (Moredun). Total protein was determined using the Bradford assay kit (Bio-Rad, Hercules, CA, USA).

RNA extraction and quantitative real-time PCR

Total RNA was extracted from colonic samples using Ribopure RNA Isolation Kit (Applied Biosystems, Carlstad, CA, USA) and quantified by Nanodrop (Nanodrop Technologies, Rockland, DE, USA). For cDNA synthesis, 1 μg of RNA was reverse-transcribed in a 20 μL reaction volume using a high capacity cDNA reverse transcription kit (Applied Biosystems). Expression of NGF, BDNF, TrkA, and TrkB was determined by quantitative real-time PCR performed with specific Taqman probes (Applied Biosystems; NGF: Rn01533872_m1, BDNF: Rn00560868, TrkA: Rn00572130_m1, TrkB: Rn01441749_m1, B-Actin: Rn00667869_m1) mixed with Taqman Universal Master Mix II for 40 cycles (95 °C for 15 s, 60 °C for 1 min) on a 7900 real-time PCR system (Applied Biosystems). Rat submaxillary gland and neocortex were used as positive controls for the gene expression of TrkA, TrkB and NGF, and BDNF, respectively. B-Actin expression served as an endogenous control for normalizing the mRNA levels of the target gens. Expression levels were analyzed by the $2^{-\Delta\Delta CT}$ method.

Chemicals

Ovalbumin (Grade V; A5503) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in saline solution. K252a [(9S,10R,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6] benzodiazocine-10-carboxylic acid methyl ester; Tocris Bioscience, Ellisville, MO, USA] was reconstituted in 8.75% ethanol in milli-q water. CCh (Sigma-Aldrich) stock solution and further dilutions were prepared in distilled water. N^G-nitro-L-arginine (Sigma-Aldrich) was prepared directly in carbogenated Krebs solution.

Statistics

All data are expressed as mean ± SEM. Motility results are presented as raw data (g of force) or frequency of contractions (number min^{-1}; *in vivo* recordings). EC_{50} for CCh was calculated by non-linear regression to a sigmoidal equation (GraphPad Prism 4.01, San Diego, CA, USA). Comparisons between multiple groups were performed with two-factor ANOVA. When the two way ANOVA revealed significant effects of treatments, data were reanalyzed with one-way ANOVA followed, when necessary, by a Student–Newman–Keuls multiple comparison test to detect differences between experimental groups. P values < 0.05 were considered statistically significant.

RESULTS

Colonic mucosal mast cell count and RMCPII content

The number of RMCPII-positive cells in the colon showed no significant differences between experimental groups despite the treatment received (Fig. 1A). Nevertheless, a two-way ANOVA analysis revealed an effect of OVA treatment increasing RMCPII content ($P = 0.022$), although only the OVA-K252a group achieved statistical significance ($P < 0.05$ *vs* vehicle-vehicle; Fig. 1B). K252a, *per se*, showed a tendency to increase the levels of RMCPII, although statistical significance was not achieved ($P = 0.13$).

Localization of NGF by immunohistochemistry

Within the colon, immunoreactivity for NGF was detected mainly in the submucosal and myenteric plexuses (Fig. 2C,D). A diffuse staining was observed in the epithelium, both on the villi and, occasionally, in the crypts. Within the villi, there were scarce cells, of undetermined type, showing NGF-like immunoreactivity (Fig. 2A,B). No labeling was detected in the muscle layers. No differences in the staining pattern or intensity were observed between OVA- and vehicle-treated animals or associated with the treatment with K252a. Immunoreactivity was absent in sections in which the primary antibody was omitted, thus confirming the specificity of the staining. Staining was intense and well localized in positive controls from mouse submaxillary glands.

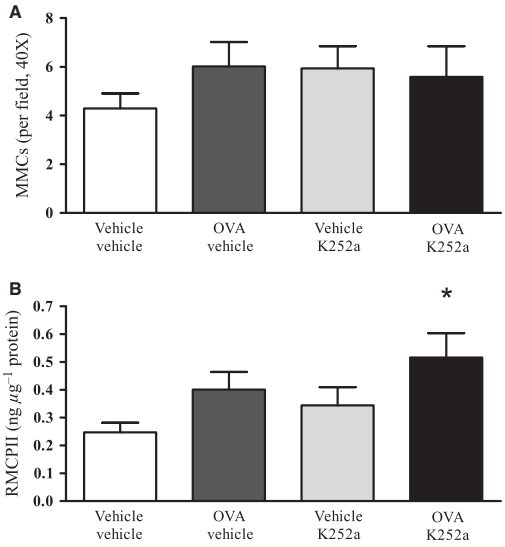

Figure 1 Colonic density of mucosal mast cells (A) and rat mast cell protease II content (B) in the different experimental groups. Data are mean ± SEM; n = 5–10 per group. *$P < 0.05$ *vs* vehicle-vehicle.

Co-localization of RMCPII and NGF, proNGF or TrkA

In vehicle-treated animals, double immunofluorescence studies demonstrated that the vast majority (63.6 ± 10.4%) of RMCPII-positive cells (identified as MMCs) co-expressed TrkA-like immunorreactivity (Figs. 2E–G). Similar ratio of RMCPII-TrkA co-localization was observed after OVA exposure (75.0 ± 5.7%). Apart from RMCPII-positive cells, other scarce cells within the lamina propria showed TrkA-labeling, indicating that not only MMCs express the receptor in the rat colonic mucosa.

No RMCPII-positive cells (MMCs) showed co-staining for NGF or ProNGF.

NGF, BDNF, TrkA, and TrkB expression in the colon

Overall, colonic expression levels of NGF, TrKA and TrkB were relatively low, with no significant differences in expression levels among the different experimental groups (Fig. 3). However, it was noticeable that K252a and OVA, *per se*, increased TrkA expression

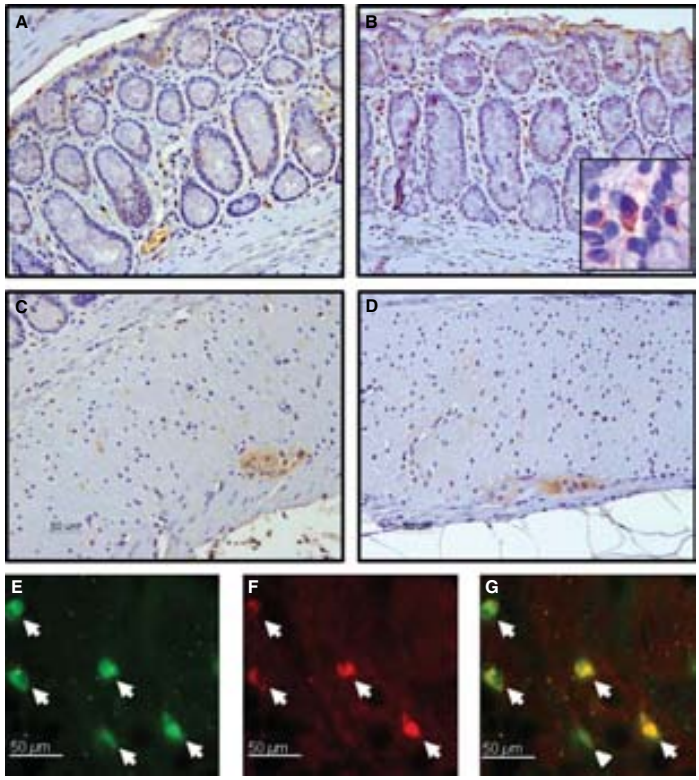

Figure 2 (A–D) Photomicrographs showing nerve growth factor (NGF)-like immunoreactivity in colonic tissues. (A, C) vehicle-vehicle-treated rat. (B, D) Oral ovalbumin (OVA)-vehicle-treated rat. Labeling for NGF was detected in the submucosal and myenteric plexuses, surface of the villi and crypts, and in scarce, unidentified cells within the villi. Insert in (B) shows a higher magnification of the NGF-immunoreactive cells observed within the colonic crypts. Note that no differences in the staining pattern or intensity are observed between OVA- and vehicle-treated animals. (E–G) Dual label immunofluorescence showing the presence of TrkA in mucosal mast cells (MMCs) of the rat colon. (E) Representative image of anti- rat mast cell protease II (RMCPII) labeling (green) of cells (identified as MMCs) in the colonic mucosa. The arrows indicate positively labeled cells. (F) Same field as in E showing labeling for TrkA (red). The arrows indicate positively labeled cells. (G) Merged image of E and F showing extensive colocalization (yellow) of RMCPII (MMCs) and TrkA immunoreactivities. The arrows indicate double labeled MMCs, whereas the arrowhead indicates a MMC negative for TrkA.

levels by 32% and 26% respectively when compared to the expression levels in the control group although these effects were not evident in OVA-K252a-treated animals (Fig. 3B).

In control tissues (rat submaxillary gland) expression levels of NGF, TrkA and TrkB were, respectively, 6-, 14- and 100-fold higher than those observed in the colon in control conditions.

Brain-derived neurotrophic factor was not detectable in the colon (ct values higher than 40), although high expression levels were found in the positive control (rat neocortex).

Colonic contractility *in vitro*

Spontaneous colonic contractile activity, as assessed *in vitro*, was similar in vehicle- and OVA-exposed animals (vehicle: 0.53 ± 0.06 g; OVA: 0.51 ± 0.03 g; $P > 0.05$; Fig. 4A). K252a decreased spontaneous activity in similar proportion in vehicle- (0.40 ± 0.05 g; $P = 0.07$ *vs* vehicle-vehicle; Fig. 4A) or OVA-exposed animals (0.35 ± 0.05 g, $P < 0.05$ *vs* OVA-vehicle; Fig. 4A).

In control conditions, CCh elicited a concentration-dependent contractile response with an estimated EC_{50} of 1.8 ± 1.3 mmol L^{-1}. Overall, a two-way ANOVA analysis revealed an OVA effect ($P = 0.031$) enhancing the contractile responses to CCh, leading to a left-shift of the concentration-response curve and a fivefold reduction in the estimated EC_{50} (0.39 ± 0.1 mmol L^{-1}; Fig. 4B). Treatment with K252a did not affect the responses to CCh, neither in vehicle- nor in OVA-exposed animals (EC_{50}; vehicle-K252a: 1.7 ± 1.0 mmol L^{-1}; OVA-K252a: 0.16 ± 0.04 mmol L^{-1}; Fig. 4B).

In colonic strips obtained from vehicle-vehicle animals, blockade of NO synthesis by the addition of L-NNA to the organ bath increased spontaneous activity over pretreatment values ($P = 0.036$; Fig. 4C). Similar effects were observed in tissues from OVA-vehicle- or vehicle-K252a-treated rats, although motor effects of L-NNA were enhanced in OVA-vehicle-treated animals (Fig. 4C). However, in animals treated with OVA plus K252a, L-NNA-induced increased spontaneous activity was not longer observed (Fig. 4C).

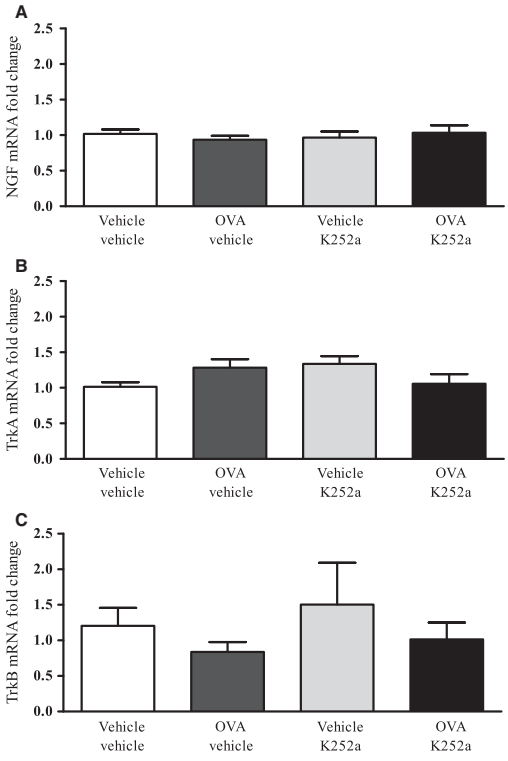

Figure 3 Real-time PCR analysis of mRNA for NGF (A), TrkA (B) and TrkB (C). Data are mean ± SEM; n = 5–9 per group.

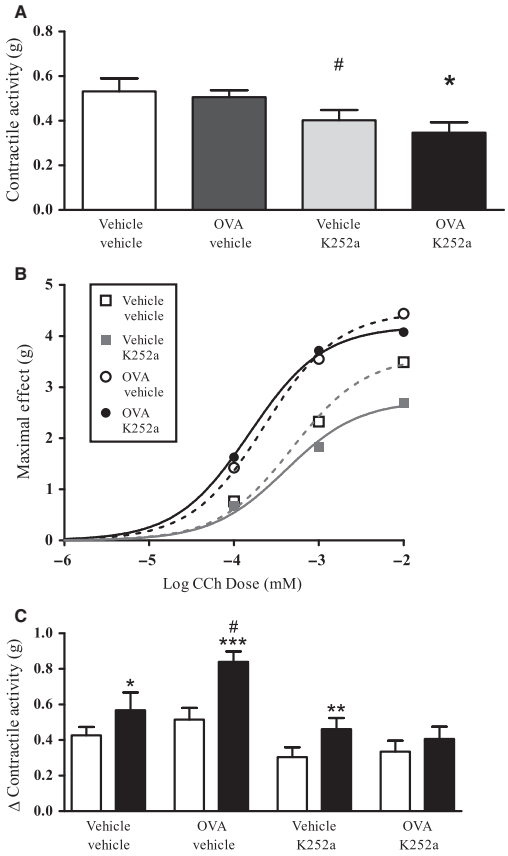

Figure 4 Effects of oral ovalbumin (OVA) and K252a on colonic contractility *in vitro*. (A) Colonic spontaneous contractile activity in the different experimental groups. Data are mean ± SEM; n = 5–10 per group. $^{*}P < 0.05$ *vs* OVA-vehicle; $^{\#}P = 0.074$ *vs* vehicle-vehicle. (B) Concentration-response curves for carbachol. Note that oral exposure to OVA leads to a left-shift of the concentration-response curve, an effect not modified by treatment with K252a. Data represent mean values (symbols) and non-linear regression curves. n = 5–10 per group. (C) Effect of NO blockade with N^{G}-nitro-L-arginine (L-NNA) on spontaneous contractility in the different experimental groups. Data represent spontaneous contractility before (open bars) and after the addition of L-NNA (closed bars). Data are mean ± SEM; n = 5–10 per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* respective contractile activity before the addition of L-NNA (Paired t-test); $^{\#}P < 0.01$ *vs* other L-NNA-treated groups (ANOVA).

Colonic motility *in vivo*

As previously described,[22] strain-gauge recordings of spontaneous colonic motility in vehicle-vehicle-treated rats exhibited two different types of contractions; LFHA contractions with superimposed HFLA contractions (Fig. 5F). Overall, exposure to OVA altered spontaneous colonic motility by affecting both types of contractions (Fig. 5). Treatment with OVA tended to increase the frequency of LFHA contractions (vehicle: 0.67 ± 0.14 contractions min^{-1}; OVA: 0.93 ± 0.08 contractions min^{-1}; $P = 0.08$; Fig. 5A) and also enhanced the amplitude (vehicle: 1.92 ± 0.25 g; OVA: 2.78 ± 0.20 g; Fig. 5B). As it refers to HFLA contractions, exposure to OVA did not alter the frequency but increased the amplitude (vehicle: 0.37 ± 0.05 g; OVA: 0.59 ± 0.03 g; Fig. 5D). However, in animals treated with K252, part of these effects of OVA exposure on spontaneous colonic motility was no longer observed. K252a inhibited the OVA-increased frequency of LFHA contractions (0.49 ± 0.14 contractions min^{-1}; $P < 0.05$ *vs* OVA-vehicle; Fig. 5A) although it did not affect the amplitude. A similar effect of K252a on the frequency, but not the amplitude, of LFHA contractions was observed on vehicle-exposed animals (0.28 ± 0.09 contractions min^{-1}; $P < 0.05$ *vs* vehicle-vehicle; Fig. 5A). Concerning to HFLA contractions, treatment with K252a showed a tendency to reverse the increase in

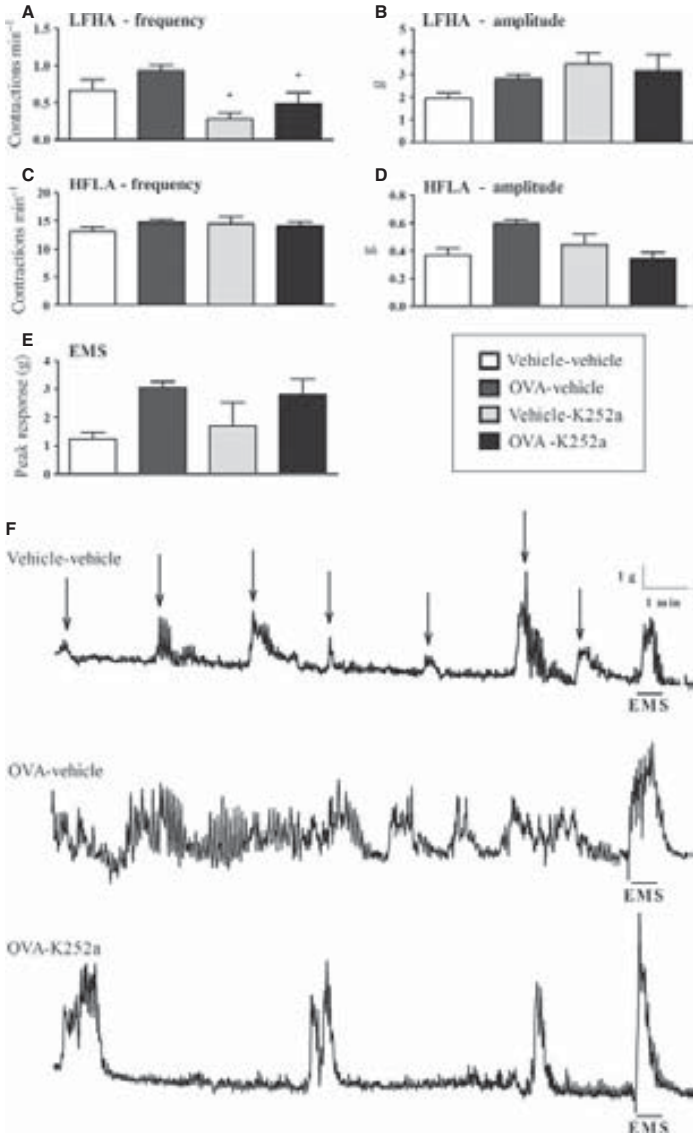

Figure 5 (A–D) Effects of oral ovalbumin (OVA) and K252a treatment on colonic motility *in vivo*. (A, B) Frequency (A) and amplitude (B) of low-frequency and high-amplitude (LFHA) colonic contractions in the different experimental groups. Note that oral exposure to OVA tends to increase the frequency of LFHA colonic contractions and treatment with K252a reduces it in both vehicle- and OVA-treated rats. *$P < 0.05$ *vs* respective vehicle (C, D) Frequency (C) and amplitude (D) of high-frequency and low-amplitude (HFLA) colonic contractions in the different experimental groups. Note that oral exposure to OVA leads to an increase of the amplitude of HFLA colonic contractions, an effect prevented by treatment with K252a. Data are mean ± SEM; $n = 3$–5 per group. (E) Colonic response to electrical mucosal stimulation (EMS) (30 V, 4 Hz, 30 s) showing that exposure to OVA increases EMS-elicited motor responses in a K252a-independent manner. Data are mean ± SEM; $n = 3$–5 per group. (F) Representative tracings showing spontaneous colonic motility and response to EMS in a vehicle-vehicle-, OVA-vehicle- and OVA-K252a-treated animal. The arrows indicate LFHA contractions. Note how OVA exposure increases the frequency of LFHA contractions, an effect prevented by K252a treatment.

the amplitude of HFLA contractions after OVA exposure, as suggested by a significant interaction between treatments in a two-way ANOVA (0.34 ± 0.05 g; $P < 0.05$; Fig. 5D).

In control conditions EMS elicited a LFHA-type response that coincided with the stimulation time (Fig. 5F). The contractile response to EMS was increased by exposure to OVA (vehicle: 1.24 ± 0.23 g; OVA: 3.05 ± 0.23 g; $P = 0.05$; Fig. 5E) in a K252a-independent manner (2.80 ± 0.54 g; Fig. 5E).

DISCUSSION

This study shows that, in the rat model of chronic exposure to oral OVA, changes in colonic motility might be related to an altered activity of the NGF-TrkA

pathway. Although NGF expression levels were not changed, motor responses following the treatment with K252a suggest that NGF-dependent signaling pathways are involved in colonic spontaneous motor activity and mediate OVA-induced enhancement of NO-dependent inhibitory tone *in vitro*. Moreover, our results suggest that colonic NGF is not MMC-derived, although these cells express TrkA receptors and, therefore, represent a target for NGF within the colonic mucosa.

The results of this study confirm that oral OVA activates MMCs in the colon, as indicated by the increase in RMCPII levels within the colonic wall, similarly to that described previously in the same model.[4] Data derived from animal models of IBS have demonstrated the importance of MMCs as effector cells mediating the array of pathophysiological changes that characterize IBS in humans. For instance, degranulation of MMCs seems to be a key step in the onset of visceral hyperalgesia and the alterations of epithelial barrier function observed both in animal models and the human disease.[23–26] In the model of chronic exposure to oral OVA in rats, although these salient features of IBS have not been characterized, observations reveal that MMCs also play a role on the changes of colonic smooth muscle contractility, thus supporting the validity of the model as an appropriate approach to IBS-like altered colonic motor responses.

Results obtained show that OVA-exposed rats have colonic contractility dysfunction, including increased responses to carbachol and L-NNA *in vitro* and enhanced spontaneous contractility and EMS-elicited responses *in vivo*. These results confirm previous observations in this model,[4] resembling that observed in IBS patients and other animal models of the disease.[5–8] Tissue histological examination excluded muscle hypertrophy as a potential cause for this carbachol and EMS enhanced responses (data not shown), thus suggesting an increased excitability of the smooth muscle to cholinergic stimulation. A possible explanation for these OVA-induced colonic motor alterations could be related to an excited-activated state of MMCs. This is suggested by the higher tissue concentration of RMCPII observed in OVA-treated animals and supported by evidences *in vivo* implicating mast cell degranulation in the onset of cecocolonic motor alterations in rats.[27] MMC mediators act on nerve ending of intrinsic and extrinsic primary afferent neurons forming neural networks within both the submucosal and myenteric plexus, leading to a local amplification of effector responses.[28] Therefore, OVA-induced colonic motor alterations might arise as a result of altered afferent nerve input into myenteric motor circuits due to a tonic activation of MMCs.

In animal models of IBS, both mast cells and NGF have been implicated in colonic epithelial barrier function, propulsive motor activity and sensitivity to colorectal distension.[12,24] However, the exact source(s) of colonic NGF remain elusive and the potential role of mast cells as the main source of intestinal NGF is controversial.[24,29] In order to elucidate these points, immunohistochemistry for the neurotrophin was performed in colonic tissues. Although we were able to see specific NGF staining, with similar distribution patterns as those previously reported,[17] we did not find any obvious difference in staining, intensity or distribution, among the different experimental groups. This was further confirmed by real time PCR, showing similar expression levels of NGF among experimental groups. Moreover, during double labeling studies, we were unable to detect NGF immunoreactivity in MMCs, identified as RMCPII-positive cells within the colonic mucosa. This contrasts with animal data suggesting that NGF is released by mast cells upon degranulation [24,30] and with data from colonic biopsies of patients with functional and inflammatory gastrointestinal disorders localizing NGF in MMCs.[14,31] However, our data agree with a recent study in a rat colitis model in which NGF immunoreactivity was not associated with MMCs.[29] Whether or not this represents a species-related difference (human *vs* rat) and/or experimental model-dependent variations in the colonic source of NGF warrants further studies. Interestingly, Stanzel *et al.* evidenced that NGF was synthesized mainly by epithelial cells and hypothesized that MMCs could represent a source of pro-NGF, in agreement to that suggested also by studies on cultured rat peritoneal mast cells.[32] Based on these data, we also attempted, although unsuccessfully, to localize proNGF in colonic MMCs. Overall, our observations indicate that MMCs are not a cellular source of NGF in the rat colon. Nevertheless, results obtained suggest a functional link between MMCs and NGF and, in particular, indicate that MMCs are a target for NGF. Firstly, we were able to demonstrate the presence of TrkA receptors on a high proportion of colonic MMCs (by 60%). Secondly, K252a treatment tended to increase colonic RMCPII levels, thus suggesting that these receptors are functional and might mediate MMC degranulation upon stimulation with NGF. Indeed, the NGF ability to degranulate mast cells has been previously demonstrated, both *in vivo* [33] and *in vitro*.[34]

In this study, we aimed also to elucidate the functional implication of NGF in the alterations of

colonic smooth muscle contractility that characterize oral OVA exposure in rats. A role for NGF on IBS-like gastrointestinal motor alterations in animal models has been previously suggested.[12] In order to further assess this involvement of NGF we used a pharmacological approach based on the blockade of the NGF high-affinity receptor, TrkA, with K252a.[35] In our conditions, treatment with K252a resulted in a decrease of spontaneous colonic motor activity both *in vivo* and *in vitro* and prevented the enhancement of the nitrergic inhibitory tone secondary to OVA exposure *in vitro*. Interestingly, direct addition of K252a to the organ bath also decreased spontaneous colonic contractility (data not shown), thus reinforcing the results obtained with the treatment with K252a and suggesting and effective blockade of TrkA in *in vivo* conditions. From these observations, it is feasible to speculate that a tonic NGF-dependent stimulation might be necessary to maintain basal spontaneous contractility at optimal conditions. Taking into consideration that rat enteric neurons express the high-affinity receptor TrkA, as previously described and also confirmed in this study by immunohistochemistry (data not shown),[36] we can hypothesize that K252a is likely to bind to TrkA receptors on myenteric neurons preventing NGF-mediated effects within the ENS and thus, affecting motor activity.

The dose and pattern of administration of K252a followed here has already been used, showing biological effects *in vivo* indicative of an effective blockade of TrkA.[18–20] Therefore, it is feasible to assume that the responses observed here are related to an effective blockade of TrkA receptors. However, K252a not only binds to TrkA but also to other neurotrophins receptors, mainly TrkB and TrkC, and other kinases, such as the Ca^{2+}/calmodulin kinase II[37] or the myosin light chain kinase,[38] which are implicated in the contractile activity of intestinal smooth muscle.[39] From our observations, it cannot be ruled out that the effects observed might be associated, at least partially, to K252a effects on these targets. Nevertheless, several observations suggest that the responses to K252a are likely to be associated with the blockade of TrkA receptors. Firstly, a recent study demonstrated that the pharmacological blockade of TrkA with K252a, the treatment with TrkA antisense oligonucleotides and the *in vivo* immunoneutralization of NGF were equally effective preventing chronic stress-induced visceral hypersensitivity to colorectal distension in rats.[17] Secondly, *in vivo* immunoneutralization of NGF normalized postinfectious gut dysmotility in *T. spiralis*-infected rats (a model of postinfectious IBS),[12] as observed here in the OVA model with K252a. Overall, these observations suggest that K252a-mediated effects within the gastrointestinal tract are related to the modulation of NGF-TrkA-dependent mechanisms.

Although up-regulation of NGF and its high-affinity receptor TrkA has been demonstrated during colonic acute inflammation,[29,31,40] expression results in animal models of IBS are discordant.[24,26,41] In the present study, only marginal changes in TrkA, and no changes in NGF expression levels were observed among experimental groups. This could seem surprising, as we show that NGF/TrkA-dependent pathways are implicated in the OVA-induced colonic dysmotility in the rat. However, a previous study assessing changes in mRNA neurotrophins levels in mice with colitis showed an increase during the early phases, returning to control levels one-week after the induction of inflammation, thus suggesting a rapid and short-term regulation of these factors during pathological conditions.[42] Taking into consideration that neurotrophins have both acute and long-term biological effects,[43] it is feasible to speculate that NGF/TrkA mRNA colonic levels in the OVA-exposed rats at the time of euthanasia may be not be representative of those along the full period of treatment, even though the persistence of the colonic dysmotility. In addition, there is also the possibility that the OVA-induced increase in MMC mediators release exerts its effects on the ENS through pathways involving NGF/TrkA-dependent mechanisms although these are not directly up-regulated by OVA. In any case, NGF/TrkA expression results should be interpreted cautiously since the interaction between TrkA and other neurotrophin receptors (namely the p75 and the neurotrophin receptor homolog) leads to an enhanced activity of the signaling pathways, without increasing the amounts of NGF and/or TrkA receptors *per se*.[44] It is feasible to assume that the marginal changes observed in TrkA expression after OVA or K252a treatment (32% and 26% increase, respectively) might have consequences at the protein level yet to be demonstrated. On the one hand, K252a-induced changes in TrkA expression could represent a compensatory mechanism to the receptor blockade. On the other hand, OVA-induced changes might be secondary to the OVA-mediated stimulation of MMC and/or other cell types, including enteric neurons, as discussed above. Interestingly, these changes in TrkA expression were not longer observed in animals receiving OVA and K252a. Although we cannot explain the mechanisms behind this effect, this observation further supports an interplay between OVA effects and the NGF-TrkA pathway.

In summary, the present study suggests that NGF-TrkA-dependent mechanisms are implicated in basal

colonic contractility and also in OVA-induced colonic motor alterations in rats. In addition, our results show that MMCs express TrkA receptors and, therefore, represent a target for NGF, rather than being a source of the peptide, in the rat colon. Overall, this study highlights a potentially important role for NGF-TrkA-dependent signaling pathways on colonic motor alterations, as observed for instance in FGDs. Nerve growth factor receptors antagonists could represent a therapeutic target for the treatment of gastrointestinal disorders characterized by altered colonic motility.

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DISCLOSURE

No competing interests declared.

AUTHOR CONTRIBUTIONS

FJ designed and performed experiments, analyzed data and wrote the paper; VM designed, performed experiments, analyzed data and wrote the paper; PV designed experiments and wrote the paper.

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- ABSTRACTS AND MEETINGS

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