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Role of the extracellular matrix molecule, hyaluronan, in the intestinal neuromuscular function adaptation to ischemia/reperfusion injury

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SUMMARY

The gastrointestinal circulation is a highly organized and well-regulated vascular network that provides a high-rate blood flow to a large exchange surface area. For this reason, the gut is one of the most ischemia-sensitive tissues in the body and intestinal ischemia/reperfusion (IR) injury is associated with a high morbidity and mortality. Intestinal IR injury represents, indeed, an important clinical problem which may occur as a consequence of embolism, arterial or venous thrombosis, shock, abdominal surgery, trauma, intestinal transplantation, necrotising enterocolitis in the human premature new-born as well as chronic inflammatory diseases [1–3]. The interruption of blood supply, occurring with the initial ischemia, causes severe depletion of tissue energy resources and accumulation of toxic metabolites, which rapidly lead to microcirculatory failure, cell necrosis and apoptosis [4]. Paradoxically, restoration of blood flow, although essential to rescue ischemic tissues, initiates a cascade of events that may lead to additional cell injury, known as reperfusion injury, exacerbating vascular and tissue damage through the stimulation of an intense systemic inflammatory response [5]. The IR insult has severe consequences for the metabolically active intestinal mucosa, which undergoes shedding, barrier dysfunction, and bacterial translocation [6]. Other enteric cell types, including smooth muscle cells, enteric glial cells and neurons, may also deteriorate. While the mucosal and muscular layers undergo only transient damage and their function is recovered with a *restitutio ad integrum* in the successive hours, myenteric neurons can be irreversibly compromised by the ischemic/reperfusion injury [7]. A long-lasting neuropathy may result, with slowing of transit and difficult digestion of food [8]. The neuronal loss involves the major enteric neurotransmitter systems: i.e. cholinergic excitatory and nitrergic inhibitory neuronal pathways [9]. The exact mechanisms involved in intestinal IR injury are largely unknown, but pro-inflammatory cytokine release, inflammatory cell infiltration, production of reactive oxygen species (ROS) and increased expression of nitric oxide (NO) are certainly involved in these events. Production of such molecules may also harm distant tissues and culminate in multiple organ dysfunctions [10].

The mutual interactions among different cell populations in the enteric microenvironment (i.e. neurons, glial cells, smooth muscle cells, interstitial cells of Cajal), infiltrating inflammatory cells and the saprophytic microflora, may account for the structural and functional changes occurring in myenteric circuitries, underlying alterations of the neuromuscular function during IR. Although the molecular mechanisms are still largely unexplored, extracellular matrix components provide an important scaffold for the enteric microenvironment and may influence the integrity of enteric neuronal circuitries during both physiological and pathological conditions. ECM consists of a well-organized structure of different molecules, such as collagens, elastin, fibronectin (FN), laminins, glycoproteins, proteoglycans (PGs) and glycosaminoglycans (GAGs) [11]. Hyaluronan (HA), an unbranched glycosaminoglycan (GAG) component of the extracellular matrix, participates in the maintenance of normal gut conditions, for example, by enhancing the epithelial barrier defence and integrity and favouring the stability of a healthy gut saprophytic microflora [12,13]. However, when these conditions are altered, i.e. during an inflammatory damage, HA may participate in the development of pathological conditions by influencing the architecture and function of different cell types within the enteric microenvironment [14,15]. Indeed, accumulation of HA in the epithelial, submucosal and smooth muscle intestinal layers and in blood vessels within the submucosal layer has been observed both in experimental rodent models of colitis and in patients with IBD [14]. Due to its exceptional length and high degree of hydration, HA plays an essential role in

tissue lubrication and stability, and there are indications for its potential anti-inflammatory action [16]. HA signalling in inflamed tissue is strictly dependent upon its molecular weight. Long HA polymers, with high molecular weight, may impede development of inflammatory responses. During inflammation, however, long HA polymers are cut by hyaluronidases into small fragments, promoting immune cell activation and pro-inflammatory cytokines production, thus increasing the inflammatory response [16]. The majority of studies on the role of HA in IBD have focused on the involvement of the GAG in the development of fibrotic tissue within the submucosal and muscularis propria layers and on its chemoattractant action for leukocytes in both layers. Recently, we have suggested that HA plays a role also in the maintenance of the enteric nervous system (ENS) homeostasis, by demonstrating that, in the rat colon, HA contributes not only to the formation of an extracellular matrix basal membrane enveloping the surface of myenteric ganglia, but is also a component of a perineuronal net surrounding myenteric neurons [15]. This latter observation is remarkable, since previous studies on the distribution of EMC molecules in the ENS could not evidence any EMC component within myenteric and submucosal ganglia. Remarkably, after an inflammatory injury, the pattern of HA distribution within myenteric ganglia is highly deranged, suggesting that the GAG may take part in the alterations of myenteric neurotransmission in these pathological conditions [15].

In view of the established participation of HA in the development of pathological inflammatory conditions in the gut, this study aimed to evaluate the involvement of HA in rat small intestine neuromuscular function derangements after an *in vivo* intestinal IR injury, carried out by temporary clamping the superior mesenteric artery. The project plan was developed applying a multidisciplinary experimental approach comprising pharmacological, immunohistochemical and biomolecular assays.

The results obtained, showed that after induction of IR injury in rats, HA levels increased in both submucosal and in longitudinal muscularis layer with the myenteric plexus (LMMP) preparations, with an increasing degree of deposition from the inner submucosal to the outer muscularis layer. Such enhancement was related to an upregulation of some HA synthase isoforms, such as HAS1 and, more importantly, HAS2, the functionally relevant HAS isoform, in the submucosal and myenteric plexus, confirming the hypothesis that enteric neurons may be a source of HA. Interestingly, after administration of an inhibitor of HA synthesis, 4-methylumbelliferone (4-MU), in both the submucosal layer and LMMP of IR animals the expression of HAS2 and HA levels decreased. In the rat small intestine, I/R injury slowed-down the gastrointestinal transit, as expected. The present data suggest that endogenous HA exerts a beneficial action on the efficiency of the gastrointestinal transit after IR, since *in vivo* administration of 4-MU further reduced this functional response. In this context it is shown that endogenous HA may influence both the excitatory and inhibitory components of the peristaltic reflex. In particular, data obtained in this study suggest that inhibition of HA synthesis leads to an enhancement of excitatory contractile components, particularly tachykinergic, and to a reduction of the relaxation inhibitory responses, mainly nitrergic, thus compromising the efficiency of peristalsis. The mechanism/s underlying the ability of HA to modulate intestinal neuromuscular functional was evaluated by studying the expression of receptors undergoing HA-mediated regulation, such as TLR2, TLR4, and CD44 receptors, in both submucosal layer and in LMMP preparations. Interestingly, enhancement of HA deposition in the intestinal wall after IR was reflected by a HA-sensitive higher expression of TLR2, TLR4, and CD44 receptors in both layers, with a more remarkable increase in LMMP preparations. TLR2, TLR4, and CD44 receptor were found both in submucosal and myenteric plexus neurons and in glial cells. Increased TLR2 and TLR4 receptor expression in myenteric ganglia may sustain HA beneficial effect on the gut neuromuscular function during IR. We cannot exclude that HA-induced TLR upregulation during IR may favor the

survival and function of specific neuronal populations, since TLR2 and TLR4 activation controls the structural and functional integrity of enteric neurons in the ENS after an inflammatory injury [17,18]. Increased CD44 expression in myenteric and submucosal neurons may exert a chemoattractant effect, favoring the recruitment of mononuclear cells towards both the submucosal and myenteric plexus, as shown in experimental models of inflammatory bowel disease [19]. In this study, after IR injury a low-grade inflammatory develops as indicated by the significant increase of MPO^+ cell infiltration in the *muscularis propria* layer and, more by a more moderate enhancement in the submucosal layer and in the myenteric plexus. These chemoattractant effects were significantly reduced after 4-MU treatment, suggesting that HA participates in immunocyte recruitment, at least in part, via CD44 recruitment.

In conclusion, this study provides evidence that HA is produced within the ENS of the rat small intestine where it takes part in the control of ENS structure and function. In pathological conditions occurring after an IR injury, alterations of HA production, especially in the outer intestinal muscular layer with the myenteric plexus may contribute to neuron derangements, underlying dysmotility. It is thus possible that, during intestinal IR injury, modulation of HA homeostasis, both by regulating its synthesis and degradation, as well as the activation of receptors which undergo HA regulation, may prevent permanent and irreversible alterations of the neuromuscular function. This hypothesis is innovative and opens new scenarios in the study of the molecular mechanism/s involved in the onset and severity of gut IR injury as well as for the development of new therapeutic agents for the treatment of this disease with high clinical impact.

INTRODUCTION

The enteric nervous system

The control of the main digestive functions in gastrointestinal (GI) tract largely depends upon the presence of a complex intrinsic neuronal network, termed enteric nervous system (ENS), that extends from esophagus to the anal sphincter. The ENS has an important role in determining motility patterns, gastric secretion, blood flow, nutrient handling, and the interactions with immune and endocrine systems of the gut, acting in a relative independent manner with respect to the central nervous system (CNS) [20]. For this reason, it is often referred to as the "second brain" or the "minibrain" [21,22]. However, the ENS is not entirely autonomous, but it works as an integrated system in which afferent signals arising from the lumen are transmitted through enteric, spinal and vagal pathways to CNS, and efferent signals from CNS are driven back to the intestinal wall. This bidirectional communication guarantees the CNS monitoring of a number of gut parameters, from chemical sensing in the lumen, to sensing mechanical stress along the gut wall [23].

The human ENS contains 200-600 million neurons, a number similar to that found in spinal cord, grouped into ganglia which are connected by bundles of nerve processes to form two major plexuses innervating the effector tissues: the myenteric (or Auerbach's) plexus and the submucosal (or Meissner's) plexus [24] (Figure 1).

The myenteric plexus lies between the longitudinal and circular muscle layers, extending in the entire length of the gut as a continuous network. It primarily provides motor innervation to the smooth muscle layers and secretomotor innervation to the mucosa. Numerous myenteric neuron projections extend into submucosal ganglia and enteric ganglia of the gallbladder and pancreas [25], and another substantial number into sympathetic ganglia [26]. The submucosal plexus is located in the submucosa, between the outer circular muscle layer and the serosal side of the *muscularis mucosae*, where it plays an important part in the control of the secretory function. Large mammals can have two layers of submucosal ganglia, and sometimes a third intermediate layer between them. Submucosal ganglia and connecting fibre bundles form the submucosal plexus, which can be found in the small and large intestine, and rarely in the stomach and esophagus [20,24].

According to the morphology, neurochemical properties, cell physiology, projections to targets and functional roles, approximately 20 distinct types of neurons have been described [27], all of which can be mainly grouped into the three major classes of enteric neurons: intrinsic primary afferents neurons (IPANs), interneurons and motor neurons. The myenteric plexus shows a high density of neurons compared to the submucosal plexus with an average ratio of the primary afferents, interneurons and motor neurons of 2:1:1, respectively [27,28].

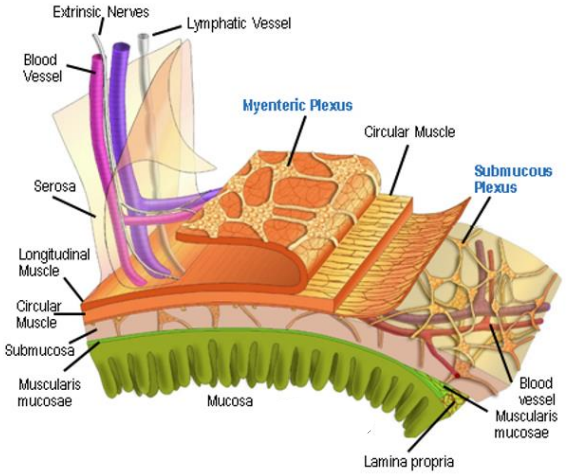

Figure 1. Organisation of the ENS in human small and large intestine. Neurons are confined in ganglia of the myenteric plexus, localized between the longitudinal and circular muscle layers, and in ganglia distributed between the circular muscle and the *muscularis mucosae* within the submucosa, depicted in the transverse section of the gut wall. The ganglia and fibers in the submucosa form the submucous plexus [29].

IPANs are sensory neurons, representing the 10-30% of total neurons in the submucosal and myenteric intestinal ganglia, that are able to detect chemical luminal stimuli, mechanical distortion of the mucosa and mechanical forces in the external musculature (e.g. tension of the gut wall) [20]. IPANs react to these signals to initiate appropriate reflex activity controlling motility, secretion and blood flow [30]. IPANs connect with each other, with interneurons and directly with motor neurons (Figure 2).

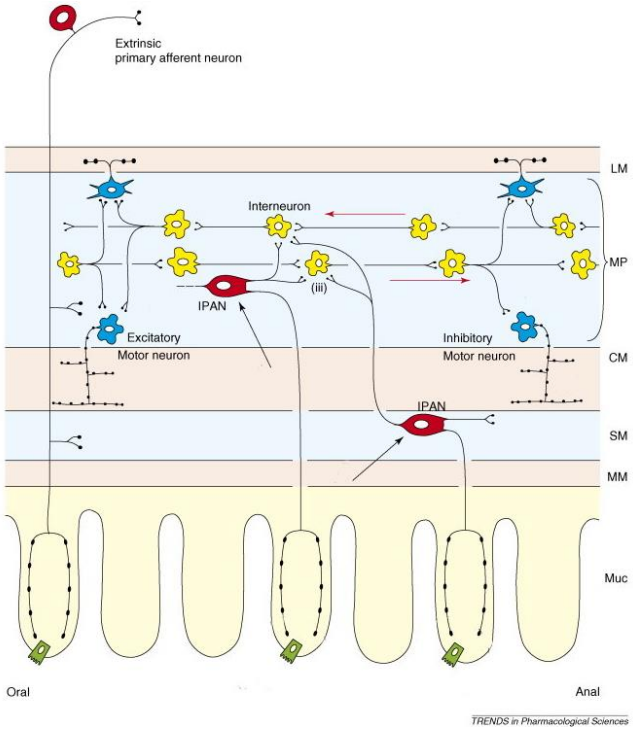

Figure 2. Neuronal connections of motility controlling circuits in the small intestine. This simplified circuit diagram shows the major circuitries involved in intrinsic motor reflexes of the small intestine. Intrinsic primary sensory neurons (IPANs, red) detect mechanical distortion and luminal chemistry and connect with excitatory and inhibitory muscle motor neurons (blue) directly and via descending and ascending interneurons (yellow). CM, circular muscle; LM, longitudinal muscle; MM, *muscularis mucosae*; MP, myenteric plexus; Muc, mucosa; SM, submucosa [31].

Uni-axonal excitatory and inhibitory motor neurons, which receive prominent fast excitatory synaptic potentials, innervate the longitudinal, circular smooth muscle layers and the *muscularis mucosae* along the entire GI tract [22]. The primary neurotransmitters for excitatory motor neurons are acetylcholine (ACh) and tachykinins. Several neurotransmitters have been identified in inhibitory motor neurons, including nitric oxide (NO), vasoactive intestinal peptide (VIP) and ATP-like transmitters, although NO is considered the primary transmitter [20]. Another important class of enteric neurons is represented by secretomotor and secretomotor/vasodilator neurons regulating electrolyte and water transport across the intestinal mucosa [32].

The next paragraphs will focus on some of these neurotransmitter pathways, which undergo adaptive rearrangements in the different studies carried out in the present thesis.

Cholinergic neurotransmission

Acetylcholine (ACh) is the major excitatory neurotransmitter in the ENS, being involved in the regulation of motor, secretory and vascular reflexes, by inducing excitatory potentials in post-synaptic effectors.

ACh is present in the greater population of the enteric neurons and synthesized in nerve terminals from choline and acetyl-CoA by choline acetyltransferase (ChAT). As a classic neurotransmitter, ACh is then translocated into synaptic vesicles by the vesicular acetylcholine transporter (VAChT) [33] and stored in synaptic vesicles until it is released on demand [34].

There are two types of receptors mediating cholinergic transmission within the ENS: the nicotinic (nAChRs) receptors, which are ligand-ion channels, and the muscarinic (mAChRs) G-protein coupled receptors [35]. Thus, ACh binding generates variable postsynaptic potentials depending on the receptor present on the cell membrane, with nAChRs mediating rapid excitatory transmission and mAChRs mediating slow excitatory transmission [36]. At the cholinergic synapse both classes of receptors are present either on effector cells (post- synaptic receptors) or on nerve terminals (pre-synaptic receptors), where they act as autoreceptors regulating release of ACh. nAChR activation is the predominant mechanism for cholinergic neurotransmission in enteric ascending reflex pathways, whilst it retains a minor role in mediating cholinergic transmission within the descending inhibitory reflex [37]. Within the ENS, activation of nAChRs entails rapid reflex propagation, producing fast responses to stimuli. Conversely, activation of mAChRs either induces cell membrane depolarization, resulting in the initiation of action potential, or cell membrane hyperpolarization, inhibiting additional action potentials by activating second messenger cascades and intracellular signalling pathways [35]. Owing to different distribution, molecular structure and intracellular signalling of mAChRs and nAChRs, these receptors may differentially influence various intestinal functions, both in physiological and pathologic conditions of the gut. Indeed, several GI disorders are associated with changes in the enteric cholinergic system. Congenital gastrointestinal aganglionosis, such as Hirschsprung's disease, show a completely lack of mAChR3-immunoreactive fibres in smooth-muscle layers and decreased mAChR3 and mAChR2 mRNA levels in the aganglionic segments of the colon, which may be responsible for the typical motility dysfunctions of this pathology [38]. In addition, abnormal levels of mAChRs expression were correlated with organic GI pathologies, including chronic inflammatory bowel disease (IBD) [38,39].

Tachykinergic neurotransmission

Tachykinins are a family of small biologically active peptides that act as a co-neurotransmitter of excitatory enteric neurons. Indeed, tachykinins represent with ACh, the main transmitters of excitatory neurons innervating the muscle, regulating intestinal motility, secretion, and vascular functions. The principal mammalian members of this family are substance P (SP), the first to be discovered in extracts of horse brain and intestine, neurokinin A (NKA) and neurokinin B (NKB) [40]. These neuropeptides are produced from the cleavage of large precursor proteins in the cell body of the neurons, where they are transported to the varicosities and released after stimulation. Tachykinins bind with high affinity to specific membrane G-protein coupled receptors, namely NK1R, NK2R and NK3R [40]. Consistent data show that NK1 and NK2 receptors are present on enteric neurons, NK1Rs are expressed also on interstitial Cajal cells (ICC), while NK2R are also highly expressed on smooth muscle cells. Although NK1Rs are considered SP-preferring, NK2Rs NKA-preferring and NK3Rs NKB-preferring receptors, all of these receptors can be fully activated by all tachykinins with moderate selectivity. NK1 and NK2 receptors mediate the transmission from excitatory motor neurons to muscle, whereas NK1 and NK3 participate in slow excitatory transmission at neuro-neuronal synapses in both ascending and descending pathways affecting motility. Moreover, the activation of NK1 and NK2 receptors on mucosal epithelium is involved in fluid secretion [41].

However, tachykinins are not only important for neurotransmission, but they also have effects on tissue growth and differentiation, immunomodulation, tumour growth, and inflammation. In this latter regard, SP, via binding to the NK1Rs in the ENS, represents a classical sensory neurotransmitter involved in visceral pain perception associated with inflammation [42]. Several studies have reported altered expression of SP in the ENS of patients with inflammatory and functional GI disorders, such as constipation, diverticulitis, inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) [43].

Nitrergic neurotransmission

Nitric oxide (NO) is a gaseous messenger molecule, which has numerous molecular targets. It controls regulatory functions such as neurotransmission and vascular tone [44], gene transcription [45] and mRNA translation (e.g. by binding iron-responsive elements) [46] and produces post-translational modifications of proteins (e.g. by ADP-ribosylation) [47]. The nerves whose transmitter function depend on NO release are called "nitrergic", and such nerves are recognized to play major roles in the control of smooth muscle tone, motility and fluid secretion in the GI tract. In mammals NO can be produced by three different isoforms of the NO synthase enzyme: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) isoforms. All NOS isoforms utilize as substrate L-arginine and molecular oxygen, and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) as co-substrates. Flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and (6R-)5,6,7,8-tetrahydro-L-biopterin (BH_4) are cofactors of all isoenzymes. All NOS proteins are homodimers. NO synthesis is accomplished via two steps. In the first step, NOS hydroxylates L-arginine to Nω-hydroxy-L-arginine, successively NOS oxidizes Nω-hydroxy-L-arginine to L-citrulline and NO [48]. All NOS isoforms bind to the intracellular Ca^{2+}-binding protein, calmodulin. nNOS and eNOS binding to calmodulin is brought about by elevated intracellular Ca^{2+} concentrations (half-maximal activity between 200 and 400 nM), while iNOS binds to calmodulin at extremely low intracellular Ca^{2+} concentrations (below 40nM) [49].

In the ENS, NO plays a pivotal physiological role in the inhibitory regulation of peristalsis [23]. All NOS isoforms have been localized in myenteric neurons of different species [50,51]. In myenteric neurons, nNOS seems to represent the main source of NO involved in the physiological modulation of non-adrenergic non-cholinergic inhibitory motor responses of the gut [52]. However, expression of iNOS, but not of nNOS and eNOS, prevails during diseases states, such as intestinal inflammation [53] and IR injury [54,55]. In these conditions, large amounts of NO can cause damages in different cellular populations, such as neurons, by the formation of peroxynitrite and nitrotyrosine [56]. This phenomenon reflects a functional plasticity of myenteric neurons that activate different NOS isoforms depending on either physiological or pathological conditions [54,57].

Plasticity in the enteric nervous system: the enteric microenvironment

Plasticity is the ability of the nervous system to rewire its connections, or to adapt its functions in response to alterations of different inputs. In the CNS, the ability of the brain to adapt to changing physiological conditions or traumatic lesions is crucial, due to a lack of a sufficient regeneration potential. The ENS also shows several features that make it a suitable and unique model of plasticity. Enteric ganglia share morphological (e.g., presence of enteric glial cells, resembling CNS astrocytes [58], and absence of collagen fibres) and functional (e.g., growth factors, neurotransmitters) similarities with the CNS, and their complex organization is unmatched in any other section of the peripheral nervous system [59]. Second, enteric ganglia maintain integrated functions in the absence of input from the CNS, which has a modulatory role. The ENS undergoes significant changes in physiological conditions during the life span, i.e during development and ageing. Plasticity in the ENS is also evident for clinical (e.g., recovery of motor function after intestinal transplantation) and experimental (e.g., denervation and pharmacological manipulations) observations, which suggest that ENS can react upon microenvironmental changes, maintaining a homeostatic control of the gut functions. Enteric neuronal plasticity may also lead to regeneration of neuronal circuitries and allow the recovery of normal intestinal functions after surgical procedures.

Several "players" are involved when considering adaptive changes in the enteric microenvironment: neurons, enteric glial cells, interstitial cells of Cajal (which are viewed as the intestinal pacemaker cells) [60] and the gut microbiota. Neuroimmune interactions also play an important role in the ENS homeostasis and plasticity [61,62]. A possible cross-talk between smooth muscle cells and dorsal root ganglion cells has also been demonstrated [63], and this finding may affect the understanding of altered visceral sensitivity and reinforce the concept of a brain–gut axis interaction. In addition, it has already been demonstrated that the gut microbiota play a crucial role in the development of innate and adaptive immunity, and in regulation of gut motility, intestinal barrier homeostasis, nutrient absorption and fat distribution [64,65]. In the following paragraphs, some of the major non-neuronal cellular components of the enteric microenvironment will be described.

Enteric glia

Enteric glia cells (EGCs) are an important non-neuronal component of the ENS, similar to CNS astrocytes, which form a cellular and molecular bridge between enteric nerves, enteroendocrine cells, immune cells, and epithelial cells, depending on their location.

During last decades, several studies have clearly shown that this cell population does not play a merely supportive function, serving only as a scaffold for neurons, but EGCs are also actively involved in most gut functions such as mucosal integrity, neuroprotection, adult neurogenesis, neuroimmune interactions, and synaptic transmission (Table 1) [66,67].

Function	Location of enteric glia	Mediator(s) released/expressed by enteric glia
Epithelial barrier function	Mucosa	S-nitrosoglutathione 15-Deoxy-$\Delta^{12,14}$-prostaglandin J2 TGF-β1 pro-epidermal growth factor
Fluid secretion	Myenteric plexus	NO
Intestinal motility	Myenteric plexus	ATP
Support of enteric neurons and neuronal survival	ENS	L-arginine Glutamine Reduced glutathione Nerve growth factor 15-Deoxy-$\Delta^{12,14}$-prostaglandin J2
Enteric neurotransmission	ENS	ATP
Neurogenesis	ENS	
Immune signalling	ENS	MHC class II, CD80, CD86 IL-1β IL-6, MCP1 Prostaglandin E2 S-100β TLR4

Table 1. Functional role of enteric glia in the GI tract [68].

In view of this role in maintaining GI homeostasis, a possible EGC role in the pathophysiology of neurodegenerative or inflammatory disorders has been widely reported [69,70]. Genetic ablation of EGCs results in a fatal haemorrhagic jejuno-ileitis, with enteric plexus disruption and the loss of epithelial layer integrity [71], while EGCs destruction by autoimmune mechanism leads to enterocolitis [72]. A reduction of the glial network were found in patients with Crohn's disease (CD) and similarly, there is a preferential loss of enteric glia in patients with slow-transit constipation [72,73]. Moreover, loss of neurons and glial cells in myenteric and submucosal plexus is characteristic of necrotizing enterocolitis (NEC), a disease occurring in premature infants [74,75].

Intestinal microbiota

In the human body, the GI tract represents one of the largest interfaces (250–400 m^2) between the host, environmental factors and antigens. The community of commensal microorganisms colonizing in or passing through the GI tract is referred to as the intestinal microbiota [76]. Bacteria are the predominant population of microorganisms, with an approximate number of $3.8x10^{13}$ [77] and an estimated number of genes of about 2-4 million, exceeding > 100-150 fold the number of human genes [78,79], more than 1000 species [79] and 7000 strains [80].

Among the different bacterial phyla detected in the human gut, *Firmicutes* and *Bacteroidetes* are the most representative, whereas other phyla such as *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia* are present in smaller quantities [81].

The composition, diversity and abundance of the human gut microbiota vary along the GI tract. The microbial density, increases from 10^1–10^8 microbial cells in the stomach and small intestine, to 10^{10}–10^{12} cells in the colon and faeces [76,82,83], indicating that the greater microbial amount of the human microbiota is located in the large intestine. The composition also differs, with predominance of Gram-positive bacteria in the upper GI tract and mainly Gram-negative microorganisms and anaerobes in the colon [84,85].

The symbiosis between the gut microbiota and the host is the result of a dynamic and beneficial equilibrium between both players.

Microorganisms harboring the human gastrointestinal tract exerts important metabolic activities by extracting energy from otherwise indigestible dietary polysaccharides, such as resistant starch and dietary fibres [86]. This activities allow the production of many compounds useful to the host, such as vitamins, gas, organic acids, bile salts, bacteriocin and short-chain fatty acids (SCFA) [87,88]. Moreover, bacteria influence absorption, distribution and metabolism of nutrients and drugs and may reinforce host innate and acquired immunity, representing a biological barrier against pathogens [89] (Figure 3). Many structural bacterial components take part in the cross-talk with the host immune system, such as lipopolysaccharide, lipoteichoic acids, peptidoglycan, flagellin, formyl peptides and unique nucleic acid structures [90]. The overabundance of microbial virulence factors (i.e. pigments, proteases, nuclease, toxins, haemophores), on reverse, represent a family of molecules detrimental to the host health [91].

The gut microbiota is also essential for normal GI motility, since it contributes to the regulation of sensory and motor functions and intestinal barrier homeostasis [83,92].

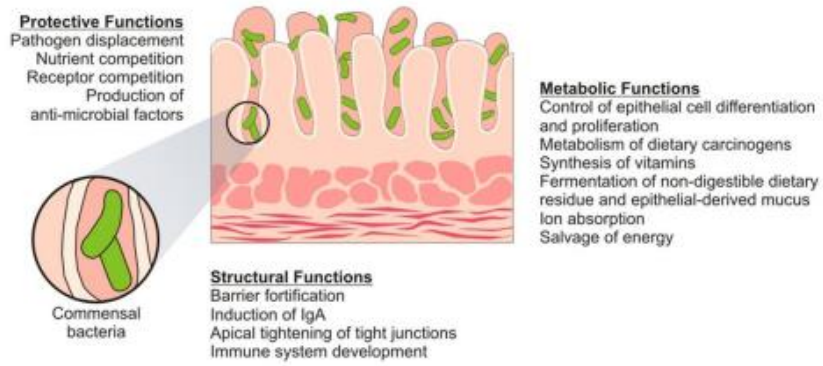

Figure 3. Functions of the intestinal microbiota. Commensal bacteria exert protective, structural and metabolic effects on the intestinal mucosa[93].

However, the gut microbiota extend its influence beyond the gut, playing a role in the bidirectional communication between the GI tract and CNS [94], the so-called "microbiota-gut brain axis". This complex network of communication between the gut, the microbiota and the brain includes the CNS, both the sympathetic and parasympathetic branches of the autonomic nervous system (ANS) and the ENS, in addition to the neuroendocrine and neuroimmune systems [93].

It is now evident that microbiota undergoes important modifications throughout the lifespan, changing composition, diversity and abundance both in the state of health and disease[95].In particular, maturation of the microbiota occurs in parallel with neurodevelopment and they have similar critical development phases, in which a broad range of different factors may act altering the brain-gut signalling and leading to development of disorders later in life.

Several studies showed that alterations of the gut microbiota composition (dysbiosis) may contribute to the onset of obesity [80,96], malnutrition [97], diabetes[98] and chronic inflammatory diseases such as IBD and IBS [99]. In the same way, microbiota alterations, through this bidirectional communication, may influence stress reactivity, pain perception neurochemistry, and several other brain-gut disorders [100].

Enteric immune system

The human immune system comprises the innate immunity, including macrophages, basophils, eosinophils, neutrophils, mast cells, natural killer cells, M cells and dendritic cells (DC), that has a standardized response to all adverse agents, and the adaptive immunity, characterized by B and T lymphocytes, that specifically recognizes each microorganism and has a specific response and memory. The GI tract represents an important immune organ with a gut-associated lymphoid tissue (GALT), formed by both inductive (Peyer's patches) and effector sites (lamina propria and sub-epithelial cells), representing approximately the 70% of the entire body immune system [101]. Moreover, the GI tract itself structurally forms a vital defensive barrier aiming to prevent the transit of external and unprocessed, potentially harmful, antigens.

In the GI ecosystem, different factors including the ENS, the gut microbial saprophytic flora and the enteric immune system, interact and influence each other in order to preserve gut homeostasis [102]. Thus, the resident population of immune cells in the gut is not only involved in the response to deleterious luminal stimuli or pathogens, but takes part also in the maintenance of homeostasis and development of normal intestinal morphology and function [103]. In this scenario, neuroimmune interactions has been shown to play an important role in the GI response to stress, inflammation from infection or to disease states, resulting in acute and chronic changes in the GI function that contribute to disease symptoms [104,105]. The mechanisms underlying the interplay between enteric neurons and immune cells is complex and involves neuro-, immuno-, and microbe- associated molecules, such as hormones, neuropeptides, cytokines, neurotrophic factors and short chain fatty acids (SCFAs) [106]. Furthermore, receptors for neurotransmitters are present on immune cells while, receptors for immune mediators are also located on neural structures (Figure 4) [107]. Another important cellular player involved in the modulation of the immune response is represented by the enteric myocyte that can also produce cytokines, thus altering neuromuscular function. Similarly to vascular and bronchial smooth muscle cells, whose plasticity has important pathophysiological implications for atherogenesis and airway remodelling [108], respectively, the production of pro-inflammatory cytokines from enteric myocytes has been shown to occur in IBD and underlies the neuromotor dysfunction symptoms associated with the disease [109]. Preclinical studies carried out on rodent gut have shown that enteric myocytes have sensors for inflammatory signals in the gut [110,111].

It must be point out that the commensal microflora exerts a fundamental role in this communication. At mucosal level, many commensal bacteria metabolites including neuromodulators, bacteriocins, bile acids, choline, and SCFAs are immunomodulatory. Ligands from commensal bacteria such as polysaccharide A, lipopolysaccharide (LPS), and lipoteichoic acid (LTA) influence the normal development and function of the mucosal immune system [112,113]. Indeed, peptidoglycans, polysaccharides, and other bacteria antigens that have a protective role for bacterial cells, allow the host immune cells to identify numerous and diverse bacteria species through the activation of pattern recognition receptors (PRR), such as the membrane associated toll-like receptors (TLR). TLRs are type I transmembrane proteins responsible for the recognition of foreign pathogens referred to as pathogen-associated

molecular patterns (PAMPs). Epithelial TLRs, once activated, can favour the release of inflammatory mediators, the production [114] of cytokines, and chemokine-induced recruitment of inflammatory cells depending on the signal that are delivered by the microbiota [112]. Interestingly, TLRs are not only engaged by PAMPs, but also by an array of endogenous molecules generated during tissue damage (damage-associated molecular patterns, DAMPs), activating firstly an acute immune response and, secondarily, tuning the subsequent adaptive immune reaction [115]. Indeed, the recognition of pathogenic antigens by specific TLRs is followed by intracellular activation of the NF-κB signaling system which results in cytokine production and T cell activation that, together with IgA secretion to the mucosal site, is essential for the development of the GALT after the birth [116] (Figure 5).

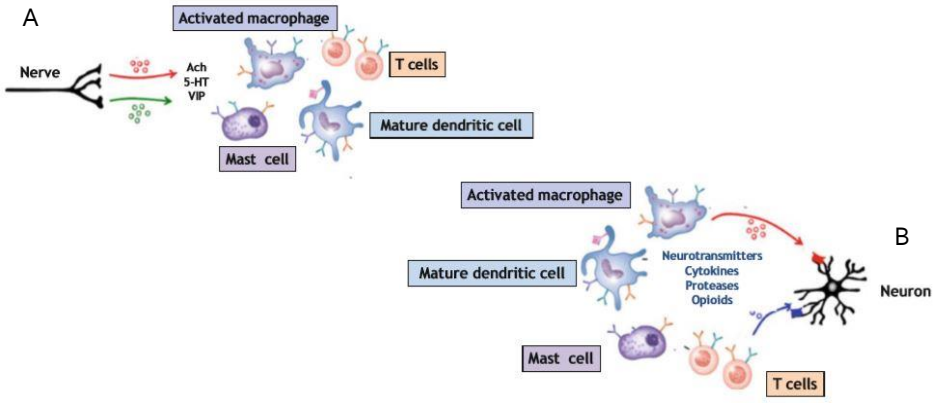

Figure 4. Neuroimmune interaction. (A) The neuroimmune synapse showing the release of neurotransmitters in the proximity of immune cells that induce changes in the phenotype and function of these cells **(B)** Resident and recruited immune mediators bind to receptors in nerves. This may result in changes in neuronal sensitivity [107].

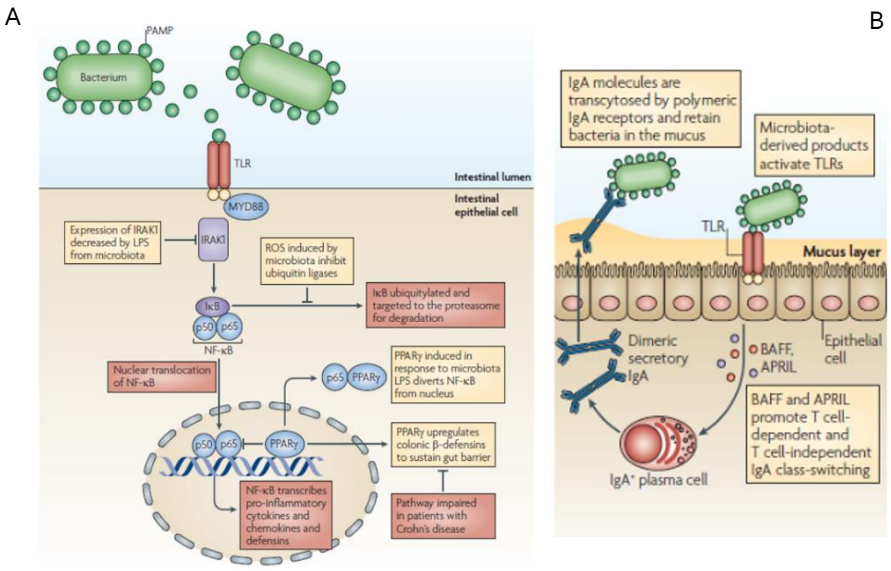

Figure 5. Modulation of the epithelial and immune intestinal cells by the microbiota. (**A**) Activation of adaptor protein and NF-κB signaling upon TLR ligation. (**B**) Production of B cell-activating factor (BAFF) and a proliferation-inducing ligand (APriL) which promote both T cell-dependent and T cell-independent IgA class-switching responses in the intestine [117].

The expression of TLRs in both the CNS and ENS [118] suggest that TLRs are not only involved in the regulation of the host immune responses but they may also have a role in central aspects of neuroinflammation, neurodevelopment and

neuroplasticity [18,119]. Among all TLRs, the most important bacterial sensor proteins are TLR2 and TLR4. Both these TLRs are expressed by enteric neurons and glia, suggesting that enteric neuronal pathways can directly sense the microbial population [18]. The importance of TLR2 in the host defence against Gram-positive bacteria has been demonstrated using TLR2-deficient ($TLR2^{-/-}$) mice, which demonstrated a high susceptibility to a challenge with *Staphylococcus aureus* or *Streptococcus pneumoniae* [120,121]. TLR2s have also a crucial role in host defence against extracellular Gram-positive bacteria exposure [122]. TLR4s may detect LPS, a major component of Gram-negative bacteria cell wall [123]. Recently, TLR2 and TLR4 signalling has been described as fundamental for ensuring intestinal integrity and protecting from harmful injuries, such as inflammation. In fact, changes in TLR2 and TLR4 expression have been reported in both IBS and IBD [124]. The absence of TLR2s seems to increase the susceptibility to intestinal injury and inflammation [125]. Polymorphisms of TLRs genes as well as a defective immune response appear to be involved in the initiation and perpetuation of chronic inflammation in IBD [126]. In addition, it has been observed that in the absence of the resident microbiota, the expression levels of some TLRs are low or absent, thus hampering proper immune responses to pathogenic stimuli [127].

Thus, it has been accepted that the balance between microbiota and immune response is fundamental not only for a healthy intestine but may also affect the bidirectional communication with the CNS making it an important target for transducing the effects of bacteria to the CNS [128].

Adaptation to a changing environment: role of the extracellular matrix

As mentioned before, several factors take place in the adaptation to microenvironmental changes in physiological and pathological conditions, both in the CNS and ENS. Some molecular mechanisms that sustain this plasticity are common to both districts. A protein typically correlated to neuronal growth and regeneration, the growth-associated protein-43, is expressed at high levels in the nervous system during development. In adult animals, its expression is lower, but still observable in brain areas showing structural or functional plasticity [129]. In the ENS, GAP-43 is strongly expressed in the myenteric and submucosal ganglia at all ages [130], thus giving evidence for a lifelong capability of the ENS to adapt to new challenges [110]. The phenomenon of plasticity has a fundamental role during all the life span, during pre- and post-natal development and aging. During the perinatal period, in the rat myenteric plexus the neuronal number per ganglionic area increases from duodenum to colon [131,132]. Conversely, the density of enteric neurons decreases with increasing age [131,132], and this variation is correlated with changes in motility, mucosal function and changes in specific neuronal phenotypes, such as IPANs (which appear to be the most age-sensitive neurons in the ENS). However, neuronal adaptation in response to changes in their microenvironment occurs even during adult life. As previously described, gut disorders, such as IBD or IBS, can lead to a remodelling of the ENS and to cytokine-induced changes in neurotrophin and neurotransmitter content and release. Inflammation also results in reactive remodeling of EGCs, characterized by an increase in GFAP expression and more "stellate" cell appearance, similar to that seen in reactive astrocytes [69]. In this scenario, extracellular matrix (ECM) molecules provide an important framework for the enteric microenvironment and may influence the integrity of myenteric neuronal circuitries during both physiological and pathological conditions. ECM molecules provide a highly organized environment filling the extracellular space and consist of different types of macromolecules. The main constituents of

ECM are fibrous-forming proteins, such as collagens, elastin, fibronectin (FN), laminins, glycoproteins, proteoglycans (PGs), and glycosaminoglycans (GAGs) [11].

In the CNS, where the role of ECM has been mostly studied, these molecules are synthetized by neurons, glia and non-neuronal cells, and are secreted into the extracellular space, where then they are associated with cell surface receptors and form heterogeneous aggregates around the neurons, the so-called perineuronal nets (PNNs). The precise composition and specific structures of the PNNs vary from tissue to tissue.

Nevertheless, ECM is not only a mere mesh-like scaffold for the cells. In the CNS, PNN takes part in the regulation of neurogenesis, gliogenesis and circuitry formation during development, and, indeed, substantial changes in its quantity and composition occur in this period [133]. Moreover, ECM continues to be essential for synapse stability, neuroprotection and regulation of neuronal plasticity during all the rest of life [134].

Synaptic dysfunctions are a critical pathological component of several brain disorders; thus, it has been proposed that ECM and PNN alterations, being involved in processes of plasticity and homeostatic maintenance, may contribute to pathogenesis of neurological and neuropsychiatric disorders [133]. In both *in vitro* and *in vivo* models, a strong correlation has been reported between an impairment in ECM molecules expression and the development of Alzheimer's disease, epilepsy, schizophrenia and drug addiction [134,135].

Less information, however, are available concerning the role of ECM in the ENS. Some studies show the distribution of several ECM molecules in the basement membranes surrounding enteric ganglions of rat and guinea pig intestines [136,137]. Recently, data obtained in our laboratory demonstrated for the first time that HA is abundantly present in colonic myenteric plexus where it form a pericellular coat of condensed matrix surrounding myenteric neurons, similar to the mesh-like structure called perineuronal net (PNN), that is associated with some classes of neurons in the central nervous system (CNS)[138]. In contrast with previous study [137], HA was detected not only on the myenteric ganglia surface but also in the perineuronal space surrounding myenteric neurons. This accumulation of the PNN may be due to the binding between HA and its receptors on neuronal surface, such as CD44 or RHAMM-l, or, directly, via one of the three transmembrane HA synthases (HAS1-3), which can retain HA on cell surface [133].

It is now well established that ECM molecules take part in the regulation of cell migration and differentiation into diverse phenotypes cells, not only during intestinal development but also in adult cell renewal [139].

Given the presence and the influence of ECM, especially of HA, in GI tract, it become particularly interesting to investigate if ECM molecules may participate also in synaptic dysfunction in GI pathological conditions.

HA and gut homeostasis

Hyaluronan: synthesis, properties and functions

Hyaluronan is a relatively simple component of the extracellular matrix, an unbranched GAG composed of repeating polymeric disaccharides of d-glucuronic acid and N-acetyl-d-glucosamine linked by a glucuronidic β(1→3) bond [140]. The number of repeat of disaccharides in a completed HA molecule can reach 10000 or more, with a molecular weight of ~4000 kDa. Due to its exceptional length and high degree of hydration, hyaluronan makes the ECM an ideal environment in which cells can move and proliferate. Hyaluronan is ubiquitously expressed in the ECM and on cell surfaces of all body fluids and tissues of vertebrates, as well as on surface coats of bacterial species and certain algae [141]. HA levels are maintained through regulation of the enzymatic activity of HA synthases (HAS) and

hyaluronidases, which are responsible for its synthesis and degradation. HA is produced by a family of three hyaluronan synthase isoenzymes (HAS1, HAS2, and HAS3) that synthetize HA on the inner surface plasma membrane and translocate the final polymer to the extracellular space. In general, HAS2 represents the main HA synthetic enzyme in adult cells and its activity reasonably undergoes fine regulation. However, HAS1 and HAS3 seem to have peculiar roles even if not completely clarified.

In spite of its relative simple structure, HA possesses many physiological roles, regulating different cell behaviours such as cell adhesion, motility, growth and differentiation [142]. Several studies have shown that the majority of HA biological effects are size-dependent: HA can exist as large polysaccharides, medium fragments and small oligosaccharides. HA molecules of high molecular weight (HMW) are involved in the maintenance of physiological conditions, whereas fragmented polymers accumulate during tissue injury and function in ways distinct from the native polymer, inducing inflammatory responses and triggering repair processes [141]. There is accumulating evidence that free radicals and an enhanced activity of hyaluronidases are responsible for HA fragmentation. The resulting degradation products can stimulate the expression of inflammatory genes by a variety of immune cells at the injury site [143].

It has been suggested that also different HAS isoforms may synthesize HA with different chain length. *In situ*, all three HAS proteins synthesize HA chains of high molecular mass (≥4 × 106 Da). However, *in vitro*, HMW HA (≥4 MDa) is synthesized only by HAS2, while HAS3 and HAS1 synthesize smaller polydisperse chains of an average molecular mass of 800 and 100 kDa, respectively [144]. It seems that the difference in size of HA synthesized by the different HAS isoforms may be dependent on (1) the intrinsic enzymatic property, (2) the intracellular environment and (3) additional accessory molecules involved. Interestingly, stimuli such as growth factors and cytokines can regulate the expression of HAS isoforms *in vitro*, and upregulation of HASs, together with increased accumulation of HA, has been found after tissue damages [15,145].

In both physiological and pathological conditions, to accomplish its important functions, HA interacts with specific proteins called hyaladerins, or membrane receptors like CD44 and RHAMM, and toll like receptors (TLRs) such as TLR2 and TLR4 [16] (Figure 6).

CD44 is considered a primary receptor for HA, existing in at least 10 different isoforms. Its interaction with HA plays an important role in a variety of cell functions like inflammation, development, tumor growth and metastasis. Upon binding with CD44, HMW and LMW HA show distinct inflammatory and angiogenic effects. Native HMW polymers demonstrated anti-inflammatory and anti-angiogenic effects, suppressing cell migration, proliferation and sprout formation. On the other hand, HA oligosaccharides exhibited pro-angiogenic and pro-inflammatory responses, stimulating cell mobility and proliferation [146].

HA is also able to induce the expression of inflammatory mediators, such as chemokines and cytokines, through the interaction with the previously described TLRs or the TLR adaptor protein MyD88, independently from CD44 activation. The abrogation of TLR2 and 4 as well as MyD88 abolished completely the activation of chemokines in macrophages in *in vitro* model [147]. Another study has shown that fragmented HA, via TLR2 and TLR4 signaling, specifically activates dendritic cells *in vitro*, with subsequent priming of allogeneic T cells. All these data suggest a focus on the role of HA as an immune regulator in both physiological and pathological conditions [148].

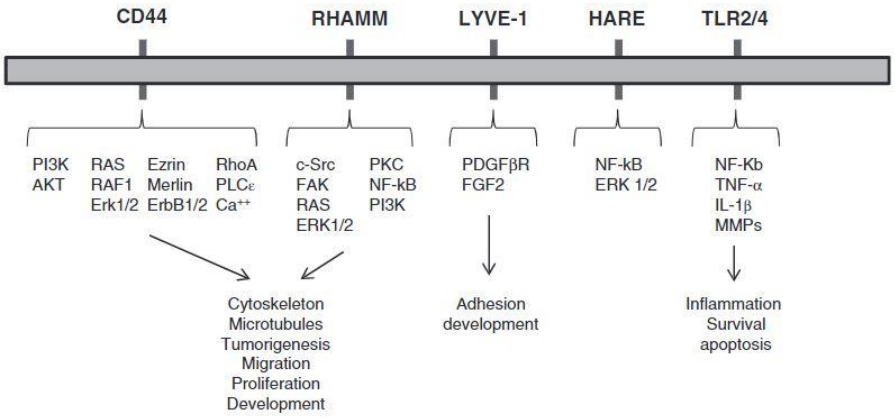

Figure 6. HA receptors. Schematic representation of the signalling cascade of HA receptors and their involvement in cell and tissue functions [16].

HA production in health and pathological conditions in the gut

HA is an important extracellular matrix component in the vertebrate intestinal tract, with a concentration four times higher in the colon than in small intestine [149]. In both healthy human and mouse guts, HA is prominently located immediately beneath the barrier epithelium, where it is deeply involved in the maintenance of gut homeostasis. As hydrophilic molecule, it ensnares water and regulates fluid exchange to and from the blood, interacting with lymphatic and blood microvessels that control water and solute transport [12]. As mentioned before, HA has also important functions in multiple host defence mechanisms. HA participates in innate immune responses in the intestine, promoting leukocyte recruitment in the intestinal extravascular space during gut injury, directly via interaction with the surface receptor CD44 [12]. Interestingly, HA of medium average molecular weight (35 kDa), increased the epithelial expression of a murine orthologue of human β-defensin (HBD2) in mice via TLR4 activation [13]. HBD2 is a naturally produced antimicrobial peptide with a broad antimicrobial spectrum against bacteria, fungus protozoa, and viruses [150]. In addition, HBD2 is one of the enteric defensins, which favour the shaping of the intestinal microflora composition [151], and HBD2 dysregulation has been reported in IBD [152].

HA can also act to systemically decrease inflammation and promote epithelial repair. Studies on rodent models demonstrated that administration of medium molecular weight HA protects from damage during experimentally induced colitis, through the activation of TLR4 receptors that drive COX2 production and promotes recovery [153]. In a recent study administration of nanoparticles consisting of medium molecular weight HA complexed with bilirubin, restored the epithelial barrier, reduced the damage score and increased the overall richness and diversity of the saprophytic microbiota in a murine model of colitis [154]. Several reports have, however, demonstrated that endogenous HA deposition may be highly altered during gut inflammation. In these conditions, small fragments of HA may promote immune cell activation as well as production of pro-inflammatory cytokines, thus favouring an increased inflammatory response. Accumulation of HA in the epithelial, submucosal and smooth muscle intestinal layers and in blood vessels within the submucosal layer has been observed, both in experimental animal models of colitis and in the intestine of patients with IBD [155]. These findings are consistent with other data concerning the increased HA deposition in different tissues, such as liver, kidney and lung, during inflammation [156].

The majority of studies have focused on the involvement of HA in the development of fibrotic tissue within the submucosal and *muscularis mucosae* layers or in vascular smooth muscle cells [19,155,157].

Recently, study conducted in a model of DNBS induced colitis in mouse revealed the presence of HA in the myenteric plexus where myenteric neurons were shown to express HAS2 enzyme, supporting the hypothesis that colonic myenteric neurons may be a source for HA [15]. Interestingly, the presence of HAS2 mRNA and protein has recently been demonstrated in developing rat cortical neurons, consolidating this hypothesis [158]. In addition, several reports demonstrated that in pathological condition, such as IBD, ECM deposition is significantly altered [15,155,159–161]. In particular, during colitis, HA deposition in the whole wall of the rat colon and the perineuronal net structure were significantly altered and this alteration is associated with increased neuronal HAS2 and HAS3 expression, in analogy with data obtained in human colon biopsies from IBD patients and in murine dextran sodium sulfate (DSS)-induced colitis [15,155,160,162]. It has been proposed that the HAS2 increased expression in colitis serves to retain inflammatory cells in the proximity of myenteric ganglia, since it is related to a prominent leukocyte infiltration and increase of MPO activity in the *muscularis propria* [15]. This hypothesis is supported by the ability of myenteric ganglia in culture to produce HA "cable-like" structures, which confer to HA the ability to attach serum components, such as the heavy chain of inter-alpha trypsin inhibitor, which are known to increase the adhesiveness of HA to leukocytes [12]. In microvessel endothelial cells and submucosal smooth muscle cells obtained from IBD patients HA cable-like structures, deriving from medium molecular weight HA, were shown to recruit mononuclear leukocytes via CD44 receptors [19]. Further fragmentation of HA may sustain the inflammatory response by stimulating inflammatory cytokine production by monocytes [163].

Moreover, a drastic reduction of HuC/D positive neurons, the presence of nuclear aggregates, cytosolic vacuolization, smaller area and changes in HuC/D immunoreactivity distribution were also observed in colitis and is indicative of neuronal damage [54,159,164]. Since PNN was shown to retain a neuroprotective role against neurotoxic molecules, preventing, for example, amyloid-β toxicity deposition or oxidative stress, in selective vulnerable neuron types [165], it might be possible that the altered deposition of HA is caused by a fragmentation of the GAG after the inflammatory injury and that it participates in the alterations of myenteric neuron structure, as observed after degradation of the PNN in cortical mouse slices, thus causing changes in motor function [165,166].

Given the increasing amount of data concerning the role of HA in inflammatory processes, it could be interesting to investigate its involvement in other pathological conditions affecting the GI tract, such as, for instance, damages derived by an ischemic/reperfusion injury.

Ischemia and reperfusion injury

The GI circulation is a highly organized and well-regulated vascular bed, composed by vessels that pass through the muscular, submucosa and mucosal compartments of the GI wall. This vascular network provides a high-rate blood flow to a large exchange surface area. For this reason, the gut is one of the most ischemia-sensitive tissues in the body [167]. In particular, celiac artery, superior mesenteric artery (SMA) and inferior mesenteric artery are the most involved in the ischemic insult [168].

In 2020 the American Gastroenterological Association classified intestinal ischemia into three major categories based on its clinical features: acute mesenteric ischemia (AMI), chronic mesenteric ischemia (CMI), also known as intestinal angina, and colonic ischemia (CI), also known as ischemic colitis.

The AMI is not considered as a single condition, but represent a complex of diseases, associated with a high morbidity and mortality (60-80%), which may occur as a consequence of embolism, arterial or venous thrombosis, and non-occlusive mesenteric ischemia, associated with conditions such as cardiovascular surgery, sepsis and administration of alpha-adrenergic agents or digitalics. Lesions caused by ischemia were also related to intestinal transplantation and necrotising enterocolitis in the human premature new-born or in chronic inflammatory diseases [1,168–170]. All these conditions originate from local and systemic responses and develop because of both the hypoxic and the reperfusion components (Figure 7).

The decrease in blood flow occurring with the initial hypoxia, results in cellular damage and death of the superficial part of the mucosa. The intestinal epithelium, due to the presence of basolateral Na- K-ATPase used to drive secretion and absorption, has a particularly high energy demand and together with submucosal layer accounts for approximately 70% of the blood flow and is therefore particularly sensitive to interruption of blood flow. Epithelial cells lose their attachment to the basement membrane with a progressive loss of cells extending from the villus tips toward the crypt base as the duration of ischemia increases and causing a histopathologically distinct lesion called Gruenhagen's space [171,172]. The loss of mucosal integrity leads to severe barrier dysfunction and translocation of not only commensal but also pathogen microorganism and endotoxins to the mesenteric lymph nodes and portal blood causing the devasting systemic consequence known as systemic inflammatory response syndrome. Both bacteria-derived and apoptotic epithelial cells-derived mediators promote the activation of neutrophils and monocytes through Toll-like receptor 4 (TLR4) interaction causing the subsequent release of a cytokines storm capable of harming any distant organ, thus promoting multiple organ dysfunction [173]. In this view, several studies showed that alteration of commensal bacteria reduces intestinal expression of TLR2 and TLR4 and has less local and systemic damage following IR injury [174,175]. However, a dual role of commensal bacteria has been proposed. Although microorganism deletion improves the survival at early stage in a mouse model of ischemia, it fails to ameliorate the overall survival 96h after IR injury since it reduces proliferation of intestinal epithelial cells. This findings suggest that importance of microbial flora for a correct epithelial restitution [176].

The perpetuation of these ischemic phases results in progressive alterations culminating in necrosis. However, if the cause of ischemia is corrected before irreversible alteration such as necrosis and apoptosis occur oxygen return allows for establishment of energetic metabolism, toxic products removal, and progressive return of normal cell functions. In this phase the reperfusion is essential for the rescue of the injured tissue. Paradoxically, restoration of the blood supply causes additional cell damage and amplifies the inflammatory response exacerbating organ injury and apoptosis. The exact mechanisms involved in intestinal reperfusion injury are largely unknown, but both reactive oxygen species (ROS) and nitric oxide (NO) production are certainly involved in these events [177].

During normal condition, the oxygen derived free radicals, generated from xanthine-oxidase metabolism, electron transport chains of the mitochondria, endothelial cells, prostaglandins and activated neutrophils, are neutralized by antioxidant enzymes. During reperfusion, an excessive amount of free extremely unstable radicals, called reactive oxygen species (ROS) and able to react with all the cellular components, were produced. In particular, the conversion of xanthine dehydrogenase to xanthine oxygenase generates superoxide free radicals as a by-product of the oxidation

of purines to uric acid as well as neutrophil enzyme, such as myeloperoxidase and NADPH oxidase, produce toxic oxygen radicals [178,179]. The absence of an efficient dismutation of superoxide anion by the activity of superoxide dismutase (SOD) allows its transformation in hydroxyl radical and its reaction with nitric oxide to produce the free radical peroxynitrite. Proteins, nucleic acid, and particularly cell membrane are susceptible to peroxidation induced by the action of the peroxynitrite, thus causing severe organ dysfunction by direct damage or by indirect injury to the vascular endothelium [169]. Organ failure is also worsened by the vasoconstriction of mesenteric vessels induced by damaged endothelial cells-derived nitric oxide [180,181]. Although an excess production of NO by inducible NO synthase isoform participate in the pathophysiology of ischemia and was observed in several rat model of mesenteric IR injury, NO produced by constitutive NO synthase may also exert a protective role at the onset of the insult acting as a mediator or messenger [182–184].

In addition to the effect of ROS and NO, the restoration of blood flow leads to an increased metabolism of arachidonic acid with the production of prostaglandins, thromboxane, prostacyclins and leukotrienes which might influence vascular permeability, platelet aggregation and leukocyte recruitment. As a result, together with an increased expression of adhesion molecules such as selectins, integrins and immunoglobulins, the phenomenon of disseminated intravascular coagulation (DIC), which exacerbates the damage to the microcirculation, is frequently observed [185–187].

All these mechanisms involved in reperfusion step converge to the activation of nuclear factor-κB which regulates the expression of a variety of modulator and contributes to the maintenance of inflammatory state. A particularly important role in the activation of this pathways might be due to the activation of complement. It is well established that the complement system plays a significant role in the pathogenesis of ischemia reperfusion injury [188]. The depletion or inhibition of complement can attenuate several of the known mediators of reperfusion injury, including neutrophil activation, cytokine production, adhesion molecule, SOD and iNOS expression [168].

From a structural viewpoint, the IR insult has severe consequences for the metabolically active intestinal mucosa, which undergoes shedding, barrier dysfunction, and bacterial translocation [189]. Other enteric cell types, including smooth muscle cells, enteric glial cells and neurons, may also deteriorate. Myenteric neurons are especially sensitive and can be irreversibly compromised [7]. Indeed, an IR injury causes pathological alterations of myenteric ganglia, with dysmotility and a slowing of GI transit, suggestive of a long-lasting neuropathy [9]. Among the different enteric neurotransmitter pathways, there is good evidence that energy deprivation in GI tract can preferentially depress cholinergic neuronal pathways involved in intestinal reflexes [190,191]. Both spontaneous and electrically evoked endogenous acetylcholine overflow from the guinea pig ileum was altered during glucose and oxygen deprivation, mimicking an *in vitro* ischemic damage [191–193]. These effects were correlated with a depression of the cholinergic neuro-effector transmission [191,194]. As regards the neuronal component, the sensitivity of enteric cholinergic neurons to IR damage may be explained considering the strict dependence of neuronal acetylcholine synthesis on the oxidative phosphorylation. Furthermore, the ability of the IR injury to influence the excitation-contraction coupling at a post-junctional level explains the depression of electrically induced smooth muscle contraction in this condition [191].

Nitrergic inhibitory pathways seem also to play a distinctive role in the development of myenteric neuron derangement during IR role. In the gut, the three isoforms of NOS, the endothelial NOS (eNOS), the neuronal NOS (nNOS), and the inducible NOS (iNOS), have been identified in different cells, comprising myenteric neurons of different species [51]. Nitrergic neurons play a fundamental role in the physiological regulation of peristalsis: nNOS is the predominant NOS

in the normal intestine as it is constitutively expressed, whereas iNOS is only expressed in response to cytokines and growth factors. nNOS containing myenteric neurons seem to be selectively targeted by in vivo IR injury, and damage or loss of function of nNOS-immunopositive neurons may underlie intestinal motility derangement, suggesting that NO produced by nNOS is protective against the metabolic insult [9]. Conversely, intestinal IR damage has been associated with upregulation of iNOS, which may replace nNOS in the synthesis of NO in myenteric neurons [54].

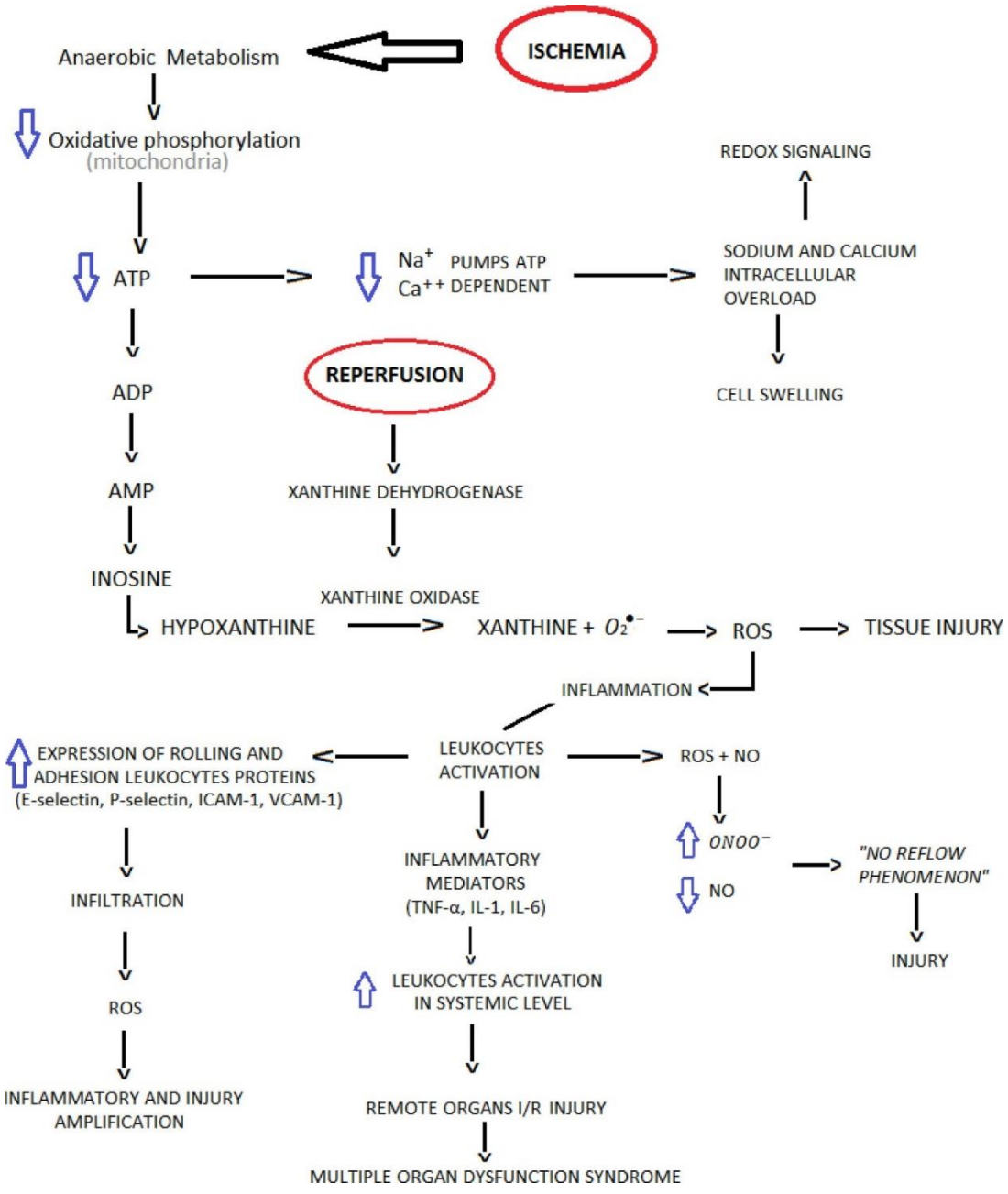

Figure 7. Mechanisms of ischemia-reperfusion injury. Schematic representation of the activation of hypoxic and reperfusion components during IR insult [195].

AIMS

The gastrointestinal tract is one of the most ischemia-sensitive tissues in the body, and intestinal ischemia/reperfusion (IR) injury is associated with a high morbidity and mortality. Intestinal IR injury represents, indeed, an important clinical problem which may occur as a consequence of embolism, arterial or venous thrombosis, shock, abdominal surgery, trauma, intestinal transplantation, necrotising enterocolitis in the human premature new-born or chronic inflammatory diseases [1–3]. The initially occurring interruption of blood supply, causes severe depletion of tissue energy resources and accumulation of toxic metabolites, which rapidly lead to microcirculatory failure, cell necrosis and apoptosis [4]. Paradoxically, restoration of blood flow, although essential to rescue ischemic tissues, initiates a cascade of events that may lead to additional cell injury known as reperfusion injury, which exacerbates vascular and tissue damage through the stimulation of an intense systemic inflammatory response [5]. The IR insult has severe consequences for the metabolically active intestinal mucosa, which undergoes shedding, barrier dysfunction, and bacterial translocation [6]. Other enteric cell types, including smooth muscle cells, enteric glial cells and neurons, may also deteriorate. In particular, myenteric neurons are especially sensitive and can be irreversibly compromised [7]. The neuronal loss may result in difficult food digestion [8]. Indeed, an IR injury causes pathological alterations, particularly a slowing of transit, suggestive of a long-lasting neuropathy involving the major enteric neurotransmitter systems: i.e. cholinergic excitatory and nitrergic inhibitory neuronal pathways [9]. The exact mechanisms involved in intestinal IR injury are largely unknown, but pro-inflammatory cytokine release, inflammatory cell infiltration, production of reactive oxygen species (ROS) and increased expression of nitric oxide (NO) are certainly involved in these events [173]. Production of such molecules may also harm distant tissues and culminate in multiple organ dysfunctions [10].

The intestinal neuronal circuitries display a considerable ability to adapt to a changing microenvironment, which comprises several cellular "players" such as neurons, enteric glia, smooth muscle cells, immune cells and the intestinal saprophytic microflora. The mutual interactions among all these cellular populations may account for the structural and functional changes occurring in myenteric circuitries, underlying alterations of the neuromuscular function during IR. However, the molecular mechanisms are still largely unexplored [89,196]. Hyaluronan (HA), an unbranched glycosaminoglycan (GAG) component of the extracellular matrix, may participate in development of pathological inflammatory gastrointestinal conditions [14,15]. The majority of studies on the role of HA in IBD have focused on the involvement of the GAG in the development of fibrotic tissue within the submucosal and *muscularis propria* layers and on its chemoattractant action for leukocytes in both layers. Our group has recently demonstrated the presence of HA as a major component of a perineuronal framework within myenteric ganglia, sustaining myenteric neuron homeostasis, similar to the perineuronal net (PNN) observed in the central nervous system (CNS) [15,197,198]. The PNN is responsible for the phenomenon of plasticity, controlling the communication between neurons, synaptic ion sorting, lateral mobility of protein on the neuronal surface, and protects neurons from oxidative stress and neurotoxins [198]. The disruption of this HA-based structure leads to the development of neurological disorders in the CNS. Accordingly, we have demonstrated that after an experimentally-induced colitis in rats a drastic perturbation of perineuronal HA is evident within colonic myenteric ganglia [15] and is associated with myenteric neuron damage [199]. HA signalling in inflamed tissue is strictly dependent upon its molecular weight. Long HA polymers, with high molecular weight, may impede development of inflammatory responses. During inflammation, however, long HA

polymers are cut by hyaluronidases into small fragments, promoting immune cell activation and pro-inflammatory cytokines production, thus increasing the inflammatory response [16].

In view of the established participation of HA in the maintenance of gut homeostasis during inflammation, the aim of this study was to evaluate the involvement of HA in small intestine derangements after an *in vivo* intestinal IR injury, carried out by temporary clamping the superior mesenteric artery in adult rats. The project plan was developed resorting to a multidisciplinary experimental approach, comprising pharmacological, immunohistochemical and biomolecular assays.

Overall, these investigations may allow to clarify whether HA may participate in the derangements of enteric networks and of the intestinal neuromuscular function in a preclinical model of intestinal IR injury.

MATERIAL AND METHODS

Animal models

Male Wistar rats (Envigo, San Pietro al Natisone, Udine, Italy), weighing between 250 and 300 g, were housed under strictly controlled environmental conditions (temperature 22 ± 2°C; relative humidity 60-70%) and maintained 12/12hr light/dark cycle. The animals were allowed free access to standard diet and tap water. All the experimental protocols were approved by Animal Care and Use Ethics Committee of University of Insubria and followed the European Union Council Directive 2010/63, recognized and adopted by the Italian Government (Decree No. 26/2014).

Ischemia-reperfusion injury

Rats were anaesthetized with intraperitoneal injection of sodium thiopental (100 mg/kg) diluted in sterile phosphate-buffered saline (PBS). After laparotomy, a loop of the small intestine was exteriorized, and the superior mesenteric artery (SMA) was occluded for 60 min with an atraumatic microvascular clamp. The clip was then gently removed, the abdominal wall was sutured, and animals returned to a cage after recovering from anaesthesia (IR). Animals were euthanized 24hr after reperfusion, when the major histological and functional changes have been evidenced. A control group of un-operated animals (CTR) and sham-operated group (SHAM), which underwent the same visceral manipulation except for the SMA occlusion, were used. In addition to these group and in order to evaluate the involvement of hyaluronan in ischemia-reperfusion injury, CTR, SHAM and IR groups were treated with 4-Methylumbelliferone (4-MU) (Sigma Aldrich, Milan, Italy), an inhibitor of hyaluronan synthesis. A single dose of 25 mg/kg 4-MU was administrated intraperitoneally 24hr before euthanasia. Segments of the small intestine ≈5 cm oral to the ileo-caecal junction, were rapidly excised and rinsed with an ice-cold Tyrode's solution for successive experiments.

Immunohistochemistry

Immunohistochemical study was performed on 3µm formalin-fixed, paraffin embedded full-thickness small intestine sections deparaffinised and rehydrated through alcohol series to water. Samples were stained with haematoxylin–eosin (HE) for morphologic evaluation. Moreover, analysis of neutrophil infiltration was performed using the avidin-biotin-peroxidase method [200] with a polyclonal antibody anti-MPO (Ventana Medical System, Tucson, Arizona). Endogenous peroxidase activity was blocked by immersing sections for 10 min in a solution of 3% hydrogen peroxide in water. Primary antibody was incubated overnight at 4°C. Specific biotinylated secondary antibody and avidin-biotin-peroxidase complex were consecutively applied, each for 1 h at room temperature. The immunohistochemical reaction was developed with diaminobenzidine–hydrogen peroxide reaction [201]. Sections were counterstained with haematoxylin. Neutrophil infiltration was evaluated only in whole well oriented sections of intestine, counting MPO^+cells in four high-power fields (×400, diameter 0.55 mm) for each mucosal, submucosal, *muscularis propria* and myenteric plexus. MPO value has been reported as the average of MPO^+cells for field in each layer.

Immunofluorescence experiments were also carried out on 3µm-thick sections to evaluate hyaluronan distribution and concentration across the gastrointestinal wall. After rehydration, 1:100 diluted HABP (Hokudo, Sapporo, Japan), which recognizes HA saccharidic sequences and is able to localize HA in tissues, were applied overnight at 4°C followed by a

FITC-Streptavidin (Molecular Probes, Eugene, Oregon) incubation RT for 1 hr. A section undergoing only the FITC-Streptavidin staining was used as negative control. Coverslips were mounted using mounting medium with DAPI (Vectashield; Vector Laboratory, Burlingame, Canada). Images were acquired at confocal microscopy using a Leica TCS SP5 confocal laser scanning system (Leica Microsystems GmbH, Wetzlar, Germany) and the density index of HA of mucosal, submucosal and LMMP layers were measured at identical exposure time conditions using ImageJ software (version 1.48a). The data was obtained on 10 fields per preparation at 40x magnification and normalized per myenteric ganglia area.

Whole-mount preparations

Small intestine segments were fixed at room temperature for 3 hr in a solution of 0.2 M phosphate-buffer (PB) containing 4% formaldehyde and 0.2% picric acid. Tissues were then stored at 4° in PBS containing 0.05% sodium merthiolate (Thimerosal, Sigma-Aldrich). For the whole-mount preparation, LMMP and submucosal layer were peeled away from the remaining gut wall and were incubated for 2 hr for nonspecific binding blocking in PBS containing 1% Triton X-100 (Sigma Aldrich) and 10% normal horse serum (NHS) (Celbio, Euroclone, Milan, Italy). A consecutive double staining was performed as follows: primary antibody was added 4°C ON, followed by secondary antibodies labelling for 2h at RT; these steps were subsequently repeated for the second target of interest. Table 2 shows the appropriate dilution of primary and secondary antibodies used.
Preparations were mounted onto glass slides, using Vectashield mounting medium.

Antibody	Producer	Diluition
Primary antibodies		
HABP, biotin	Hokudo (BC41)	1:100
Rabbit Anti- HAS1	Bioss (bs-2946R)	1:100
Goat Anti- HAS2	Santa Cruz (sc-34068)	1:100
Mouse Anti-HUC/D, biotin	Invitrogen (A-21272)	1:100
Rabbit Anti-TLR2	ABclonal (A11225)	1:50
Rabbit Anti-TLR4	ABclonal (A5258)	1:50
Rabbit Anti-CD44	ABclonal (A16807)	1:100
Goat Anti-ChAT	Chemicon (AB144P)	1:50
Rabbit Anti-nNOS	Santa Cruz (sc-648)	1:50
Rabbit Anti-iNOS	Santa Cruz (sc-8310)	1:200
Rabbit Anti- Substance P	Immunostar (20064)	1:200
Rabbit Anti- Vasoactive Intestinal Peptide	Immunostar (20077)	1:200
Secondary antibodies and streptavidin complexes		
FITC-conjugated streptavidin	Molecular Probes (SA1001)	1:200
Cy3-conjugated streptavidin	Amersham (PA43001)	1:500
Anti-rabbit Alexa Fluor 488	Molecular Probes (A21206)	1:200
Anti-goat Cy3	Jackson (705-165-147)	1:400

Table 2. Primary and secondary antibodies.

Images from 5 animals per group were acquired using a Leica TCS SP5 confocal laser scanning system at 40X magnification. The number of nitrergic (iNOS, nNOS), tachykinergic (SP) and VIPergic (VIP) neurons were evaluated

as the percentage of co-stained HuC/D$^+$ neuronal cells on the number of total HuC/D$^+$ myenteric neurons. The percentage of HAS1 and HAS2 positive neurons was obtained in the same manner for both myenteric and submucosal neurons. In addition, the density index of HA, ChAT, VIP and SP for the LMMP and HAS2 for the submucosal layer was measured using ImageJ software as described above.

ELISA assay

In M, SM, and LMMP preparations obtained from all the experimental groups, levels of HA were evaluated using a Hyaluronan Quantikine ELISA Kit (R&D Systems, Minneapolis, Minnesota), following the manufacturer's instructions. Briefly, frozen tissues from 6 rats per group were lyophilized overnight and then suspended in a solution of PBS and cell lysis buffer 2 (1:1) (R&D Systems) ON at RT, under gentle agitation. 500µl of Cell Lysis Buffer 2 each 100 mg of fresh tissue were used at this stage. Then, debris were removed by centrifugation and supernatants were collected for ELISA assay. Absorbance (Abs) values were recorded using a microplate reader (Infinite 200pro; Tecan Trading AG, Männedorf, Switzerland) at 450 nm and corrected for optical imperfections subtracting the Abs readings at 570nm. HA levels were expressed as ng of HA per mg of dry tissue.

RNA isolation and quantitative RT-PCR

Total RNA from small intestine SM and LMMP segments was extracted with TRIzol (Invitrogen, Carlasbad, California) and treated with DNase I (Invitrogen), to remove possible traces of contaminating DNA. 2 µg of total RNA were then retrotranscribed using the High-Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, California). Quantitative RT-PCR (qRT-PCR) was performed with QuantStudio 3 PCR Systems (Applied Biosystems). TaqMan Gene Expression Mastermix (Applied Biosystems) was used to detect HAS1 (Rn00597231_m1), HAS2 (Rn00565774_m1), CD44 (Rn00563924_m1) and the housekeeping gene β-actin (Rn00667869_m1) mRNA levels, following the manufacturer's instructions. To evaluate the expression of HIF-1α, TLR2 and TLR4 standard qRT-PCR was carried out with Power Sybr Green Universal PCR Master Mix (Applied Biosystems), as indicated by manufacturer's instructions. Primers were designed using Primer Express software (Applied Biosystems) on the basis of available sequences deposited in public database (Table 3) and were used at a final concentration of 500 nmol/L for each. The $2^{-\Delta\Delta Ct}$ method was applied to compare the relative gene expression. Experiments were performed at least four times for each different preparation.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
β-actin	TGACAGGATGCAGAAGGAGA	TAGAGCCACCAATCCACACA
HIF-1α	AAGCACTAGACAAAGCTCACCTG	TTGACCATATCGCTGTCCAC
TLR2	CCGAAACCTCAGACAAAGCG	ACAGCGTTTGCTGAAGAGGA
TLR4	TGAGATTGCTCAAACATGGC	CGAGGCTTTTCCATCCAATA

Table 3. Primer sequences.

Western immunoblot analysis

To assess protein levels, small intestine SM and LMMP segments were homogenized at 4°C by using stainless steel beads (Next Advance, Troy, New York) 30 minutes at 24000rpm in Mixer Mill MM301 (Retsch GmbH, Hann, Germany) in ice cold T-PER (ThermoScientific, Waltham, Massachusetts) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10% protease inhibitor cocktail and 1ug/mL aprotinin (Sigma Aldrich). After a sonication step (4x4 seconds at 100% power) the homogenate was centrifuged at 12000 g for 30 min at 4° C. The supernatant was collected, while the resulting pellet was re-suspended and incubated for 15 min at RT in a Tris-HCl 20mM buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10% protease inhibitor cocktail and 1 ug/mL aprotinin. Quantification of protein concentration in the supernatant and in pellet was performed by using the Bradford method. After protein quantification samples were diluted in Laemli sample buffer (Tris-HCl 300 mM, pH 6.8, glycerol 10%, SDS 2%, β-mercaptoethanol 0.04%). Sample aliquots were then heated for 5 min at 95°C to mediate protein denaturation. Protein were separated on 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted to nitrocellulose membranes (Merck Millipore,Milan, Italy). Membranes were then incubated in a blocking solution consisting of 5% fat-free milk dissolved in TBS-Tween 20 0.1%, 2 hr at room temperature in order to saturate aspecific sites. Membranes were incubated with primary antibodies against TLR2, TLR4 and CD44 overnight at 4 °C, followed by a second incubation at room temperature with horseradish peroxidase (HRP)-linked appropriate secondary antibodies. Before the experiments, both the pellet and supernatant fractions obtained from the homogenization step were tested to allow the correct localization of the protein of interest. Dilutions and reactivity of primary and secondary antisera are reported in Table 4. The antibody/substrate complex was visualized by chemiluminescence (LiteAblot, Euroclone) and acquired by using Alliance Q9 Advanced System (Uvitec Ltd, Cambridge, United Kingdom). In each membrane β-actin were used as protein loading controls. Experiments were performed at least four times for each different preparation. Signal intensity was quantified by densitometric analysis using Alliance Q9 AVD software.

Antibody	Producer	Dilution
Primary antibodies and streptavidin complexes		
Mouse actin	Cell Signalling Technology (3700S)	1:1000
Rabbit Anti-TLR2	ABclonal (A11225)	1:1000
Rabbit Anti-TLR4	ABclonal (A5258)	1:1000
Rabbit Anti-CD44	ABclonal (A16807)	1:1000
Secondary antibodies and streptavidin complexes		
Anti-mouse igG HRP-linked	Cell Signalling Technology (7076S)	1:2000
Anti-rabbit IgG HRP-linked	Amersham (NA934)	1:5000

Table 4. Primary and secondary antibodies

Gastrointestinal transit

Gastrointestinal transit was measured by evaluating the distribution of fluorescein isothiocyanate (FITC)-labeled dextran from the stomach to the colon. An intragastric gavage of 200 µl FITC-dextran (Sigma-Aldrich) (250kDa; 6.25mg/ml dissolved in 0.9% saline) were administered in all the groups. Control, IR and sham-operated animals, with and without 4-MU treatment, were euthanized 90 min after administration and the entire GI tract was collected and

divided into 15 segments: a single stomach segment (sto), 10 equal-length segments of small intestine (S1–S10), a single caecum segment (CEC), and 3 equal-length segments of colon (C1–C3). Luminal contents from each sample were collected and clarified by centrifugation (12,000 g for 10 min). The cleared supernatants were fluorimetrically measured in duplicate for FD250 intensity using 0.9% saline solution as a control. The signal was assayed at 492/521 nm using the Infinite 200pro system. Data were expressed as the percentage of fluorescence for each segment compared to the total fluorescence along the gastrointestinal tract. The efficiency of FITC-dextran transit was determined by calculating the geometric center (GC) for the distribution of the fluorescent probe using the following equation [202]:

GC = Σ (% of fluorescence signal per segment x segment number)/100

Excitatory and inhibitory *in vitro* motor responses

Intestinal motor response was evaluated by measurement of tension changes of small intestine samples from all the experimental groups. Segments of full-thickness small intestine (1 cm) were rapidly excised, washed with Krebs solution, cleared of connective tissue and mounted in isolated baths containing 10 ml of continuously oxygenated (95% O2 and 5% CO2) and heated (32 ± 1°C) Krebs solution. Each segment was positioned along the longitudinal axis end attached to an isometric force displacement transducer (MDE Research GmbH, Walldorf, GE). Mechanical activity was recorded with a PowerLab acquisition data system 8 (AD Instruments, Oxford, UK) and elaborated with a LabChart 8.0 program (AD Instruments, UK). An initial load of 1 g was applied to each intestinal specimen. Tissues were allowed to equilibrate for 45 min prior to the start of the experiments.

Each segment was exposed to increasing concentrations (0.01-100 μM) of the muscarinic agonist, carbachol (CCh) (Cayman Chemical, Ann Arbor, Michigan). Subsequently, the concentration-response curves were constructed cumulatively and plotted into a nonlinear regression model (fitted to a sigmoidal equation) to calculate EC50 and maximal tension (E_{max}) values. Moreover, neuronally mediated contractions were obtained by Electric Field Stimulation (EFS, 0–40 Hz; 1-ms pulse duration, 10-s pulse train, 40 V) using platinum bipolar co-axial electrodes, attached to an MDE electronic stimulator (MDE Research). Contractile responses were expressed as g tension/g tissue weight of the small intestine. Frequency–response curves were repeated in the presence of tetrodotoxin (Tocris Biosciences, Bristol, UK) (TTX; 1 μM) to identify if the response was of neuronal origin.

Non-adrenergic non-cholinergic (NANC) neuromuscular responses were measured at a frequency of 10 Hz after an incubation period of 20 min with atropine (Cayman Chemical) (1 μM) and guanethidine (Cayman Chemical) (1 μM). To evaluate the nitrergic component of the inhibitory neurotransmission, tissues under NANC conditions were additionally incubated with 100 μM of L-Nω-Nitroarginine methyl ester chloridrate (L-NAME, a non-selective NOS inhibitor) (Sigma Aldrich), or 10 μM 1400W (a selective iNOS inhibitor) (Sigma Aldrich) for 20 minutes before a 10 Hz EFS. The on-inhibitory response was quantified by calculating the area under the curve (AUC), defined as the integrated area under waves and normalized per g tissue weight. The effects of NOS inhibitors were expressed as percentage variation compared to the total relaxation in NANC w/o inhibitors.

To evaluate the tachykinergic component of the small intestine contraction, the 10 Hz EFS-mediated off-contraction was assessed in presence of L-NAME, under NANC conditions. The off-responses were expressed as g tension/g tissue weight of small intestine segments.

Statistical analysis

All results were reported as mean ± standard error of the mean (SEM), except for the geometric center, which is presented as median and range (minimum–maximum), of at least 5 experiments. Statistical significance was calculated by one-way ANOVA followed by Tukey's post-hoc test or by two-way ANOVA for multiple variables, where appropriate. The statistical significance (P values < 0.05) was assessed using GraphPad Prism version 7.00 (GraphPad Software, La Jolla, California).

RESULTS

General observation

Histological assessment of IR injury

After occlusion of the terminal branch of the superior mesenteric artery, the corresponding small intestinal segment became purple and returned to a normal pink color after blood flow restoration. Once awake, animal recovered from anesthesia were active, ate normally and did not show any sign of distress. A gross visual inspection of the ischemic regions did not reveal any abnormality compared to sham-operated and un-operated control animals at all times. The administration of 25 mg/kg of 4-MU did not induce significant gross morphology changes in small intestinal segments obtained from animals subjected to IR injury, sham-operated and controls, versus the respective untreated groups. However, in all experimental groups treated with the HA synthesis inhibitor, intestinal segments and mesenteric attachments were looser than in the untreated groups. After 24 hours of reperfusion following ischemia, myenteric neurons both in the submucosal and myenteric plexus showed signs of cellular suffering, displaying swollen soma, cytoplasm vacuolization and ill-defined cellular membrane with respect to control preparations (Figure 8 C). Nuclear inclusions were sometimes observed. The smooth muscle layer was also affected by the IR injury, displaying cytoplasmic vacuolization and spaces between cells in some regions of *muscularis propria* with respect to control segments (Figure 8 A, C). Nuclear inclusions were sometimes present. Mucosa and serosal epithelium did not display prominent histological abnormalities. In sham-operated animals, histological features of neurons or muscle cell cellular suffering were rarely observed. In control and sham-operated groups, 4-MU treatment did not modify the architecture of myenteric neurons (Figure 8 B). After the 4-MU treatment, cytoplasmic vacuolization and spaces were still visible in some regions of the *muscularis propria* and within the myenteric plexus. Moreover, myenteric neurons of 4-MU treated animals showed signs of nuclear suffering and cellular shrinking, although to a minor extent, than in the IR untreated group. (Figure 8 C, D).

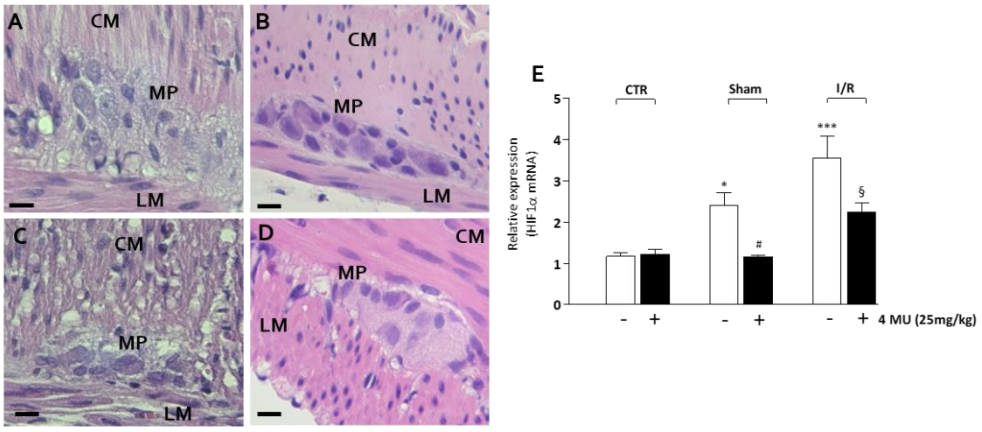

Figure 8. (A-D) Smooth muscle cells in the circular (CM) and longitudinal (LM) layers and myenteric plexus (MP) of CTR (**A**), CTR 4-MU treated (**B**), IR (**C**) and IR 4-MU treated (**D**) rat small intestine (HE, original magnification 600x; bar: 0.01mm). **(E)** RT-PCR quantification of HIF1α mRNA in rat small intestine LMMPs in the different experimental groups. Values are expressed as means±SEM. N=5 rats/group. **P*<0.05, ****P*<0.001 vs CTR; #P<0.05 vs Sham; §P<0.05 vs IR by one way ANOVA followed by Tukey's test. Relative gene expression was determined by comparing $2^{-\Delta\Delta Ct}$ values normalized to β-actin.

Neutrophil infiltration, myeloperoxidase activity and HIF-1α levels

Immunohistochemical analysis of MPO activity revealed an increase in the number of neutrophil cells per field in the submucosal and *muscularis propria* layers and showed a trend towards an increase in the myenteric plexus of animals subjected to ischemia followed by 24 hours of reperfusion with respect to control preparations (Figure 9 D-F). Also in the sham-operated group, the number of neutrophils increased significantly in the submucosal layer and showed a trend towards an increase in the myenteric plexus and *muscularis propria* layer, with respect to controls (Figure 9 D- F). Neutrophil infiltration in the mucosa was not significantly different compared to controls (Figure 9 C). In the IR group, after 4-MU treatment, the number of neutrophils infiltrating the mucosa, the *muscularis propria* and myenteric ganglia was significantly reduced with respect to the IR untreated group, and was also reduced, but not to a significant extent in the submucosal layer (Figure 9 A, B, C-F). In the IR group, the number of infiltrating MPO^+ cells also decreased in the submucosal layer, although not to a significant extent with respect to the untreated IR group (Figure 9 D).

In the control and sham-operated groups, 4-MU treatment reduced neutrophil infiltration in all intestinal layers, reaching significant values only in the mucosa of control preparations, with respect to the relative un-treated groups (Figure 9 C-F).

In small intestine LMMPs obtained from the sham-operated and IR groups, the expression of HIF-1α mRNA significantly increased with respect to control preparations. After 4-MU treatment, HIF-1α mRNA levels were significantly reduced in both the sham-operated and IR group, reaching values not significantly different those obtained in control preparations (Figure 8 E).

In vivo 4-MU treatment regulates HA levels in rat small intestine after IR injury

In accordance with previously described results on the presence of HA in the small intestine [15], the evaluation of hyaluronan distribution in the whole wall paraffin-embedded section showed a strong staining of HABP in the *lamina propria* of villous and in the submucosal layer, with a particularly remarkable expression in *tunica media* and *tunica adventitia* of large and medium-sized vascular vessels together with a well-structured staining around and within both submucosal and myenteric ganglia. A fainter labelling was found in the *muscolaris propria* (Figure 10 A-C). In all the experimental groups no significant alterations in the mucosal HA level were observed, as indicated also by the measurements of labelling density index values. Similar results were obtained after the 4-MU treatment (Figure 10 A-C, D). Conversely, in the submucosal layer, a higher HA level was found in IR groups with respect to CTR and sham-operated animals, (Figure 10 B, E). No significant differences were detected in the sham-operated groups instead. Moreover, after IR injury, the treatment with 4-MU restored HA staining to control values (Figure 10 C, E).

As regard the *muscolaris propria* layer, a significant increase in HA level was observed after the IR injury with respect to control animals. After the treatment with 4-MU, the IR animals displayed a reduced HA expression with respect to the untreated group. A less but still significant enhancement was detected in *muscolaris propria* of sham-operated group compared to controls (Figure C, F).

In LMMP whole-mount preparations of control animals, HA staining was detected on the surface of myenteric ganglia and along interconnecting fibre strands (Figure 11 A, E). In addition, a faint HA labelling was found in myenteric neuron cytoplasm and in the perineuronal space, as demonstrated by co-staining with the pan neuronal marker HuC/D (Figure 11 B-D, F-H). After 4-MU treatment, HA labelling in myenteric ganglia was fainter, as suggested by a decrease,

although not significant, in the density index (Figure 11 Q). The intensity and distribution of HA staining in the myenteric plexus of sham-operated animals was not significantly different with respect to control preparations with or without the 4-MU treatment (Figure 11 E-H, Q).

After IR injury, HA density index in myenteric ganglia significantly increased with respect to control and sham-operated groups, as shown in panels I, J and Q of Figure 11. In addition, HA staining in myenteric neuron cytoplasm and in perineuronal space was conserved and displayed higher intensity with respect to controls (Figure 11 J, L). HA labelling in LMMP preparations obtained from 4-MU-treated rats subjected to IR injury was faint and the density index was significantly reduced with respect to the IR group, reaching values not significantly different from those obtained in control and sham-operated animals (Figure 11 M, N, Q).

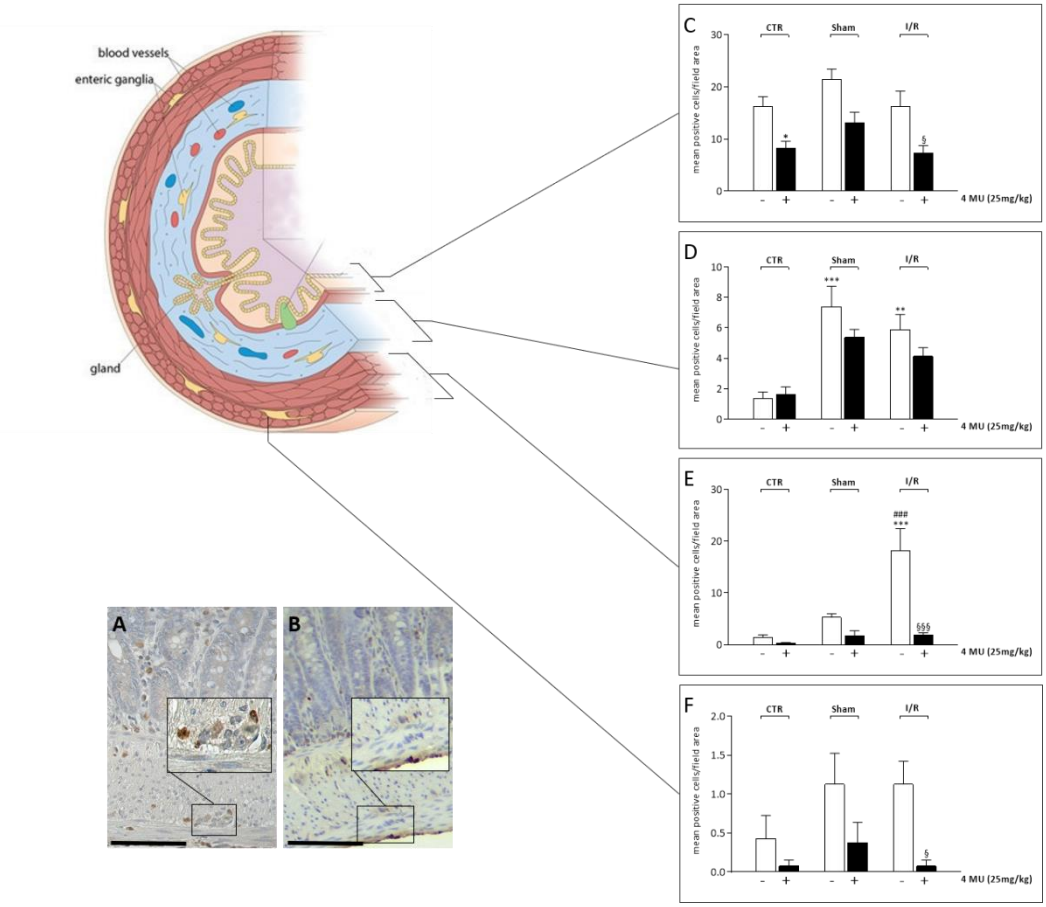

Figure 9. (**A**-**B**) MPO immunohistochemical staining of whole wall rat small intestine obtained from IR (**A**) and 4-MU treated IR animals (**B**). Neutrophils are well marked (brown) and their count is easy for all the layers. (**C**-**F**) number of neutrophils infiltrated in mucosa (**C**), Submucosa (**D**), *muscolaris propria* (**E**) and myenteric plexus (**F**) of all the experimental groups. Values are expressed as means±SEM. N=5 rats/group. **P*<0.05, ***P*<0.01, ****P*<0.001 vs CTR; ###P<0.001 vs Sham; §P<0.05, §§§P<0.001 vs IR by one way ANOVA followed by Tukey's test.

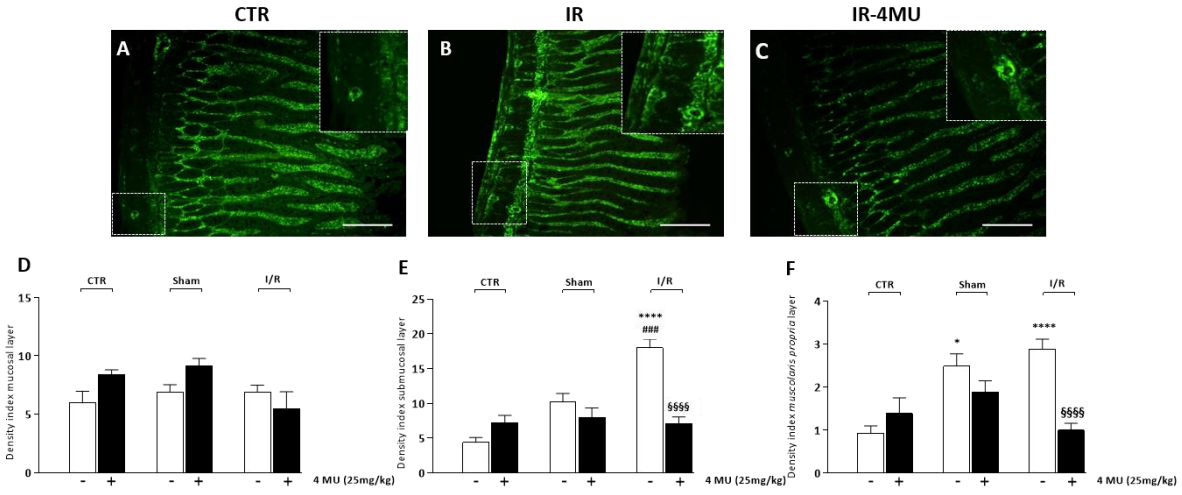

Figure 10. (**A-C**) Representative confocal microphotographs showing HA staining of the whole-wall rat small intestine in control (**A**), after ischemia followed by 24 hours of reperfusion (**B**) and in the IR group treated with 4-methylumbelliferone (**C**). Bar 100 µm. (**D-F**) HA density index of HA in paraffin-embedded mucosa (**D**), submucosa (**E**) and *muscolaris propria* (**F**) preparations obtained by all the different experimental groups with and without treatment of 4-MU, as indicated on bottom of bars. Data are reported as mean±SEM. ****P<0.0001 vs CTR, ###P<0.001 vs Sham, §§§§P<0.0001 vs IR by one-way ANOVA with Tukey's post hoc test, N=5 rat/group.

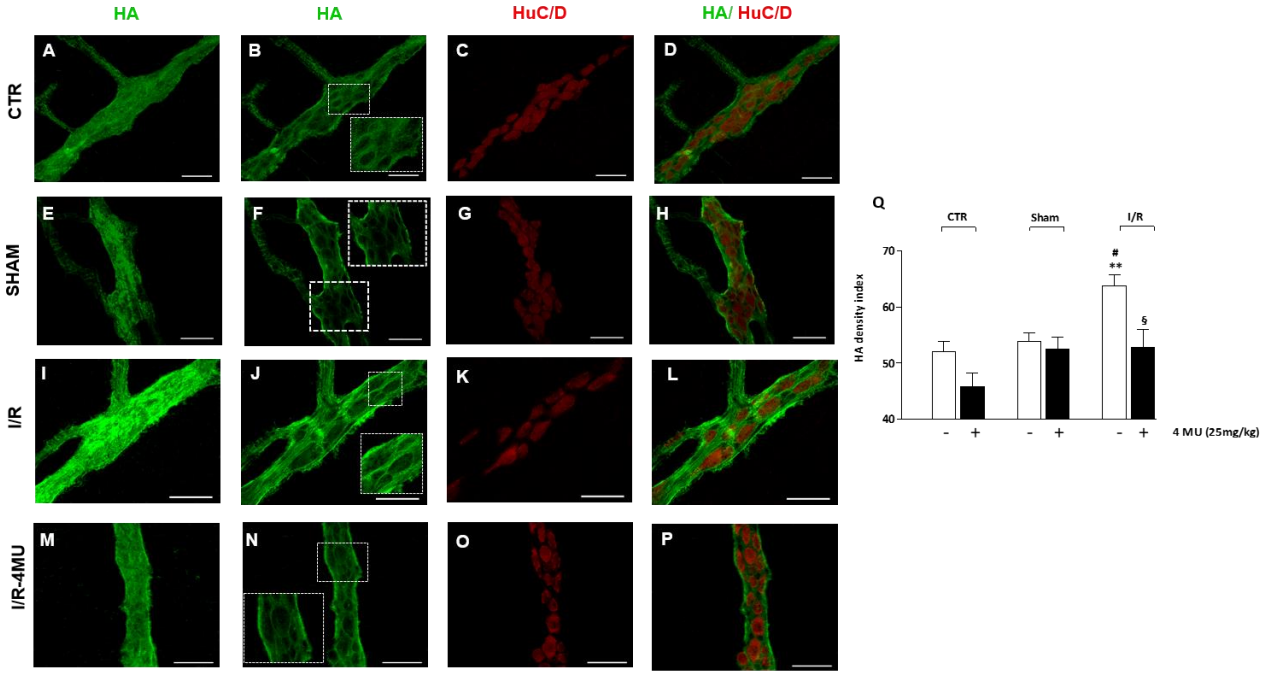

Figure 11. In vivo 4-MU treatment regulates HA levels in rat small intestine LMMPs after IR injury. (**A-P**) Representative confocal microphotographs showing HA staining of the rat small intestine myenteric plexus in control conditions (**A-D**), sham-operated animals (**E-H**), after ischemia followed by 24 hours of reperfusion (**I-L**) and in the IR group treated with 4-methylumbelliferone (**M-P**). In all groups, HA intensely stained the surface of the myenteric ganglia and interconnecting fibers (**A, E, I, M**). In myenteric ganglia median sections, HA immunofluorescence was prevalently found in neuronal soma and in the perineuronal space (**B, F, J, N**), as demonstrated by double-staining with the pan-neuronal marker, HuC/D. Bar 50 µm. **(Q)** HA density index of HA in LMMP preparations obtained the different experimental groups with and without treatment of 4-MU, as indicated on bottom of bars. Data are reported as mean±SEM. **P<0.001 vs CTR, #P<0.05 vs sham-operated, §P<0.05 vs IR by one-way ANOVA with Tukey's post hoc test, N=5 rat/group.

In good agreement with HABP staining, ELISA assay revealed an increased HA levels in submucosal layer of animals subjected to IR injury. In particular, we assessed an IR-induced HA enhancement of around 45% with respect to CTR and sham-operated groups. This HA deposition was significantly reduced by administration of 4-MU, reaching values not significantly different compared to control preparations (Figure 12 B). Data obtained from sham-operated animals were not significantly different from those obtained in control preparations, both with and without 4-MU treatment (Figure 12 B). Similarly, in LMMP preparations obtained from sham-operated animals, HA levels measured by ELISA assay were not significantly different from those obtained in control preparations, both with and without 4-MU treatment (Figure 12 C), whereas a 478% increase of the GAG levels was obtained from the IR group versus control and sham-operated groups (Figure 12 C). HA levels in the IR 4-MU treated group remained significantly elevated with respect to controls and sham-operated groups but were reduced with respect to the untreated IR group (Figure 12 C). Regarding the mucosal layer, no differences were detected in HA levels in all the experimental groups (Figure 12 A).

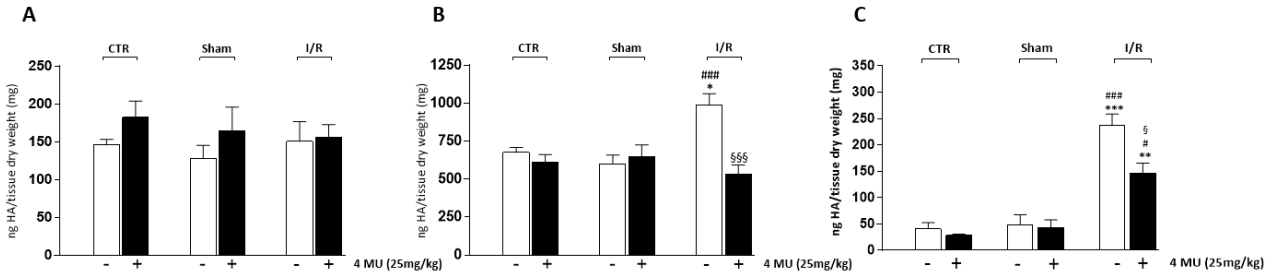

Figure 12. (**A-C**) Quantification of HA levels by ELISA assay in small intestine mucosa (**A**), submucosa (**B**) and *muscolaris propria* (**C**) preparations obtained from the different experimental groups with and without treatment of 4-MU, as indicated on bottom of bars. HA levels are expressed as ng of HA normalized per mg of dry tissue weight. *P<0.05 and ***P<0.001 vs CTR; #P<0.05 and ###P<0.001 vs sham-operated, §P<0.05 and §§§P<0.001 vs IR by one-way ANOVA with Tukey's post hoc test, N=6 rat/group.

Influence of 4-MU treatment on IR induced changes of HAS1 and HAS2 expression in rat small intestine

In rat small intestine LMMP preparations obtained from all experimental groups, immunohistochemical experiments revealed the presence of HAS1 in myenteric ganglia and along interconnecting fibers (Figure 13 A-I). HAS1 staining was detected in the cytoplasm and cytosolic membrane of large-, medium- and small-sized ovoid neurons. Intense HAS1 labelling was also found in enteric glial cells surrounding myenteric ganglia (Figure 13 A-I). In LMMP preparations obtained from the IR group, the number of $HAS1^+$ myenteric neurons was significantly higher than in the control and sham-operated groups and such enhancement was reduced, although not significantly with respect to the IR untreated group (Figure 13 J). In the 4MU-treated IR group, the number of $HAS1^+$ myenteric neurons remained significantly higher versus controls, but not versus sham-operated animals (Figure 13 J). In the sham-operated and IR groups, HAS1 mRNA levels significantly increased with respect to control preparations (Figure 13 K). In the IR group, HAS1 mRNA levels were also significantly higher than in preparations obtained from sham-operated animals. In the sham-operated group after 4-MU treatment, HAS1 mRNA levels were still significantly higher with respect to controls and similar to those obtained in the respective untreated groups. In the IR group after 4-MU treatment, HAS1 mRNA levels were

significantly higher with respect to controls, but not versus the sham-operated group, and were slightly, but not significantly, reduced with respect to the IR untreated group (Figure 13 K).

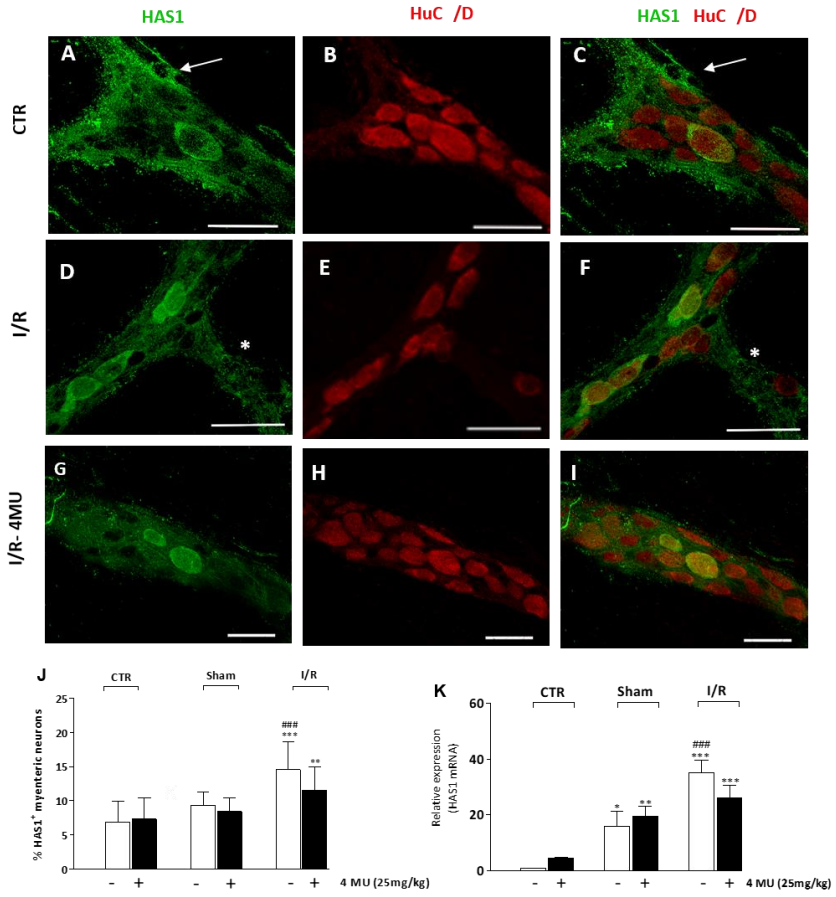

Figure 13. Enhancement of HAS1 expression in the rat small intestine myenteric plexus is regulated by 4-MU treatment. (**A-I**) Confocal images showing co-localization of HAS1 with the pan neuronal marker HuC/D in myenteric neurons of CTR animals (**A-C**), after IR injury (**D-F**) and in IR group treated with 4-MU (**G-H**). HAS1 stained the soma and cytoplasmic membranes of ovoid myenteric neurons, interconnecting fibers (*) and enteric glial cells (arrow). Panel **J** shows the percentage of myenteric neurons co-staining for HuC/D and HAS1. Bar 50 μm. **P<0.01, ***P<0.001 vs CTR, $^{###}$P<0.001 vs sham-operated by one-way ANOVA with Tukey's post hoc test. N= 5 rat/group (**K**) HAS1 mRNA levels in LMMP preparations obtained from the different experimental groups. Histograms show HAS1 relative gene expression determined by comparing $2^{-\Delta\Delta Ct}$ values normalized to β-actin. *P<0.05, **P<0.01, ***P<0.001 vs CTR, $^{###}$P<0.001 vs sham-operated by one-way ANOVA with Tukey's post hoc test. N=5 rat/group.

HAS2 immunoreactivity was found in the cytoplasm, nuclear and cytosolic membranes of few medium-sized ovoid neurons of control rat small intestine myenteric plexus (Figure 14 A-I). The number of HAS2$^+$ myenteric neurons significantly increased with respect to sham-operated and control animals after ischemia followed by 24 hours of reperfusion (Figure 14 D-F, J). In this latter condition, 4-MU treatment induced a slight reduction of the number of HAS2$^+$ myenteric neurons with respect to the untreated IR group, which, however, remained significantly higher than in controls and sham-operated animals (Figure 14 J). In LMMP preparations obtained from animals undergoing IR, HAS2 mRNA levels significantly increased with respect to both control and sham-operated preparations (Figure 14 K). 4-MU treatment did not modify HAS2 mRNA expression in both control and sham-operated preparations. In the IR group, 4-MU treatment significantly reduced IR-induced enhancement of HAS2 transcripts with respect to the relevant

untreated IR group, reaching values not significantly different versus those obtained in control and sham-operated rats (Figure 14 K).

HAS3 mRNA levels were not measurable by qRT-PCR in LMMPs of all experimental groups.

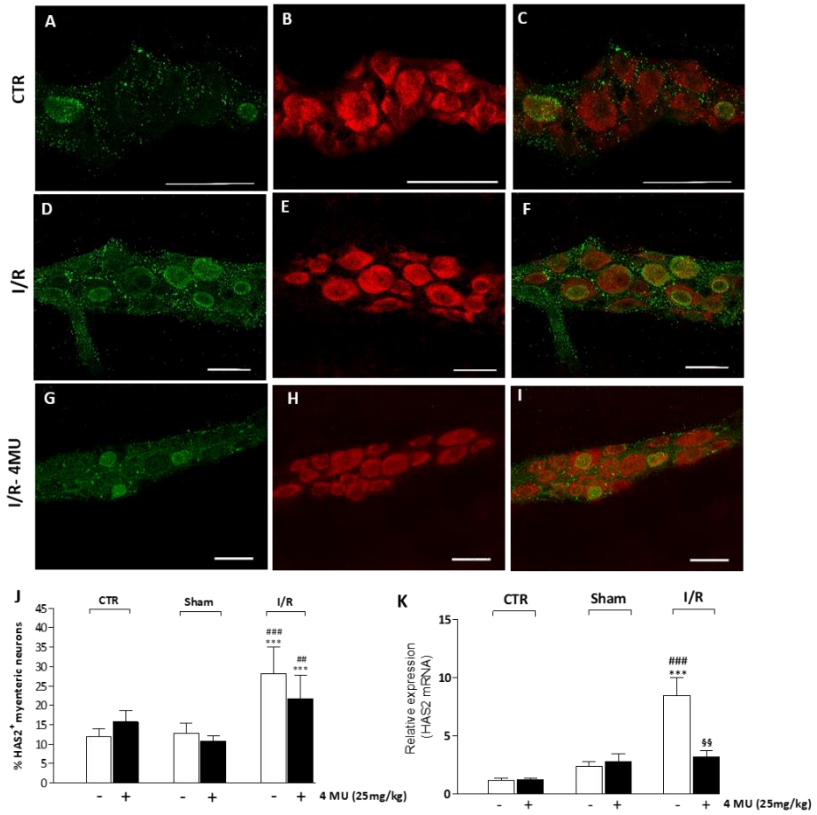

Figure 14. Enhancement of HAS2 expression in the rat small intestine myenteric plexus is regulated by 4-MU treatment. (**A-I**) Confocal images showing co-localization of HAS2 with HuC/D in myenteric neurons of CTR animals (**A-C**), after IR injury (**D-F**) and in IR group treated with 4-MU (**G-I**). HAS2 stained the soma of ovoid myenteric neurons. Panel **J** shows the percentage of myenteric neurons co-staining for HuC/D and HAS2. Bar 50 μm. ***P<0.001 vs CTR, ##P<0.01 and ###P<0.001 vs sham-operated by one-way ANOVA with Tukey's post hoc test. N= 5 rat/group. (**K**) mRNA levels of HAS2 in LMMP preparations obtained from the different experimental groups. Histograms show HAS2 relative gene expression determined by comparing $2^{-\Delta\Delta Ct}$ values normalized to β-actin. ***P<0.001 vs CTR, ###P<0.001 vs sham-operated, §§P<0.01 vs IR by one-way ANOVA with Tukey's post hoc test. N=5 rat/group.

Similarly, to the results obtained in the myenteric plexus, in all experimental groups, immunohistochemical experiments conducted on rat small intestine submucosal plexus preparations revealed the presence of HAS2 in the cytoplasm, nuclear and cytosolic membranes of few medium-sized ovoid neurons animals (Figure 15 A-F). In submucosa whole-mount preparations obtained from the IR group, the density index of HAS2 submucosal neurons was significantly higher than in the control and sham-operated groups (Figure 15 D-F, G). Such enhancement was significantly reduced after 4-MU treatment with respect to the relative IR group (Figure 15 G). Accordingly, in submucosal layer preparations, HAS2 mRNA levels significantly increased after the IR injury with respect to both control and sham-operated preparations (Figure 15 H), and such enhancement was significantly reduced by 4-MU treatment, reaching values not significantly different versus those obtained in control and sham-operated groups. 4-MU treatment did not modify HAS2 mRNA expression in both control and sham-operated preparations (Figure 15 H).

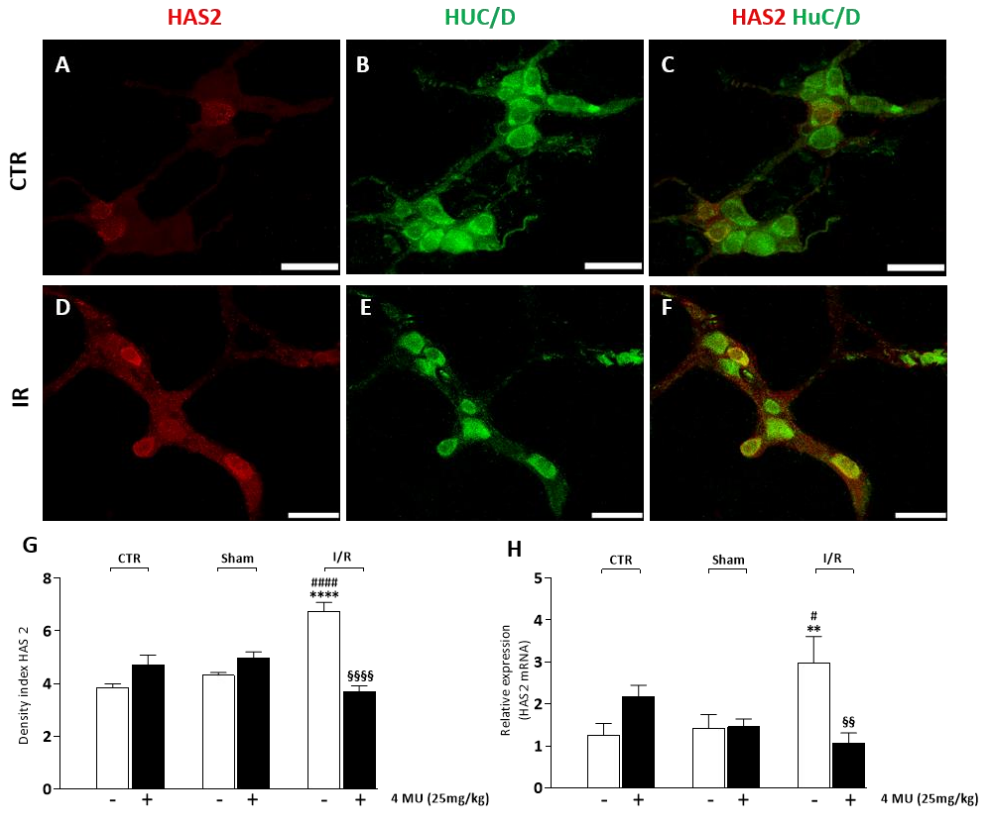

Figure 15. Enhancement of HAS2 expression in the rat small intestine submucosal plexus is regulated by 4-MU treatment. (**A-F**) Confocal images showing co-localization of HAS2 with HuC/D in submucosal neurons of CTR animals (**A-C**) and after IR injury (**D-F**). HAS2 stained the soma of ovoid myenteric neurons. Bar 50 µm. (**G**) Density index of submucosal neurons from whole-mount preparations of all the experimental groups. ****P<0.0001 vs CTR, ####P<0.0001 vs sham-operated, §§§§P<0.0001 vs IR by one-way ANOVA with Tukey's post hoc test. N= 5 rat/group. (**H**) mRNA levels of HAS2 in submucosal preparations obtained from the different experimental groups. Histograms show HAS2 relative gene expression determined by comparing $2^{-\Delta\Delta Ct}$ values normalized to β-actin. **P<0.01 vs CTR, #P<0.05 vs sham-operated, §§P<0.01 vs IR by one-way ANOVA with Tukey's post hoc test. N=5 rat/group.

Modulation of HA molecular targets in in rat small intestine after IR injury

Submucosal layer

qRT-PCR, immunoblotting and immunofluorescence experiments were performed in submucosal layer, confirming the presence of receptors regulated by HA. TLR2, TLR4 and CD44 labelling was detected in the cytoplasm of submucosal neurons and among the interconnecting fiber strands. Moreover, all the antibody-stained enteric glial cells in agreement with other reports (Figure 16 A-I; 17 A-I).

In the submucosal plexus after an ischemic insult following by 24 hr of reperfusion, TLR2 expression significantly increased with respect to control group. In this condition, the administration of 4-MU reduced, although not significantly, the level of TLR2 transcript compared to the relevant untreated group. 4-MU treatment did not modify TLR2 mRNA expression in both control and sham-operated preparations (Figure 16 L). These results were confirmed by protein expression analysis. Animals subjected to IR injury exhibit an enhanced level of TLR2 protein, which was decreased by 4-MU, whereas in the control and sham-operated groups, TLR2 protein levels were unaffected by the treatment with the HA synthesis inhibitor (Figure 16 O).

None of the experimental groups revealed significant IR injury- or 4-MU- mediated regulation of TLR4 mRNA and protein expression (Figure 16 M, P).

The protein levels of CD44 receptor, were significantly higher in IR group compared to controls and sham-operated animals (Figure 16 Q), although significant IR-induced changes in mRNA expression level were not found (Figure 16 N). 4-MU administration to IR rats induced a significant decrease of the protein with respect to the untreated IR group (Figure 16 Q).

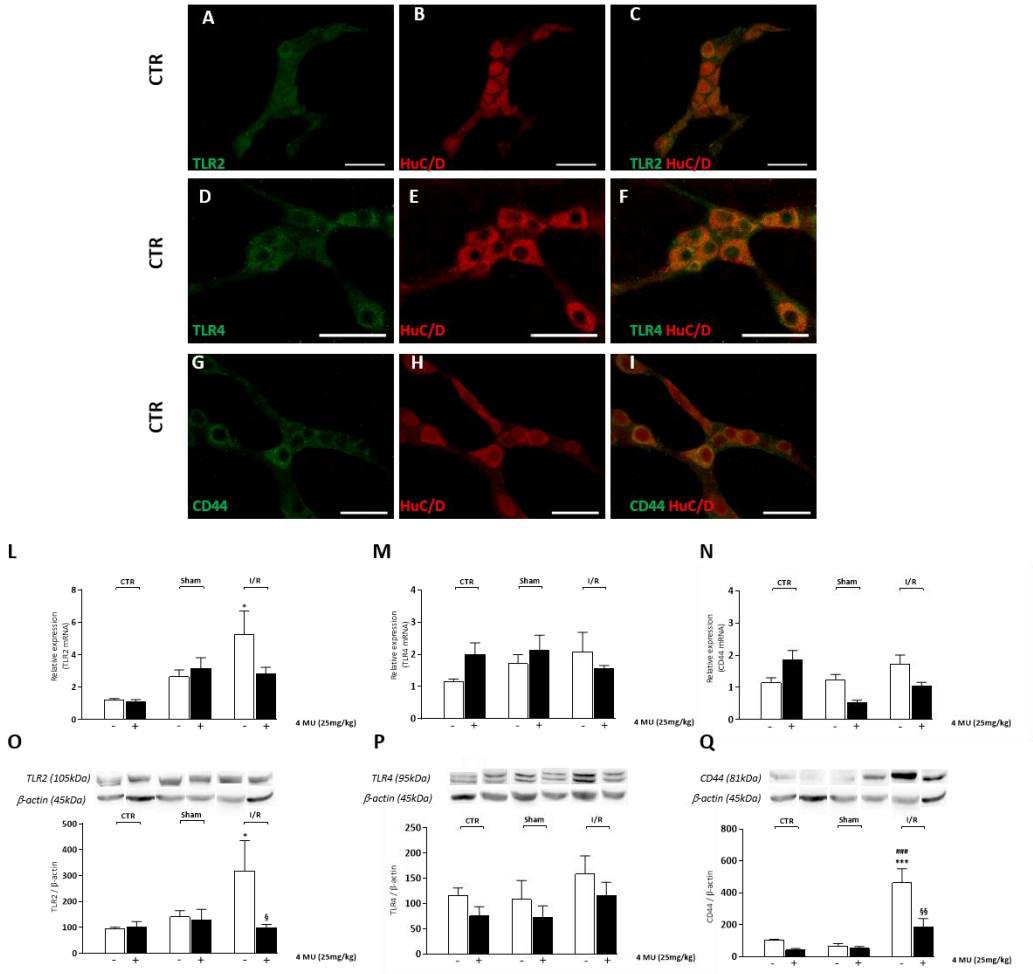

Figure 16. Expression of TLR2, TLR4 and CD44 in the rat small intestine submucosal layer. (**A-I**) Confocal images showing distribution of TLR2 (**A-C**), TLR4 (**D-F**) and CD44 (**G-I**) in HuC/D$^+$ submucosal neurons of CTR animals. Bar 50 µm. (**L-N**) Relative expression of TLR2 (**L**), TLR4 (**M**) and CD44 (**N**) in submucosal layer preparations obtained in all experimental conditions. *P<0.05 vs CTR by one-way ANOVA with Tukey's post hoc test. Values are expressed as mean±SEM, N= 5 rat/group. (**O-Q**) Protein expression of TLR2 (**O**), TLR4 (**P**) and CD44 (**Q**) in submucosa preparations obtained from all experimental groups. *P<0.05, ***P<0.001 vs CTR, ###P<0.001 vs sham-operated, §P<0,05, §§P<0,01 vs IR by one-way ANOVA with Tukey's post hoc test. Values are expressed as mean±SEM, N= 5 rat/group.

LMMP

In rat small intestine LMMP preparations, significantly higher TLR2 mRNA and protein levels were observed in IR animals compared to both control and sham-operated groups. Both transcript and protein levels were reduced after 4-MU administration. 4-MU did not affect TLR2 mRNA levels in control and sham-operated animals (Figure 17 L, O). Both the qRT-PCR and immunoblotting results revealed a remarkable increase in TLR4 mRNA and protein expression levels compared to control and sham-operated groups. In IR animals, 4-MU treatment reduced both values to those obtained in controls (Figure 17 M, P). The IR injury induced an increased mRNA expression of CD44 receptor compared to the control group which was not regulated by the treatment with 4-MU (Figure 17 N). This enhancement was confirmed by immunoblotting experiments, showing a higher amount of the receptor protein in IR animals with respect to CTR and sham-operated group. In none of the experimental conditions 4-MU significantly affected the protein expression, although in the IR group a trend towards a reduction was observed (Figure 17 Q).

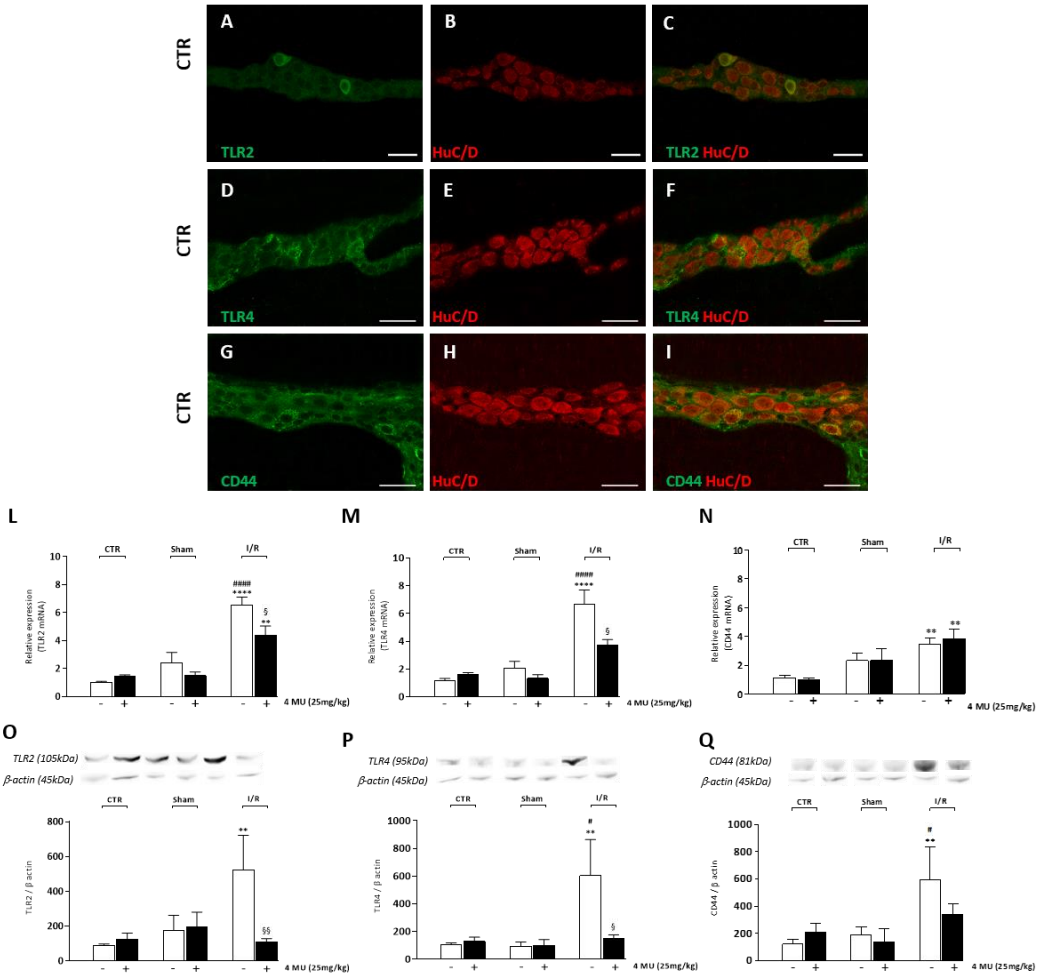

Figure 17. Expression of TLR2, TLR4 and CD44 in the rat small intestine LMMPs. (**A-I**) Confocal images showing distribution of TLR2 (**A-C**), TLR4 (**D-F**) and CD44 (**G-I**) in HuC/D$^+$ myenteric neurons of CTR animals. Bar 50 μm. (**L-N**) Relative expression of TLR2 (**L**), TLR4 (**M**) and CD44 (**N**) in LMMP preparations obtained from all the experimental conditions. **P<0.01, ****P<0.0001 vs CTR, ####P<0.0001 vs sham-operated, §P<0,05 vs IR by one-way ANOVA with Tukey's post hoc test. Values are expressed as mean±SEM, N= 5 rat/group. (**O-Q**) Protein expression of TLR2 (**O**), TLR4 (**P**) and CD44 (**Q**) in LMMP preparations obtained from all the experimental conditions. **P<0.01 vs CTR, #P<0.05 vs sham-operated, §P<0,05, §§P<0,01 vs IR by one-way ANOVA with Tukey's post hoc test. Values are expressed as mean±SEM, N= 5 rat/group.

Efficiency of the gastrointestinal transit after IR injury with and without 4-MU treatment

The efficiency of gastrointestinal transit was evaluated by calculating of the percentage of fluorescence distribution along the gastrointestinal tract 1 hr after the administration of a FITC-dextran bolus by oral gavage. The results revealed that in the IR group, the GI transit significantly decreased, as shown by the higher content of non-absorbable FITC-labelled dextran in the upper part of the gastrointestinal tract (Figure 18 A). Accordingly, IR injury induced a significant reduction of the value of the geometric center of fluorescence distribution (GC) with respect to values obtained in control and sham-operated animals. In sham-operated animals, the GC was not significantly different from the value obtained in control animals. 4-MU treatment did not significantly modify both the efficiency of the gastrointestinal transit and the value of the GC in control and sham-operated groups. In the IR group, treatment with 4-MU, induced a significant reduction of the GC with respect to both control and sham-operated untreated animals (Figure 18 B). In addition, in the IR group, 4-MU treatment induced a significant delay in gastric emptying with respect to both control and sham-operated groups (Figure 18 C).

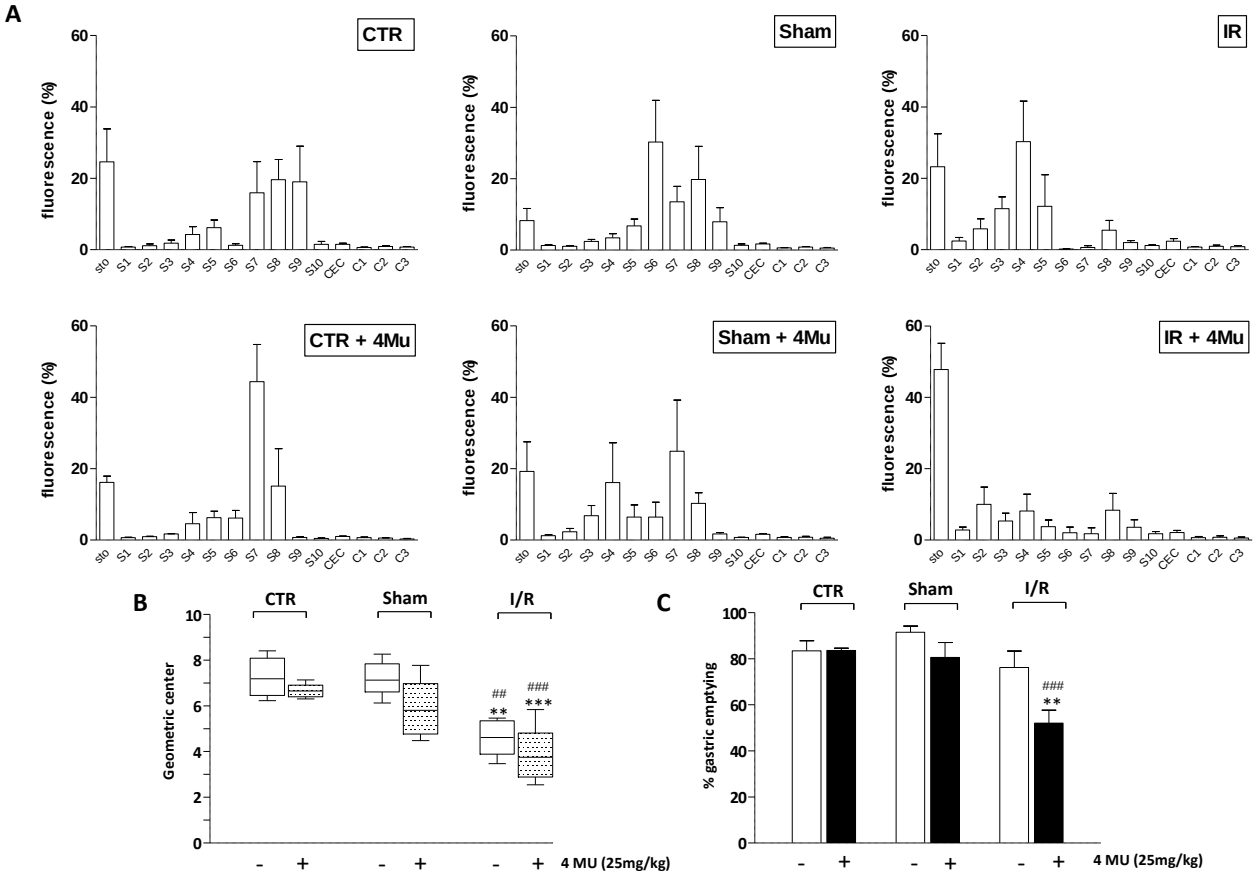

Fig 18. 4-MU treatment affects G/I transit and gastric emptying after IR injury in the rat. (**A**) Percentage of non-absorbable FITC-dextran distribution along the gastrointestinal (GI) consisting of stomach (Sto), small bowel (S 1–10), caecum (Cec) and colon (C 1–3); (**B**) geometric centre (GC) of non-absorbable FITC-dextran distribution; **(C)** Percentage of gastric emptying in the different experimental groups. Data are reported as mean±SEM for panels (A) and (C) and as median, minimum, maximum, upper and lower quartiles for panel (B). **P<0.01 and ***P<0.001 vs CTR, ##P<0.01 and ###P<0.001 vs sham-operated and by one-way ANOVA with Tukey's post hoc test. N=5 rat/group.

IR injury alters excitatory neuromuscular contractility: effect of 4-MU treatment

The effects of IR injury on the rat small intestine neuromuscular function were examined *in vitro* by measuring tension changes of the longitudinal muscle contractile activity, which underlies the preparative phase of the peristaltic reflex and is synchronous with circular muscle contraction during peristalsis, thus influencing the whole propulsive bowel activity [203]. In all experimental groups, small intestine segments showed spontaneous contractile activity consisting of phasic contractions, displaying a significantly reduced amplitude (tension) in the sham-operated (7,09 ± 0,740) and IR (7,56 ± 0,834) groups with respect to control animals (10,43 ± 0,920) (Figure 19 A). In all experimental groups, 4-MU treatment did not modify the amplitude of spontaneous contractions, which remained significantly lower in the sham-operated (7,13 ± 0,592) and IR treated groups (6,67 ± 0,774) with respect to the control group (Figure 19 A). The frequency of spontaneous basal contraction was similar in all the experimental groups (Figure 19 B).

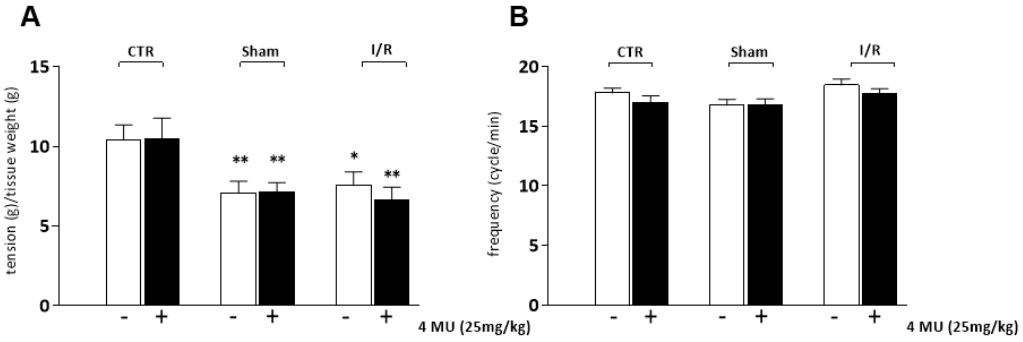

Figure 19. Basal spontaneous contractile activity of small intestine from all experimental groups. (**A**) Basal tension of all the experimental groups. Data are expressed as mean±SEM. *P<0.05, **P<0.01 vs CTR. (**B**) Frequency of spontaneous contraction of all the experimental groups measured as cycle/min. Data are expressed as mean±SEM.

To evaluate changes in the excitatory neuromuscular response, cumulative concentration–response curves to the non-selective cholinergic agonist, carbachol (CCh), were performed on longitudinally oriented small intestine segments from all experimental groups. IR induced a significant downward shift of the concentration-response curve to CCh with a decrease in maximum response (E_{max}) compared to values obtained in control and sham-operated. In the sham-operated group the contractile response curve to CCh was superimposable with the response observed in the control group (Figure 20 A). In the control group, 4-MU treatment induced a significant increase in the E_{max} with respect to the value obtained in untreated controls (Figure 20 A). The concentration-response curves to CCh were not significantly modified both in the sham-operated and in the IR group after 4-MU treatment (Figure 20 A). The potency of CCh in inducing concentration-dependent contractile responses in the control group was [0.17 (0.10-0.28) µM, n=5)], and was not significantly different from CCh potency values obtained in the other experimental groups [CTR-4MU: 0.34 (0.25-0.46) µM, n=5; sham-operated: 0.20 (0.15-0.27) µM, n=5; sham-operated-4MU: 0.15 (0.11-0.20) µM, n=5; IR: 0.11 (0.036-0.32) µM, n=5; IR-4MU: 0.15 (0.06-0.36) µM, n=5]. To further investigate potential changes in the excitatory contractile function, the effect of EFS was evaluated at increasing frequencies of stimulation on small intestine preparations. In the IR and sham-operated groups, EFS-elicited contractions were significantly reduced with respect to controls (Figure 20 B). In the control and sham-operated groups, 4-MU treatment did not modify the responses to EFS compared to those observed in the respective untreated groups. EFS-induced contractions in the 4-MU treated sham-operated group remained significantly lower than in controls. In the IR group, after 4-MU treatment, EFS-evoked

responses of the small intestine longitudinal muscle were higher with respect to those obtained in the respective untreated group, remaining lower compared to those obtained in the control group. EFS-mediated responses were of neuronal origin, since in all groups addition of the neuronal blocker tetrodotoxin (1 μM) totally abolished the EFS-induced responses. In small intestine myenteric plexus, distribution of ChAT immunoreactivity was similar in all experimental groups as shown by the unchanged density index (Figure 20 C, D).

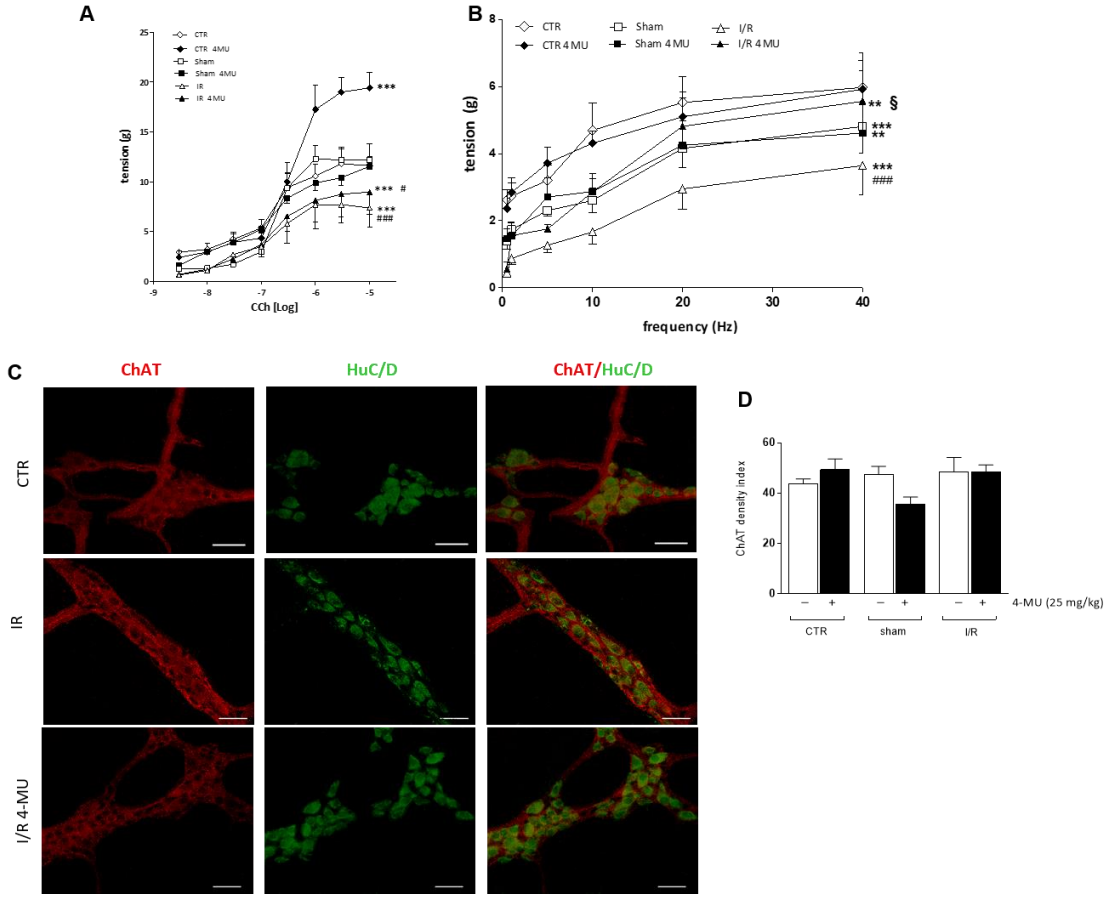

Figure 20. 4-MU treatment influences excitatory contractility in the rat small intestine. (**A**) Concentration–response curves to carbachol (CCh) of isolated rat small intestine segments in the different experimental groups (N=5 rats per group). (**B**) Neuromuscular excitatory responses induced by EFS (0.5–40 Hz) in isolated small intestine preparations obtained from the different experimental groups N=5 rat/group. (**C**) Representative confocal photomicrographs showing the distribution of ChAT (red, marker for cholinergic neurons) and HuC/D (green, pan-neuronal marker) and (**D**) Density index of ChAT labelling in myenteric neurons of LMMP preparations obtained from all experimental groups (N=5 rat/group). Data are reported as mean±SEM. **P<0.01, ***P<0.001 vs CTR; ###P<0.001 vs sham-operated; §P<0.05 vs IR by Two-way ANOVA.

IR injury influences NANC small intestine SP excitatory neurotransmission: effect of 4-MU treatment

In small intestine whole-mount preparations from all experimental groups, immunoreactivity to SP, a neuropeptide member of the tachykinin family with higher affinity for NK1, than for NK2 or NK3 receptors [43], was generally faint in the soma of myenteric neurons and more intense in nerve varicose fibers within myenteric ganglia and in interconnecting trunks along the longitudinal muscle (Figure 21 A). The percentage of SP^+ myenteric neurons and the density index of SP labelling in myenteric ganglia were similar in control animals with and without 4-MU treatment (Figure 21 B, C). In the sham-operated group, the number of SP^+ myenteric neurons as well as the density index was slightly, but not significantly, increased with respect to controls; after 4-MU treatment both values were slightly reduced (Figure 21 B, C). In the IR group, the number of SP^+ myenteric neurons was significantly higher compared to the control and sham-operated groups and the density index of SP labelling was significantly higher compared to the control groups. Both enhancements were significantly reduced by 4-MU treatment (Figure 21 B, C). To evaluate the contribution of non-cholinergic excitatory neurotransmitters to the small intestine motor function, post-stimulus excitatory off-responses were assessed in the presence of L-NAME and in non-adrenergic non-cholinergic conditions, to unmask tachykinergic nerve-evoked contractions [43]. The tachykinin-mediated response was not significantly different in control and sham-operated groups, neither before nor after the 4-MU-treatment (Figure 21 D). After IR, tachykinin-mediated contractions were significantly decreased with respect to both control and sham-operated animals and reached values similar to those observed in control 4-MU treated rats (Figure 21 D).

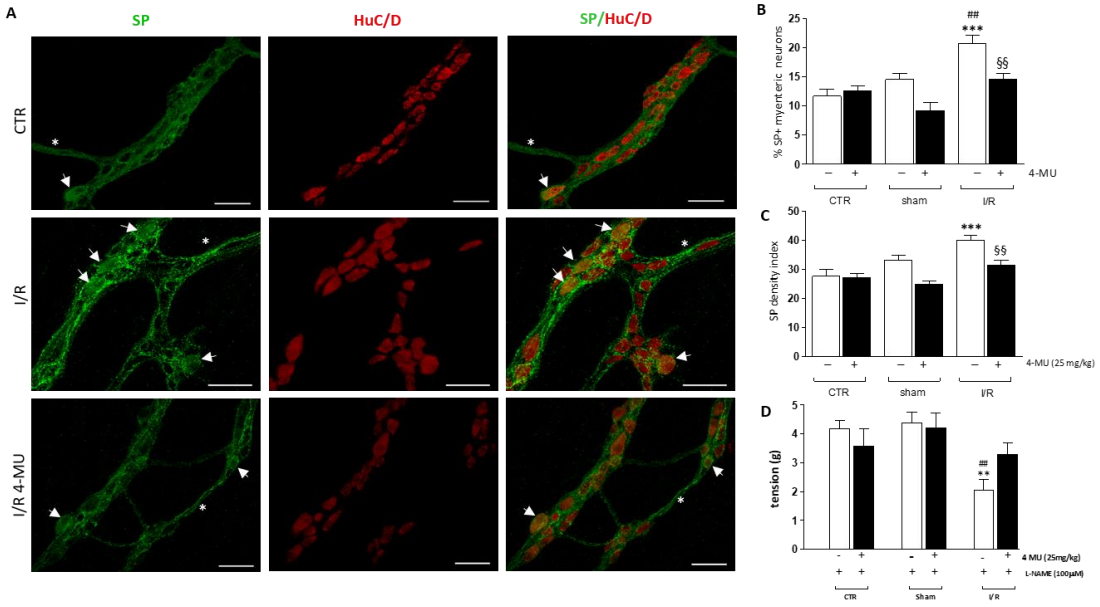

Figure 21. 4-MU modulation of tachykinergic neurotransmission in the rat small intestine after IR injury. (**A**) Representative confocal microphotographs showing the distribution of SP (green) and HuC/D (red) in rat myenteric plexus obtained from CTR, IR and IR-4MU groups. Scale bars = 50 μm. Arrows indicate SP^+-HuC/D^+ neurons, asterisk interconnecting fibers. (**B**) Percentage of SP^+-HuC/D^+ neurons with respect to total HuC/D^+ neurons and (**C**) density index of SP immunoreactivity in small intestine LMMP whole-mount preparations in the different experimental groups (N=5 rats per group). (**D**) Tachykinergic-mediated contractions evoked by 10 Hz EFS in small intestine segments in the different experimental groups (N=5 rats per group), under NANC conditions, in absence or presence of L-NAME. Data are reported as mean±SEM. Difference significance: **P<0.01, ***P<0.001 vs CTR; ##P<0.01 vs sham-operated; §§P<0.01 vs IR.

IR injury influences the inhibitory neuromuscular response: effect of 4-MU treatment

Non-adrenergic non-cholinergic (NANC) induced responses to 10 Hz transmural stimulation were evaluated in the small intestine of all groups in the presence of guanethidine and atropine. In these conditions, control and sham-operated groups, with or without 4-MU treatment, exhibited similar inhibitory relaxation responses (Figure 22 A). In IR and 4-MU-treated IR groups, NANC relaxation responses significantly decreased with respect to control values (Figure 22 A). In all the experimental groups, NO substantially contributed to the inhibitory responses, as demonstrated by the ability of the non-selective NOS inhibitor, L-NAME, to significantly reduce NANC-evoked relaxations at 10 Hz stimulation frequency by 50% with respect to the values obtained in the absence of the inhibitor (Figure 22 A). In addition, in all groups 4-MU treatment did not influence the extent of NANC relaxation in the presence of L-NAME.

Pretreatment with the selective iNOS inhibitor did not influence NANC relaxations in control and 4-MU treated control groups (Figure 22 A). In the sham-operated group and 4-MU treated sham-operated group, 1400W significantly reduced NANC relaxations with respect to values obtained in the absence of the iNOS inhibitor. In the IR group, the iNOS inhibitor decreased NANC relaxations with respect to the untreated IR group and the 1400W-treated control group, but not versus the 1400W-treated sham-operated group. After 4-MU treatment in the IR group the reduction of NANC in the presence of 1400W was not significant with respect to the untreated IR group (Figure 22 A). In the 4-MU treated IR group, NANC relaxations in the presence of 1400W were significantly lower than in the 1400W-treated control group ($P<0.05$) and did not significantly differ to the values obtained in the 1400W treated sham-operated group. In each experimental group, the effect of 1400W on NANC relaxations was not affected by 4-MU treatment. To further investigate the possible effects of 4-MU treatment on IR-induced changes in the enteric nitrergic neurotransmission, nNOS and iNOS distribution was evaluated by immunofluorescence in the myenteric plexus of all experimental groups. Immunohistochemical co-localization with HuC/D showed that the number of nNOS+ myenteric neurons was similar in all the experimental groups (Figure 22 B, C). nNOS+ neurons displayed morphological changes in both IR and 4-MU treated IR groups consisting in a significantly enhanced area/soma ratio and total area with respect to control ($P<0.001$) and sham-operated groups ($P<0.01$ and $P<0.001$) (Figure 22 D, E). In the small intestine of the IR group, the number of $iNOS^+$ neurons significantly increased with respect to control and sham-operated animals. Such enhancement was significantly reduced after 4-MU treatment compared to the untreated group (Figure 22 F, G).

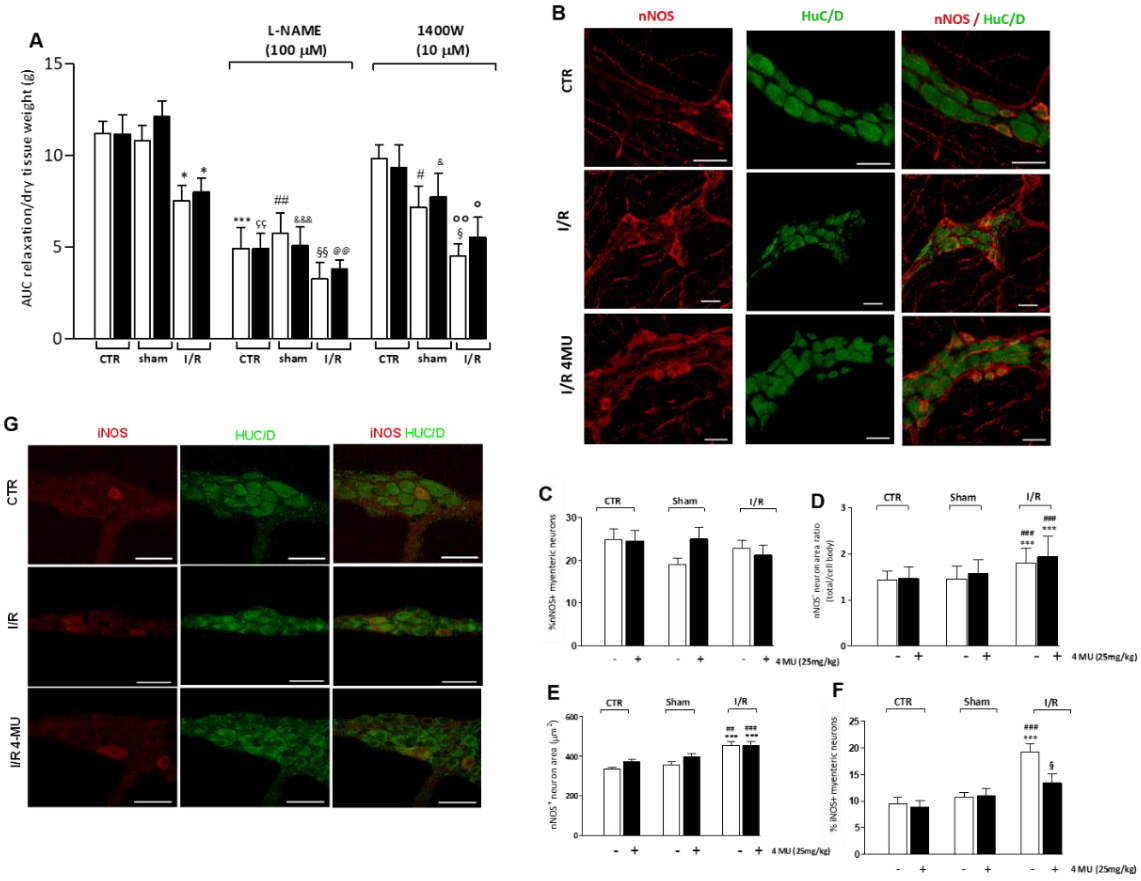

Figure 22. Effect of 4-MU treatment on inhibitory neurotransmission in the rat small intestine. (**A**) 10 Hz EFS-evoked NANC on-relaxation responses of the rat small intestine longitudinal muscle in CTR, sham-operated and IR (blank columns) and in the same groups after 4-MU treatment (black columns) in the absence and presence L-NAME and 1400W (N=5 rats per group). Data are reported as mean±SEM. Statistical significance: *P< 0.05, ***P<0.001 vs CTR; ^{ç}P<0.05, ^{çç}P<0.01 vs CTR 4-MU; $^{\#\#}$P<0.01 vs sham-operated; $^{\&\&}$P<0.01 vs sham-operated 4-MU; §§P<0.01 vs IR; @P<0.05 vs IR 4-MU; °P<0.05 and °°P<0.01 vs 1400W-treated CTR, by one-way ANOVA followed by Tuckey's post hoc test. (**B**) Representative confocal photomicrographs showing the distribution of nNOS immunoreactive myenteric neurons (red) and their co-localization with pan neuronal marker HuC/D (green). (**C**) percentage of HuC/D+-nNOS+ neurons with respect to total HuC/D+ neurons in small intestine LMMP whole-mount preparations in the different experimental groups, (N=5 rats per group). (**D-E**) Morphological analyses of nNOS immunoreactive myenteric neurons in rat small intestine of the different experimental groups. (**D**) Ratios of total cell areas, including dendrites, to cell body area and (**E**) nNOS+ cell profile total areas, including dendrites. Values are expressed as means±SEM. ***P<0.001 vs control, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs sham-operated by one way ANOVA followed by Tukey's test. (**F**) Percentage of HuC/D+-iNOS+ neurons with respect to total HuC/D+ neurons in ileal LMMP whole-mount preparations in the different experimental groups, (N=5 rats per group). Values are expressed as means±SEM. ***P<0.001 vs. control, $^{\#\#\#}$P<0.001 vs sham-operated and §P<0.05 vs IR by one-way ANOVA followed by Tukey's test. (**G**) Representative confocal photomicrographs showing the distribution of iNOS immunoreactive myenteric neurons (red) and their co-localization with pan neuronal marker HuC/D (green).

HA modulates VIPergic neurotransmitter pathways in the rat myenteric plexus after IR.

Vasoactive intestinal peptide (VIP) acts as an inhibitory neurotransmitter in the descending reflex of peristalsis and exerts fundamental roles in neuronal maintenance and protection during several pathophysiological conditions, including intestinal ischemia/reperfusion injury [20,204]. LMMP whole-mount preparations obtained from all experimental groups revealed a generally faint staining of VIP in the soma of myenteric neurons and a more intense signal in nerve varicose fibers within myenteric ganglia and in interconnecting trunks along the longitudinal muscle (Figure 23 A). In control and sham-operated animals, with and without 4-MU treatment, the percentage of VIP$^+$ myenteric neurons and the staining density index were not significantly different (Figure 23 B, C). The number of VIP$^+$ myenteric neurons significantly increased after IR injury, as well as the density index. Both enhancements were significantly reduced by 4-MU treatment (Figure 23 B, C).

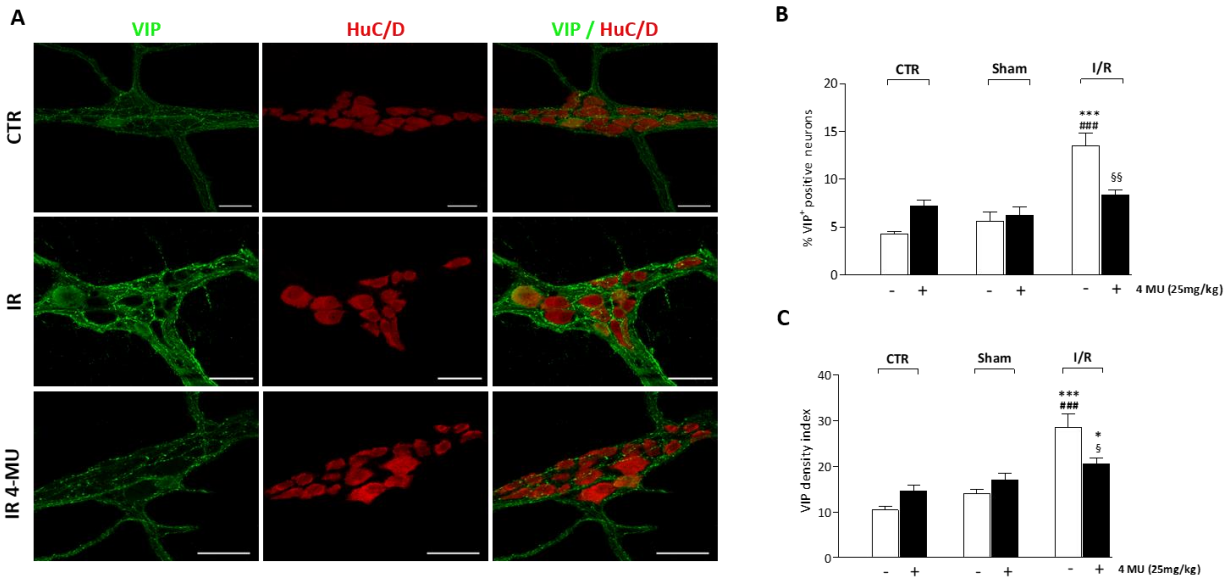

Figure 23. 4-MU modulation of VIPergic innervation in the rat small intestine after IR injury. (**A**) Representative confocal microphotographs showing the distribution of VIP (green) and HuC/D (red) in rat myenteric plexus obtained from CTR, IR and IR-4MU groups. Scale bars = 50 µm. Arrows indicate VIP$^+$-HuC/D$^+$ neurons, asterisk interconnecting fibers. (**B**) Percentage of VIP$^+$-HuC/D$^+$ neurons with respect to total HuC/D$^+$ neurons and (**C**) density index of VIP immunoreactivity in small intestine LMMP whole-mount preparations in the different experimental groups (N=5 rats per group). Data are reported as mean±SEM. Difference significance: **P<0.01, ***P<0.001 vs CTR; $^{\#\#}$P<0.01 vs sham-operated; §§P<0.01 vs IR.

DISCUSSION

Intestinal IR injury represents a pathological condition of the gastrointestinal tract causing harmful consequences to the different cellular components of the intestinal layers. In particular, important alterations may occur in myenteric neurons, which represent the enteric cell population with highest sensitivity to IR injury [8]. However, the mechanisms underlying enteric neuron rearrangements in these conditions are still largely unknown. In the present study, functional, morphological and biomolecular data have been provided to demonstrate that HA, a glycosaminoglycan (GAG) component of extracellular matrix, may participate in changes of the rat small intestine ENS architecture and neurochemical coding as well as of the neuromuscular function after an *in vivo* IR injury induced by a temporary occlusion of the superior mesenteric artery. In the rat small intestine both submucosal and myenteric neurons represent a source for HA production, as demonstrated by HA-binding protein staining in both submucosal and myenteric ganglia as well as by the presence of HA synthesizing enzymes, HAS1, and HAS2, the main biologically active isoform, within both plexuses. These data agree with previous data obtained in our laboratory on the distribution and function of HA in the rat colon [15]. In rat small intestine submucosal and myenteric ganglia, HA participates in the external architecture, forming an ECM coat in or next to the basement membrane. Within enteric ganglia, the GAG forms also a pericellular structure surrounding myenteric neurons, which resembles the perineuronal net (PNN) associated with some neuronal populations in the central nervous system (CNS). The PNN is formed by different EMC molecules and proteins lying on a backbone of HA and takes part in the regulation of neurogenesis, gliogenesis and neuronal circuitry formation during development [138].

After IR injury, in the submucosal and *muscularis propria* layers, on the surface and in the perineuronal space of myenteric ganglia, HA labelling density index significantly increased with respect to normal controls and sham-operated animals. Accordingly, after IR injury HA levels, measured by ELISA assay in the different intestinal layers, remained unchanged in the mucosal layer and significantly increased in the submucosal layer and in longitudinal muscle preparations containing the myenteric plexus (LMMP) preparations, with the highest increase found in this latter portion of the intestinal wall. Overall, these data suggest that IR-induced HA deposition in the intestinal wall may target specific gut layers and cell types, and that the submucosal layer as well as the *muscularis propria* and, in particular, myenteric ganglia, may represent peculiar targets for HA mediated effects in these conditions. These observations agree with previous reports about the accumulation of HA after reperfusion following ischemia, both in the peripheral tissues and in the CNS, supporting the hypothesis that the GAG may participate in the pathophysiology of the IR injury [145,205]. The increased HA deposition in the small intestine of ischemic rats after 24 hours of reperfusion may depend upon an up-regulation of HA synthases, HAS1 and HAS2, as also demonstrated in post mortem tissues of patients after an ischemic stroke, and in the mouse brain after an IR injury [206,207]. Indeed, this hypothesis is supported by the increased number of $HAS1^+$ and $HAS2^+$ myenteric neurons as well as by the increased mRNA levels for both proteins in LMMP preparations. Analogously, a higher density index for HAS2 staining and increased HAS2 mRNA levels were detected in submucosal layer preparations. In sham-operated rats, HAS1 mRNA expression levels, but not the number of $HAS1^+$ myenteric neurons, significantly increased in LMMP preparations possibly owing to overexpression of the enzyme in enteric glial cells, triggered by the mild inflammatory state observed in this experimental group.

In the IR group, but not in the control and sham-operated groups, treatment with the HA synthesis inhibitor, 4-methylumbelliferone (4-MU), decreased HA density index and levels in both the submucosal layer and in LMMP preparations, whereas the inhibitor did not affect HA levels in the mucosal layer. These observations further support the hypothesis of an IR-associated HA *de novo* synthesis, which selectively involves the submucosal and the *muscularis propria* and the relevant neuronal plexuses. The effect of 4-MU on HA levels after IR in the submucosal and *muscolaris propria* layers may reflect the ability of 4-MU to downregulate HA synthases in IR conditions [208]. Although the number of $HAS1^+$ and $HAS2^+$ myenteric neurons was only slightly reduced in the IR group after 4-MU treatment, HAS1 and HAS2 mRNA levels were more importantly reduced with HAS2 mRNA levels reaching values significantly lower than those observed in the untreated IR group. In submucosal ganglia, the density index of HAS2 immunostaining and the levels of HAS2 mRNA in the submucosal layer were significantly reduced in the 4-MU-treated IR group with respect to the untreated IR group. Overall, these latter observations suggest that HAS2 expression may undergo a higher level of modulation by 4-MU and that this isoform represents the main enzyme responsible for HA accumulation after IR in our model. This hypothesis is strengthened by the limited enzymatic capability of HAS1, which has a low affinity for its substrates and catalyzes HA synthesis only when UDP-sugar precursors accumulate in the cytosol, as occurs in hyperglycemic conditions [209,210]. 4-MU-mediated inhibition of HA synthesis relays upon different mechanisms, including a reduction of the availability of UDP-glucuronic acid, one of the cytosolic HA precursors, due to UDP-glucuronyl transferases [211], and a not yet fully understood downregulation of HASes expression [212]. In addition, HIF-1α is suggested to induce HA synthesis during hypoxia via HAS2 activation. Interestingly in this study, HIF-1α mRNA was upregulated by IR injury and was downregulated after 4-MU treatment, suggesting a further molecular mechanism for the drug-induced HA downregulation [213].

The consequences of increased HA deposition in our model have been evaluated on the efficiency of the neuromuscular function in the different experimental conditions adopted. Previous studies have shown that an *in vivo* IR in mice and rats has detrimental effects of the intestinal neuromuscular function and on a prolongation of the gastrointestinal transit [7,213,214]. Accordingly, in this study, the IR damage correlated with a significant slowing of the gastrointestinal transit and a reduced gastric emptying rate which were enhanced after 4-MU treatment. This latter observation suggests that *de novo* synthesis of HA may influence myenteric plexus circuitries to sustain the neuromuscular motor function in IR condition. Indeed, the administration of 4-MU altered the neurochemical coding of both excitatory and inhibitory neurotransmitter pathways in the myenteric plexus causing remarkable changes both in the contractile and in the relaxation responses. As already demonstrated, the ischemic insult followed by 24 hours of reperfusion altered the muscarinic postjunctional contractile responses causing a reduction of carbachol-induced contractions [213,215]. However, HA synthesis inhibition did not affect the impaired post junctional cholinergic response induced by IR damage, suggesting that HA deposition does not influence muscarinic postjunctional receptors. However, in the control group the administration of 4-MU induced an increase of the CCh concentration-response curve E_{max} value, suggesting that, in normal conditions, in the *muscolaris propria* the regulation of HA synthesis may influence muscarinic postjunctional responses, possibly by favoring receptor recruitment, or by enhancing receptor coupling with effector systems in smooth muscle cells.

In the sham-operated group, TTX-sensitive EFS-induced contractions, were significantly lower than in controls and this response was further significantly reduced after IR injury, indicating that the neuronal component of the excitatory contraction was impaired both in sham-operated and IR-treated animals. Treatment with 4-MU significantly increased

the excitatory response to EFS in the IR group, and did not affect the sham-operated response, suggesting that HA may influence excitatory neuronal pathways only in specific pathophysiological conditions, i.e. after IR injury, but not in mild inflammatory conditions. Excitatory cholinergic neuronal pathways are particularly sensitive to oxygen deprivation, sustained by an ischemic/hypoxic damage, which may selectively depress excitatory cholinergic responses involved in the peristalsis [193,194,216]. In this context, we investigated whether, in LMMP preparations, the distribution of the cholinergic innervation was influenced by IR and by 4MU treatment during IR, by evaluating the distribution of Choline Acetyltransferase (ChAT), the enzyme responsible for ACh synthesis, which is considered a marker of cholinergic enteric neurons. However, we did not observe any difference in myenteric ganglia ChAT density index in all the experimental group under study, suggesting that alterations of the EFS contractile response in the IR and 4-MU-treated IR groups do not depend upon changes in the cholinergic innervation. We cannot, however, exclude the hypothesis of possible changes in the synaptic ACh turnover or in the modulation of other excitatory neurotransmitter and co-transmitters pathways such as glutamate or tachykinins. Indeed, our data suggest a possible involvement of excitatory tachykinergic pathways, since excitatory off-contractions of tachykinergic nature, evaluated in non-adrenergic non-cholinergic (NANC) conditions in the presence of the NOS inhibitor, L-NAME, were lowered in IR group and were restored to control values after 4-MU treatment. These data strongly support the hypothesis that, in IR conditions, 4-MU-mediated inhibition of HA synthesis may enhance excitatory non-cholinergic responses, which are depressed by IR injury. Apparently, this modulation is in contrast with the ability of 4-MU to reduce IR-induced enhancement of both SP^+ myenteric neuron number and SP density index in LMMP preparations. In agreement with these data, several reports show that SP release may enhance after acute brain ischemia and is involved in neuronal injury and vasogenic edema [217,218]. Our data would suggest that HA favors an enhanced expression of SP^+ myenteric neurons during IR injury. We cannot exclude that, during IR, an increased SP availability and release may sustain receptor down-regulation, leading to reduced NANC off-contractions. All the 4-MU-mediated effects on the tachykinergic excitatory component were observed only in the IR group, since in the sham-operated group, the study of the same parameters in the presence of 4-MU gave similar results to those observed in controls.

Another fundamental component of the peristaltic reflex is represented by inhibitory relaxation pathways, which may undergo significant impairment after IR injury [219]. In this study, a significant reduction of NANC on-relaxations was observed after IR, which was not influenced by 4-MU treatment. In all experimental groups, NANC relaxations were significantly reduced by the non-selective nitric oxide inhibitor, L-NAME, suggesting that NO represents a major component of the inhibitory response [220,221]. In small intestine segments obtained from both untreated and 4-MU treated control animals, NANC on-relaxations were not influenced by the iNOS inhibitor, 1400W, in agreement with the low number of constitutively iNOS expressing myenteric neurons [7,54]. In untreated and 4-MU treated sham-operated animals, the mild inflammatory state, may explain the increased sensitivity to 1400W. In the IR group, addition of 1400W significantly reduced NANC relaxation indicating that iNOS is the main NOS isoform involved in this group, as also suggested by the increased number of $iNOS^+$ myenteric neurons [54,218]. IR-induced up-regulation of $iNOS^+$ neurons was significantly lowered by 4-MU treatment, indicating that blockade of HA synthesis may influence iNOS expression in myenteric neurons. This latter observation is in good agreement with reports demonstrating that the GAG has the ability of increase iNOS mRNA expression [222]. However, in the IR 4-MU-treated group the degree of NANC on-relaxation in the presence of 1400W were similar to those observed in the IR group in the presence of the iNOS inhibitor, suggesting that the decreased number of $iNOS^+$ myenteric neurons after HA synthesis blockade does

not apparently bear consequences on the overall enzyme activity in the control of the neuromuscular function during IR. The number of $nNOS^+$ myenteric neurons was unchanged in all experimental groups, although important morphological alterations were observed in $nNOS^+$ neurons [56], which perdured after 4-MU treatment, suggesting that during IR injury HA deposition influences enteric nitrergic transmission, involving iNOS, but not nNOS enzyme. Alternative inhibitory neurotransmitter pathways, comprising purines and peptides may participate in the alterations of on-relaxation responses during IR [223,224]. In this study, 4-MU treatment reduced IR-mediated VIP^+ neuron numbers and density index enhancement, suggesting that the GAG participates in enteric VIPergic pathway upregulation in this pathophysiological condition. VIP has vasodilatory and antioxidant properties [211], and the ability of HA to favour VIP upregulation in myenteric ganglia during IR, further strengthen the hypothesis that the GAG has a neuroprotective function in our model. However, other inhibitory neurotransmitters may also take- over NO and VIP in the modulation of on-relaxations in the IR 4-MU treated group, since the extent of the inhibitory response in the absence of NOS inhibitors was similar to that observed in the IR group.

In order to evaluate the possible mechanism/s underlying HA-mediated effects on the intestinal neuromuscular function during IR injury, we carried out immunohistochemical and biomolecular analysis of the distribution and expression levels of the main receptors involved in HA biological effects, such as TLR2 an TLR4 and CD44, in the submucosal layer and LMMP from all experimental groups. In agreement with previous studies, TLR2 and TLR4 receptors were prevalently found in enteric neurons of the submucosal and myenteric plexus and in enteric glial cells [118,225]. After IR injury, TLR2 mRNA and protein levels were found to significantly increase both in the submucosal layer and in LMMP preparations. Such enhancements were significantly reduced after 4-MU treatment suggesting that HA may influence both the transcription and protein stability of TLR2. However, the inhibitory effect of 4-MU was not observed neither in the control nor in the sham-operated group, suggesting that the GAG modulation of TLR2 levels represents an adaptive response to the IR injury. TLR4 receptor expression showed a more remarkable upregulation both in the transcript and in the protein in the LMMP with respect to the submucosal layer, where both the transcript and protein levels underwent only a slightly, not-significant enhancement with respect to controls. Analogously to TLR2, in LMMPs, HA seems to selectively regulate IR-induced TLR4 upregulation, since only in the IR group, 4-Mu treatment induced a significant reduction of the receptor mRNA and protein expression levels. These results are in line with the ability of 4-MU to downregulate TLR2 and TLR4 expression during inflammation of corneal stromal cells and of mouse chondrocytes [226,227]. The major changes observed in TLR2 and TLR4 expression in LMMP preparations, with respect to the submucosal layer, strengthen the hypothesis that after an ischemic injury followed by 24 hours of reperfusion, alterations in HA homeostasis may gradually increase from the submucosal layer to the *muscularis propria* containing the myenteric plexus, bearing important consequences especially on the gut motor function [8]. In view of the cross-talk existing between the submucosal and myenteric plexus we cannot exclude, however, that activation of TLR2 and TLR 4 receptors on submucosal neurons may also influence the responses of myenteric neurons [24]. Increased TLR2 and TLR4 receptor expression in myenteric ganglia may support the beneficial effect of HA on the gut neuromuscular function during IR. In this context, we hypothesize that HA-induced TLR upregulation during IR may favor the survival and function of specific neuronal populations. Interestingly, the presence of TLR2 and TLR4 in the mouse ENS small intestine, by sustaining the structural and functional integrity of enteric neurons, has been positively correlated with the repair and recovery from an inflammatory injury [17,18]. In addition, TLRs may have favorable effects on other intestinal cell types, such as enterocytes in the mucosal layer, thus influencing the integrity of the

barrier and, indirectly, the efficiency of other intestinal functions including motility [228]. For example, HA binding to TLRs mediates the normal growth of the small and large intestine, comprising the proliferation of epithelial stem cells [229]. During experimental inflammation, such as in dextran sodium sulphate (DSS)-induced colitis in mice, HA promotes epithelial repair by favoring TLR4 recruitment to the cell membrane, suggesting the potential therapeutic action of the GAG [154,162]. In addition, *in vivo* and *in vitro* experiments showed the HA, after the interaction with TLR, favors the synthesis of beta defensin-2, an antimicrobial peptide, thus impeding translocation of potentially pathogenic microorganism in pathological conditions [13].

As regards the ubiquitously expressed CD44 receptor, we have found that it is expressed by neurons and enteric glial cells both in the submucosal and myenteric plexus [230]. In submucosal layer preparations, CD44 mRNA levels were similar in all experimental groups both in the absence and presence of 4-MU treatment. However, the protein levels were upregulated after IR and significantly downregulated by 4-MU treatment, suggesting that CD44 transduction, more than transcription, is sensitive to HA modulation during IR. Accordingly, CD44 expression decreased in the presence of 4-MU in different pathological settings, including inflammation of corneal stromal cells and in prostate cancer cells [226,231]. In LMMP preparations, CD44 transcript levels significantly increased after IR, and remained elevated in the presence of 4-MU, whereas the protein levels significantly increased after IR and tended to decrease in the presence of 4-MU. These observations mirror those obtained in the submucosal plexus suggesting a higher sensitivity of CD44 protein transduction, more than transcription, to 4-MU in IR conditions. As regards the functional significance of CD 44 expression in myenteric and submucosal neurons we may hypothesize that CD44 receptors by exerting a chemoattractant effect, favor the recruitment of mononuclear cells toward both the submucosal and myenteric plexus, possibly underlying development of neuroimmune responses. In experimental models of inflammatory bowel disease, CD44 has been shown to promote the formation of "HA cable-like" structures, deriving from medium molecular weight HA, thus favouring the engagement of mononuclear cells in the submucosal layer in a CD44-dependent manner [19]. In this study, IR injury was associated with a low-grade inflammatory response associated with a significant MPO^+ cells infiltration in the *muscularis propria* layer and to a more moderate enhancement in the number of neutrophilic cells in the submucosal layer and in the myenteric plexus. The increased MPO^+ cell recruitment was drastically reduced in the *muscularis propria* and myenteric plexus and more moderately in the submucosal layer after 4-MU treatment, suggesting that, after IR injury HA plays a fundamental role in neutrophilic recruitment, especially within the more outer structures of the gut. We cannot exclude that a reduced neutrophilic infiltration via CD44 activation in the *muscularis propria* and myenteric plexus may sustain the neuromuscular function as suggested in different murine types of colitis [232]. In sham-operated animals, however, the enhanced number of MPO^+ cells infiltrating the submucosal layer was not associated with significant changes in CD44 expression, suggesting the participation of other chemoattractant mechanisms. In order to fully disclose the functional significance of TLR2, TLR4, and CD44 HA-mediated upregulation on the neuromuscular function, however, further functional investigation resorting to specific modulators in the different experimental settings of the study are required.

In conclusion, this study suggests that, during an IR injury, HA supports the efficiency of the gastrointestinal transit by influencing both excitatory and inhibitory components of the peristaltic reflex. Our hypothesis is that changes in the efficiency of the GI transit may probably depend upon alterations in myenteric plexus chemical coding. Indeed, the present data suggest that, after blockade of HA synthesis, excitatory neuronal pathways, mainly tachykinergic, are up-regulated, while inhibitory responses remain downregulated, overall deteriorating transit efficiency. We cannot

exclude that IR-associated changes of HA synthesis, besides influenced myenteric plexus chemical coding, may also influence myenteric neuron wiring by altering the composition of the perineuronal net. Furthermore, we do not exclude that 4-MU treatment may involve other mechanisms originating from the mucosa and submucosal layer affecting the gut motor function, although the present data strongly suggest that main IR-induced changes in HA homeostasis occur in the *muscularis propria* and in the overlying myenteric plexus. The beneficial effects of HA may depend upon the ability of the IR-induced *de novo* synthetized ECM component to sustain, through the interaction with its target receptors, the function of specific enteric neurons subpopulations as well as to promote neuroimmune responses in these pathological conditions. This hypothesis is innovative and opens new scenarios in the study of the molecular mechanism/s involved in the onset and severity of gut IR injury as well as for the development of new therapeutic agents for the treatment of this disease with high clinical impact.

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