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Review article

Role of glutamatergic neurotransmission in the enteric nervous system and brain-gut axis in health and disease

Viviana Filpa [a], Elisabetta Moro [b], Marina Protasoni [c], Francesca Crema [b], Gianmario Frigo [b], Cristina Giaroni [a, *]

[a] Department of Clinical and Experimental Medicine, University of Insubria, via H. Dunant 5, I-21100 Varese, Italy
[b] Department of Internal Medicine and Therapeutics, Section of Pharmacology, via Ferrata 9, I-27100 Pavia, Italy
[c] Department of Surgical and Morphological Sciences, University of Insubria, via F. Guicciardini 9, I-21100 Varese, Italy

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ABSTRACT

Several studies have been carried out in the last 30 years in the attempt to clarify the possible role of glutamate as a neurotransmitter/neuromodulator in the gastrointestinal tract. Such effort has provided immunohistochemical, biomolecular and functional data suggesting that the entire glutamatergic neurotransmitter machinery is present in the complex circuitries of the enteric nervous system (ENS), which participates to the local coordination of gastrointestinal functions. Glutamate is also involved in the regulation of the brain-gut axis, a bi-directional connection pathway between the central nervous system (CNS) and the gut. The neurotransmitter contributes to convey information, via afferent fibers, from the gut to the brain, and to send appropriate signals, via efferent fibers, from the brain to control gut secretion and motility. In analogy with the CNS, an increasing number of studies suggest that dysregulation of the enteric glutamatergic neurotransmitter machinery may lead to gastrointestinal dysfunctions. On the whole, this research field has opened the possibility to find new potential targets for development of drugs for the treatment of gastrointestinal diseases. The present review analyzes the more recent literature on enteric glutamatergic neurotransmission both in physiological and pathological conditions, such as gastroesophageal reflux, gastric acid hypersecretory diseases, inflammatory bowel disease, irritable bowel syndrome and intestinal ischemia/reperfusion injury.

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* Corresponding author.

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1. Introduction

As a major excitatory neurotransmitter in the central nervous system (CNS), glutamate plays a fundamental role in the modulation of both physiological (e.g. memory, learning) and pathophysiological (e.g. stroke, epilepsy, neurodegenerative diseases such as Alzheimer's and Parkinson's disease, etc.) conditions (Meldrum, 2000; Genoux and Montgomery, 2007). Increasing evidence suggest that glutamate may also have a role in the regulation of a number of functions in the peripheral nervous system, including the gastrointestinal function (Gill and Pulido, 2001). Several studies have been carried out in the last 30 years in the attempt to clarify the possible role of glutamate as a neurotransmitter/neuromodulator in the gut. Such effort has provided immunohistochemical, biomolecular and functional data suggesting that the entire glutamatergic neurotransmitter machinery is present in the complex circuitries of the enteric nervous system (ENS), which participates to the local coordination of gastrointestinal functions, as well as in the brain-gut axis, a bi-directional connection pathway between the central nervous system (CNS) and the gut. This opens an exciting scenario on the possibility to target the enteric glutamatergic neurotransmission for the development of new potential pharmacological tools addressed to the treatment of gastrointestinal disorders. The present review will consider the more recent literature describing the involvement of glutamate as a neurotransmitter in the modulation of the digestive tract under physiological and pathological conditions. In this latter regard, specific paragraphs will be addressed to describe the participation of enteric glutamatergic pathways in gastroesophageal reflux, gastric acid hypersecretory diseases, inflammatory bowel disease (IBD), irritable bowel disease (IBS) and intestinal ischemia/reperfusion (I/R) injury.

2. The enteric nervous system

The ENS is a complex and extensive neuronal network, which extends from the esophagus to the anal sphincter, composed of ganglia, interconnecting fibers and neuronal fibers impinging on effector tissues, including the smooth muscle layer, epithelial lining, intrinsic blood vessels and gastroenteropancreatic endocrine cells (Furness et al., 2014). All aspects of the gastrointestinal function are under control of the ENS including: motility patterns, gastric secretion, transport of fluids across the epithelium, blood flow, nutrient handling, interaction with the immune and endocrine systems of the gut (Furness, 2012; Wood, 2012). A unique property of the ENS with respect to any other section of the peripheral nervous system is that enteric ganglia can maintain integrated functions in the absence of input from the CNS. For instance the bowel can propel intraluminal contents (peristaltic reflex) or generate the migrating myoelectric complex (MMC, whose progression during fasting along the small intestine depends on intrinsic neuronal activity) independent of extrinsic innervations (Furness, 2012). The ENS, however is not autonomous and neuronal control of the gastrointestinal tract depends on an integrated interaction between local reflexes, reflexes that pass through sympathetic ganglia and reflexes that pass from the gut and back to the CNS, via vagal, splanchnic and pelvic nerves (Furness et al., 2014; Veremulen et al., 2014). Large number of neurons, 200–600 millions in human, the same number of neurons that is found in the human spinal cord, give rise to the three major components: the subserous, the myenteric (Auerbach's located between the two smooth muscle layers) and the submucosal (Meissner's located in the submucosal layer) plexuses. This latter is absent in the esophagus and stomach (Furness, 2006; Furness et al., 2014). Approximately 20 distinct types of neurons have been described according to their morphology, neurochemical coding, cell physiology, projections to targets and functional roles. From a functional view point three major classes of neurons have been identified: intrinsic primary afferent neurons, interneurons, excitatory and inhibitory motor neurons (Furness, 2006). Intrinsic primary afferents are sensory neurons which detect mechanical distortion of the mucosa, mechanical forces in the external musculature (tension of the gut wall) or the presence of chemical luminal stimuli and initiate appropriate reflex control of functions including motility, secretion and blood flow (Clerc et al., 2002). Intrinsic primary afferents are multiple-axonal neurons with a large ovoid cell body (type II morphology) and represent the 10–30% of neurons in the submucosal and myenteric ganglia of the small and large intestine of mammals (Wood, 2012). Intrinsic primary afferents connect with each other, with interneurons and with motor neurons, and upon activation display a pronounced after-hyperpolarization that depends on a Ca^{++}-activated K^{+} conductance. Along the whole gastrointestinal tract, the longitudinal and circular smooth muscle layers and the muscolaris mucosae are innervated by uni-axonal excitatory and inhibitory motor neurons (type I morphology), which receive prominent fast excitatory synaptic potentials (Wood, 2012). The primary neurotransmitters for excitatory motor neurons are acetylcholine (ACh) and tachykinins. Several neurotransmitters have been identified in inhibitory motor neurons, including nitric oxide (NO), vasoactive intestinal peptide (VIP) and ATP-like transmitters, although NO is considered the primary transmitter (Furness et al., 2014). Another important class of enteric neurons is represented by secretomotor and secretomotor/vasodilator neurons regulating the electrolyte and water transport across the intestinal mucosa (Vanner and Macnaughton, 2004). Pharmacological and immunohistochemical studies have evidenced ACh-containing as well as VIP-containing neurons which, on their own may or may not express ACh. Submucosal neurons expressing VIP cause sodium and water secretion and, by sending collaterals to submucosal arterioles, increase blood flow (Banks et al., 2005). Several types of interneurons have been identified by means of physiological/structural investigations within the gut wall (Brookes, 2001a, 2001b). In the small intestine myenteric plexus, one type of orally projecting (ascending) and three types of anally projecting (descending) neurons have been described (Neal and Bornstein, 2008). The ascending interneurons are cholinergic and participate to the local motor reflex, as are two types of descending interneurons expressing ACh and either nitric oxide synthase (NOS) or serotonin. Another type of descending interneuron containing ACh and somatostatin participate to the conduction of MMC along the intestine. Some kinds of interneurons are also mechanoceptive and contribute to stretch-initiated reflexes (Mongardi Fantaguzzi et al., 2009). An important non-neuronal component of the ENS is represented by enteric glial

cells, which resemble CNS astrocytes. Several studies carried out in the last few years have demonstrated that enteric glial cells, in analogy with the function of astrocytes, do not only contribute to create a protective local microenvironment, but may also have a functional role in enteric information transfer by responding to a variety of neuroligands (Sarosi et al., 1998; Gulbransen and Sharkey, 2012). A distinctive feature of the ENS is that enteric neurons communicate with different cell types, which constitute the enteric microenvironment (Giaroni et al., 1999). Enteric neurons may receive and send inputs to enteric glial cells, smooth muscle cells and the interstitial cells of Cajal, which are considered intestinal pacemaker cells, and immunocytes, which play an important role in the modulation of neuroimmune interactions (Giaroni et al., 1999).

3. Glutamatergic neurotransmission

The identification of glutamate as a neurotransmitter in the CNS dates back to the middle 80s. Since then numerous studies have documented that a complex "glutamatergic neurotransmitter machinery" is responsible for regulating the synthesis, release and reuptake of the aminoacid into neurons and glial cells (Niciu et al., 2012) (Fig. 1). In the CNS, extracellular glutamate concentrations are normally tightly controlled and very low (less than 2 μM), although levels of the aminoacid in whole brain are 5 10^3-fold higher (Hawkins, 2009). Glutamate does not enter the blood brain barrier and is produced by neurons from transamination of α-chetoglutarate, originated from glycolysis, and from hydrolytic deamination of glutamine by phosphate-activated glutaminase (Kvamme, 1998; McKenna, 2007). The aminoacid is then transported by multimeric proton/glutamate antiporters, the vesicular transporters (VGLUTs) into the vesicles where it is stored (Niciu et al., 2012). Three VGLUTs have been cloned to date: VGLUT1 and VGLUT2, which are primarily expressed in glutamatergic neurons and in glial cells, and VGLUT3, that has been detected in non-glutamatergic neuronal populations (Niciu et al., 2012). Invasion of the synaptic terminal by an action potential leads to membrane depolarization, fusion of the vesicles and release of the neurotransmitter in a Ca^{++}-dependent manner (Meldrum, 2000). The synaptic release of glutamate is under control of metabotropic autoreceptors and by several heteroreceptors (Meldrum, 2000). Glutamate is actively removed from the synaptic cleft and transported into the cytosol against its concentration gradient via excitatory amino acid transporters (EAAT) primarily found on synaptically-associated astrocytic processes. Five of such high affinity transporters have been identified: EAAT1, which is restrictedly expressed by astrocytes, EAAT2 which is mostly expressed on astrocytes and, to a limited extent, in neurons, EAAT3 and EAAT4 which are exclusively neuronal, while EAAT5 is located in the retina. In rodents the homologues of the EAAT1-3 are referred to GLAST, GLT and EAAC1 (Niciu et al., 2012). Once in the astrocyte cytosol, glutamine synthase converts glutamate into glutamine, which is then transported into the extracellular fluid and then back into neurons where it is converted by the deaminase into glutamate (Danbolt et al., 2016). The fine regulation of extracellular glutamate is essential to prevent dysregulation of excitatory neurotransmission which may result in excitotoxicity (Obrenovitch and Urenjak, 1997).

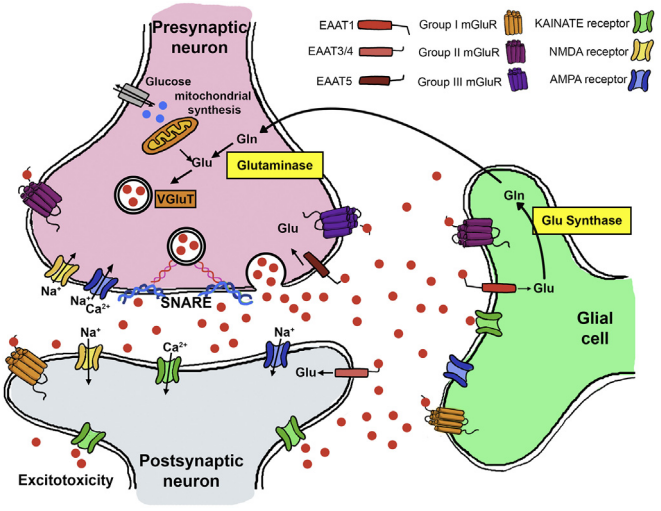

Fig. 1. Schematic representation of glutamatergic synapses. The figure shows neuronal glutamate (Glu) *de novo* synthesis from glucose and from glutamine (Gln) of glial origin. The vesicular transporter (VGLUT) transfers Glu into synaptic vesicles, whose fusion via the SNARE complex of the presynaptic membrane induces Glu release into the synaptic space. After release, Glu activates ionotropic (AMPA, NMDA and kainate) and metabotropic (mGlu1 to mGlu8) receptors located on the membranes of both postsynaptic and presynaptic neurons and on glial cells, initiating a cascade of intracellular events, such as membrane depolarization, activation of intracellular messenger cascades, modulation of protein synthesis and gene expression (not shown). Glu is then cleared from the synapse through excitatory aminoacid transporters (EAATs) located on glial cells (EAAT1 and EAAT2) and, to a lesser extent, on neurons (EAAT3 and EAAT4). Impairment to effectively clear synaptic Glu by glial EAATs may increase activation of extrasynaptic Glu receptors resulting in excitotoxicity.

The actions of glutamate are mediated by two types of receptors: ionotropic (iGlu) and metabotropic (mGlu) receptors. iGlu receptors are ion channels that flux cations (Na^+ and Ca^{++}) and are classified into three major subtypes according to their sequence homologies, electrophysiological properties and affinity to selective agonists: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors. iGlu receptors may differently contribute to excitatory post synaptic potentials elicited by glutamate: AMPA and kainate receptors induce a fast depolarizing response followed by a rapid decay, while NMDA receptors induce a more prolonged depolarization phase (Traynelis et al., 2010). Functional NMDA receptors are heterotetrameric proteins usually composed of two obligatory GluN1 subunits (there are eight different GluN1 subunits generated from alternative splicing from a single gene) and two modulatory GluN2 (denoted A-D) and GluN3 (A-B) subunits, which confer functional variability to the receptor (Traynelis et al., 2010). NMDA receptors are unique among the glutamate receptor family in that the simultaneous binding of glycine to GluN1 and glutamate to GluN2 is required for activation. Another distinctive feature of NMDA receptors is the voltage-dependent block by Mg^{++} that may be overcome by partial depolarization of the resting membrane potential, which may be induced by AMPA or kainate receptor activation (Meldrum, 2000). A further specific feature is the need for glycine as a co-agonist: each receptor unit has two glycine binding sites located on GluN1 subunits and two glutamate binding sites on GluN2 subunits. AMPA and kainate receptors assemble as homo- or heteromers from four and five subunits, GluA1-4 and GluK1-5, respectively (Traynelis et al., 2010). mGlu receptors exert their effect on intracellular signal transduction cascades by coupling to GTP-binding proteins and are classified into three major groups, Group I, Group II and Group III according to their homology sequence, pharmacological properties and to the related signal transduction pathways (Niswender and Conn, 2010). Group I receptors activate phospholipase C to produce IP_3, leading to Ca^{++} release from intracellular stores, and diacylglycerol to stimulate protein kinase C. Group II and III receptors reduce intracellular cAMP levels, by negatively coupling to adenylate cyclase. mGlu receptors prevalently encompass a regulatory role acting either presynaptically, as auto- or heteroreceptors to modulate glutamate

and other neurotransmitter release, or postsynaptically to modulate the effects of glutamate on neurons and glial cells (Niswender and Conn, 2010).

4. Glutamatergic innervation of the gastrointestinal tract

In the gastrointestinal tract, the major source for glutamate is represented by dietary glutamate which can be internalized by epithelial cells from the luminal side, with the exception of the large intestine where the aminoacid is taken into colonocytes from the arterial blood and there is little or no transfer of glutamate from the colonic lumen to the portal blood (Reeds et al., 2000; Blachier et al., 2009). Monosodium glutamate (MSG), which is used as a flavor-enhancing food additive, as well as glutamate derived from cleaved proteins are able to target epithelial cells in the luminal surface of the gastrointestinal tract and more than 95% of enteral glutamate is involved in enterocyte metabolism and biosynthetic pathways as well as in the regulation of epithelial barrier (Blachier et al., 2009; Reeds et al., 2000). Dietary glutamate may also play a role in the regulation of various digestive functions, including taste perception, process of digestion, yield of nutrient absorption and metabolism, via modulation of intrinsic and extrinsic neuronal pathways (Reeds et al., 2000; Uematsu et al., 2010; Nakamura et al., 2013), although a recent study casts doubts on the ability of enterally absorbed glutamate to modify both secretory and motor gut functions (Wang et al., 2014). Several studies carried out from the middle 80s have, however, provided evidence that glutamate does not only play an important modulatory role on the gastrointestinal epithelial function, but may also represent an enteric neurotransmitter. This hypothesis has initially been put forward on the basis of functional/pharmacological evidences suggesting that glutamate and different selective agonists and antagonists to glutamate receptors were able to influence gastrointestinal motor and secretory functions (Kirchgessner, 2001). To reinforce this concept, in the late 90s, histological and biomolecular data have been provided to demonstrate the presence of the entire glutamatergic neurotransmitter machinery, including vesicular and neuronal transporters, iGlu and mGlu receptors, in the ENS (Kirchgessner, 2001). Numerous studies have also shown that glutamate signaling is involved in the regulation of the neuronal connections between the CNS and the gut, which constitute the brain-gut axis (Hornby, 2001). Signals departing from the CNS to the gut, along this axis, modulate gastrointestinal secretory and motor functions, while chemical and electrical signals from the gastrointestinal tract provide sensory information to the CNS. Glutamate, via activation of either vagal, splanchnic or pelvic afferents, whose cell bodies are contained within the nodose vagal ganglion (NVG) and dorsal root ganglia (DRG), participates in conveying sensory inputs to brain areas involved in the regulation of different gut functions. Efferent pathways, comprising the dorsal motor nucleus of the vagus (DMV), which drive both excitatory and inhibitory inputs into the gastrointestinal tract, may also be regulated by glutamate receptor activation (Hornby, 2001; Furness et al., 2014; Moloney et al., 2015).

4.1. Enteric glutamatergic neurons

Ultrastructural and immunohistochemical evidences showed that glutamate immunoreactivity is concentrated in axonal terminals of the stomach, ileum and colon myenteric and submucous plexus of different species, including rat, guinea pig and human (Liu et al., 1997; Giaroni et al., 2003; Tsai, 2005). A large proportion of glutamatergic myenteric and submucosal neurons of the rat stomach and guinea pig ileum co-express choline acetyltransferase (ChAT) and substance P (SP), suggesting that glutamate may behave as an excitatory sensory co-transmitter with acetylcholine (ACh) and tachykinines (Liu et al., 1997; Tsai, 2005). Neurons co-expressing ChAT and SP in the submucosal plexus are considered intrinsic primary neurons and are involved in the neurotransmission of information from the mucosa to the ENS. Electrophysiological studies support the hypothesis that glutamate may behave as an excitatory neurotransmitter for intrinsic primary neurons, since direct application of the aminoacid or glycine to intraganglionic fibers induced depolarizing responses at this level both in the guinea pig ileum and colon (Liu et al., 1997; Neunlist et al., 2001). However, this excitatory effect has recently been questioned by a study showing the absence of glutamate-mediated effects on enteric neurons (Wang et al., 2014). Consistent with a sensory function, several studies resorting to immunohistochemical, electrophysiological and functional approaches have demonstrated that glutamate is also a neurotransmitter in extrinsic primary afferent fibers of the vagus nerve conveying information from the gut to the brain (Hornby, 2001; Li, 2007; Schicho et al., 2005). To further support the hypothesis that glutamate may behave as an enteric neurotransmitter, studies carried out in guinea pig ileum longitudinal muscle myenteric plexus (LMMP) preparations, showed that, in analogy with the CNS, glutamate is synthesized from glutamine in myenteric neurons (Wiley et al., 1991; Kvamme, 1998). Co-localization of glutaminase and glutamate has also been observed in nerve bundles innervating the circular and longitudinal muscle layers of the rat stomach (Tsai et al., 1994). Furtermore, as a classical neurotransmitter, glutamate is selectively stored in varicosities of enteric neuron terminals, which release the amino acid in a Ca^{++}-dependent and Ca^{++}-independent manner (Sinský and Donnerer, 1998; Reis et al., 2000; Giuliani et al., 2006).

4.2. Enteric glutamate transporters

The presence of glutamate reuptake and vescicular tranporters in enteric neurons and glial cells has been demonstrated by several groups (Table 2). Transporters regulating intrasynaptic glutamate levels are expressed in the soma and in the ganglionic neuropil of enteric neurons. In the guinea pig ileum myenteric and submucosal plexus, the neuronal high affinity glutamate transporter, excitatory amino acid carrier 1 (EAAC1; EAAT3), was mainly localized to intrinsic primary afferent neurons (Liu et al., 1997). A splice variant of the glial high affinity glutamate transporter 1 (GLT1; EAAT2) has been found in neurons of the rat myenteric plexus (Schmitt et al., 2002). There are reports demonstrating that also non-neuronal cells express glutamate transporters, which may participate to the regulation of extracellular aminoacid concentrations in the gut. In particular, immunohistochemical and in situ hybridization approaches showed that EAAC1 is the predominant transporter of the aminoacid on epithelial cells in the mouse small intestine (Iwanaga et al., 2005), where it may be involved in the initial steps of the glutamate metabolism pathways in the gut mucosa. EAAT1 is abundantly expressed on enteric glial cells within the submucosal and myenteric plexus of the mouse colon (Seifi and Swinnyy, 2016). Although there are no direct reports available at the moment to demonstrate that enteric glial cells control extracellular glutamate levels in enteric ganglia, this latter observation suggests that also in ENS circuitries, glial cells may contribute to prevent extracellular glutamate concentrations to raise, reaching neurotoxic levels in the synaptic cleft, in analogy with astrocytes in the CNS (Schousboe and Waagepetersen, 2005). Indeed, enteric glia shares many common morphological and molecular features with astrocytes, including expression of glutamine synthase (Jessen and Mirsky, 1983), glutamate (Giaroni et al., 2003) and iGlu receptors (Carpanese et al., 2014; Von Boyen et al., 2006).

Vesicular glutamate transporters (VGLUT) are expressed in

neuron terminals of both intrinsic and extrinsic origin. In guinea pig, rat and mouse ileum, VGLUT2-immunoreactivity was found both in the submucosal and in the myenteric plexus, often co-staining with ChAT, the vesicular ACh transporter and calbindin, a marker for intrinsic primary afferent neurons (Tong et al., 2001). Retrograde tracing with Fluoro-Gold established that a subset of NVG and DRG neurons, displaying somatic immunostaining for VGLUT2, innervate the bowel, reinforcing the hypothesis that glutamatergic nerve terminals in the gut originate also from extrinsic primary vagal afferent neurons (Tong et al., 2001). In the rat and mouse esophagus, non-peptidergic intraganglionic laminar endings (IGLE), which represent the only vagal mechanosensory terminals in the muscular layer and are involved in local reflexes, stained for VGLUT2 (Raab and Neuhuber, 2004). In a small proportion of extrinsic afferent neurons innervating the guinea pig rectum and distal colon, VGLUT1 and VGLUT2 co-localized with calcitonin-gene-related peptide (CGRP), which is a sensory transmitter in the gut. Non-peptide containing IGLEs expressing both VGLUT1 and VGLUT2 were also observed in the guinea pig rectum (Olsson et al., 2004; Chen et al., 2015).

Immunohistochemical and in situ hybridization studies showed that in BALB/c mice most colorectal DRG neurons and a subpopulation of myenteric neurons express VGLUT2 and to a lesser extent VGLUT1, suggesting that VGLUT2 may have a more important role in modulating glutamatergic transmission at this level (Brumovsky et al., 2011).

4.3. Glutamate receptors in the GI tract and in the brain-gut axis

All subtypes of iGlu and mGlu receptors have been localized to intrinsic and extrinsic neuronal circuitries involved in the regulation of secretory and motor functions along the gastrointestinal tract of different species, including rat, mouse, guinea pig and human (Kirchgessner, 2001; Julio-Pieper et al., 2011) (Fig. 2). In the following paragraphs a report of the most significant studies on glutamate receptor distribution both in the gastrointestinal tract and along the brain-gut axis and the relative function will be given (Table 1).

4.3.1. Glutamate receptor modulation of the esophageal function

Several studies in the literature suggest that esophageal motor responses are under control of iGlu and mGlu receptors. The propulsive activity of the esophagus body and the activity of the lower esophageal sphincter (LES) are mainly under control of vagovagal reflex pathways, although a structurally-formed ENS is present in the esophageal wall (Furness et al., 2014). Glutamate receptors may affect esophageal motility by modulating both brainstem nuclei and vagal afferents and efferent pathways. A glutamatergic mechanism, involving AMPA and NMDA receptors, more than a cholinergic contribution, has been proposed to activate vagal esophageal afferents conveying excitatory inputs into the central subnucleus of the nucleus of the solitary tract (NTSc) after esophageal distension. This hypothesis has been proposed following the observation that in urethane anaesthetized rats, NTSc discharges and reflex contractile responses after esophageal mechanical distension were strongly inhibited by AMPA and NMDA receptor antagonists, NBQX and (−)-AP7, respectively (Table 2) (Lu and Bieger, 1998). Subunits of AMPA and NMDA type have been identified in the NTS and in vagal afferents projecting into the NTS. The combination of transneuronal viral labeling and in situ hybridization technique allowed to demonstrate the presence of GluN1 mRNA within brainstem circuitries controlling esophageal swallowing, including the NTSc and the compact formation of the rat nucleus ambiguous (Broussard et al., 1994). In the cat NTS, all AMPA subtypes and GluN1 receptor subtypes are present although their distribution

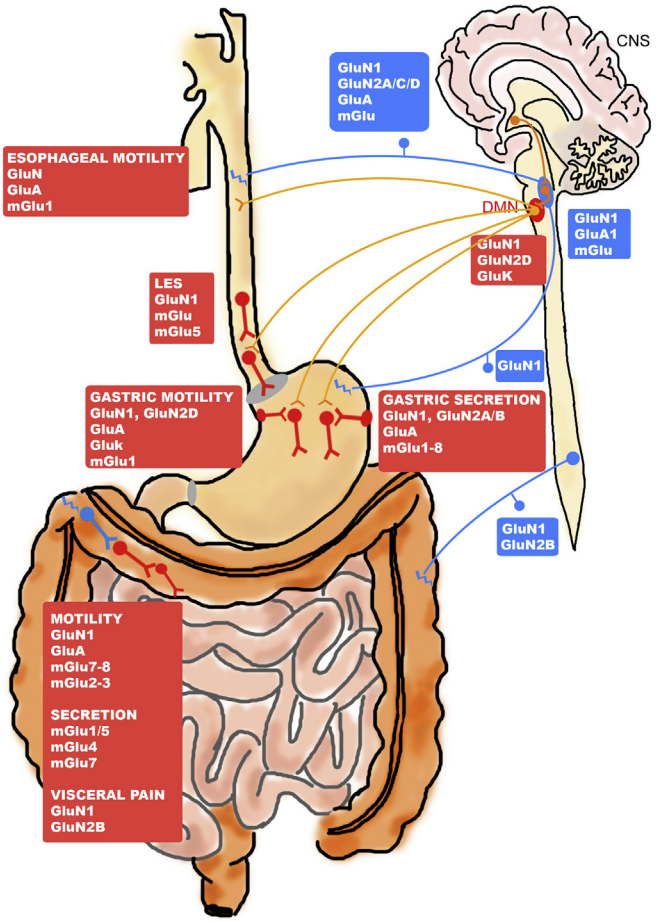

Fig. 2. Distribution and function of the major glutamate receptors along the brain-gut axis. Both ionotropic and metabotropic receptors are located in ENS circuitries (motor neurons and interneurons in red and intrinsic primary neurons in blue) involved in the motor and secretory reflex responses and in visceral pain perception. Glutamate receptors modulate also extrinsic primary afferent pathways (blue) sending sensory information to the CNS via vagal and spinal routes as well as effector pathways from the CNS (yellow) conveying excitatory and inhibitory inputs into the gastrointestinal tract. In the CNS, neurons projecting from the hypothalamus to sensory vagal nuclei (nodose vagal ganglion, NVG, blue) in the brain stem and from the NVG to effector nuclei (dorsal motor nucleus, DMV, red) modulate digestive functions via glutamatergic ionotropic and metabotropic receptors. Abbreviations: CNS, central nervous system; ENS, enteric nervous system.

may diverge in the various subnuclei, indicating that neurons in these structures are designed to respond differently to excitatory inputs (Ambalavanar et al., 1998). Triple fluorescent immunohistochemical staining of GluA1, GluN1 and neuronal nitric oxide synthase (nNOS) showed that almost all GluN1 positive neurons in the rat NTS contained GluA1 immunoreactivity and that some of the double-labeled cells also stained for nNOS, suggesting the existence of an anatomical link between iGlu receptors and NO (Lin and Talman, 2002). In a previous study, *trans*-synaptic tracing was used to demonstrate GluN1 and nNOS co-expression in second order esophageal premotor neurons of the NTSc. This set of neurons may synthesize nitric oxide (NO) in response to NMDA activation, strengthening the concept of a role for centrally produced NO in the coordination of esophageal motility (Broussard et al., 1995). NO synthesis after NMDA receptor stimulation may have important implications on the esophagus motor function since NO is the primary neurotransmitter mediating vago-vagal inhibitory reflexes involved in esophageal propulsion and LES relaxation to allow food passage (Furness, 2012). The ability of AMPA and NMDA agonists to

Table 1
Distribution of glutamate receptors and transporters in the gastrointestinal tract and in the brain-gut axis.

Receptor/ transporter	Localization	Species	Method	References
GluN1	CNS/PNS (NA, NTS, NVG, DMV/DRG)	cat, mouse, rat	IH, IHC, RT-PCR, WB	Shigemoto et al., 1992; Broussard et al., 1994; Broussard et al., 1995; Ambalavanar et al., 1998; McRoberts et al., 2001; Lin and Talman, 2002; Chang et al., 2003; Czaja et al., 2006, Slattery et al., 2006; Banerjee et al., 2009
	esophagus (WW)	cat	RT-PCR, PCR	Banerjee et al., 2009
	stomach (MP, SP,SM, WW)	rat	IH, IHC, RT-PCR	Burns and Stephens, 1995; Tsai et al., 2004; Watanabe et al., 2008
	small intestine (MP, SP)	guinea pig, rat	IH, ICC, NB, RT-PCR, WB, IHC	Burns et al., 1994, 1995; Von Boyen et al., 2006; Liu et al., 1997; Carpanese et al., 2014
	colon (MM, MP, SP)	human, rat	IH, IHC, NB	Burns et al., 1994, 1995; McRoberts et al., 2001; Giaroni et al., 2003
GluN2A/2B	small intestine (MP, SP)	guinea pig	IHC, WB	Liu et al., 1997
GluN2A	CNS/PNS (NVG/DRG)	mouse, rat	IHC, RT-PCR	Slattery et al., 2006; McRoberts et al., 2001
	esophagus (WW)	cat	RT-PCR	Banerjee et al., 2009
	stomach (MP, SM, SP, WW)	rat	RT-PCR, IHC	Tsai et al., 2004;
	small intestine (MP)	rat	RT-PCR, WB,	Von Boyen et al., 2006
GluN2B	CNS/PNS (NVG/DRG)	mouse, rat	IHC, RT-PCR	Czaja et al., 2006,Slattery et al., 2006 McRoberts et al., 2001,
	stomach (MP, SM, SP,WW)	rat	IHC, RT-PCR	Watanabe et al., 2008; Tsai et al., 2004
	small intestine (MP)	rat	RT-PCR, WB	Von Boyen et al., 2006
GluN2C	CNS/PNS (NVG)	mouse, rat	RT-PCR, IHC	Czaja et al., 2006,Slattery et al., 2006
	stomach (WW)	rat	RT-PCR	Watanabe et al., 2008
GluN2D	CNS/PNS (NVG)	mouse, rat	RT-PCR, IHC	Czaja et al., 2006,Slattery et al., 2006
	stomach (M, MP)	rat	IH, IHC, RT-PCR, WB,	Watanabe et al., 2008
GluN3A	CNS/PNS (NVG)	mouse	RT-PCR	Slattery et al., 2006
GluA1-4	small intestine (MP)	rat	ICC	Carpanese et al., 2014
GluA1	CNS/PNS (NA, NTS, NVG DMV)	cat, mouse, rat	IHC, RT-PCR	Ambalavanar et al., 1998; Lin and Talman, 2002; Slattery et al., 2006
	small intestine (MP, SP)	rat, guinea pig	RT-PCR, WB, IHC	Von Boyen et al., 2006, Liu et al., 1997
GluA2/3	CNS/PNS (NA, NTS, DMV)	cat	IHC	Ambalavanar et al., 1998;
	small intestine (MP, SP)	guinea pig	IHC, WB	Liu et al., 1997
GluA2	CNS (NVG)	mouse	RT-PCR	Slattery et al., 2006
	small intestine (MP)	rat	RT-PCR, WB	Von Boyen et al., 2006
GluA3	CNS (NVG)	mouse	RT-PCR	Slattery et al., 2006
	small intestine (MP)	rat	RT-PCR, WB	Von Boyen et al., 2006
GluA4	CNS (NA, NTS, NVG DMV)	cat, mouse	IHC	Ambalavanar et al., 1998; Slattery et al., 2006
	small intestine (MP, SP)	guinea pig	IHC, WB	Liu et al., 1997
GluK1-3	small intestine (MP)	rat	ICC	Carpanese et al., 2014
GluK1	CNS (NVG)	mouse	RT-PCR	Slattery et al., 2006
	small intestine (MP)	rat	RT-PCR, WB	Von Boyen et al., 2006
GluK2	small intestine (MP)	rat	RT-PCR, WB	Von Boyen et al., 2006
GluK3	CNS (NVG)	mouse	RT-PCR	Slattery et al., 2006
	small intestine (MP)	rat	RT-PCR, WB	Von Boyen et al., 2006
GluK4	CNS (NVG)	mouse	RT-PCR	Slattery et al., 2006
GluK5	CNS (NVG)	mouse	RT-PCR	Slattery et al., 2006
mGlu1	CNS/PNS (NTS, NVG/DRG)	dog, ferret, human rat	IHC, RT-PCR	Hoang and Lay, 2001; Page et al., 2005; Akiba et al., 2009
	esophagus (M, SM)	rat	RT-PCR	Akiba et al., 2009
mGlu1	stomach (CC, EC, PC, M, SM)	rat	IHC, RT-PCR, WB	Nakamura et al., 2010; San Gabriel et al., 2007; Akiba et al., 2009
	small intestine (SP)	guinea pig	IHC	Hu et al., 1999
	colon (SP)	guinea pig,	IHC	Hu et al., 1999
mGlu5	CNS (NTS, NVG)	dog, ferret, human, mouse, rat	IHC, RT-PCR	Hoang and Lay, 2001; Page et al., 2005; Slattery et al., 2006
	stomach (EC, M)	rat	RT-PCR	Nakamura et al., 2010
	small intestine (EGC, MP, SP)	mouse, rat, guinea pig,	IHC	Nasser et al., 2007; Hu et al., 1999; Liu and Kirchgessner, 2000
	colon (EGC, SP)	mouse, rat, guinea pig,	IHC, RT-PCR	Hu et al., 1999; Nasser et al., 2007
mGlu2/3	CNS (NVG)	ferret	IHC	Page et al., 2005
	small intestine (SP, MP)	rat	IHC	Larzabal et al., 1999
mGlu2	CNS (NTS, NVG)	dog, ferret, human, rat	RT-PCR	Hoang and Lay, 2001; Page et al., 2005;
	stomach (EC, PC, M)	rat	RT-PCR	Nakamura et al., 2010
mGlu3	CNS (NTS, NVG)	dog, rat	RT-PCR	Hoang and Lay, 2001; Page et al., 2005
	stomach (EC, PC, M)	rat	RT-PCR	Nakamura et al., 2010

(*continued on next page*)

Table 1 (*continued*)

Receptor/ transporter	Localization	Species	Method	References
mGlu4	CNS (NTS, NVG)	dog, ferret, human, rat	RT-PCR	Hoang and Lay, 2001; Page et al., 2005;
	esophagus (M, SM)	human, rat	IHC, RT-PCR	Chang et al., 2005a; Akiba et al., 2009
	stomach (EC, M, SM)	human, rat	IHC, RT-PCR	Nakamura et al., 2010; Chang et al., 2005a; Akiba et al., 2009
	colon (M, MP)	human	IHC	Chang et al., 2005a
mGlu6	CNS (NTS, NVG)	dog, ferret, rat	IHC, RT-PCR	Hoang and Lay, 2001; Page et al., 2005;
	stomach (CC, EC, M)	rat	RT-PCR	Nakamura et al., 2010
mGlu7	CNS (NTS, NVG)	dog, ferret, human, rat	IHC, RT-PCR	Hoang and Lay, 2001; Page et al., 2005
	stomach (EC, M)	rat	RT-PCR	Nakamura et al., 2010
	small intestine (MP)	mouse, rat, guinea pig, human	RT-PCR, WB	Tong and Kirchgessner, 2003
mGlu8	CNS (NTS, NVG)	dog, ferret, human, rat	IHC, RT-PCR	Hoang and Lay, 2001; Page et al., 2005;
	small intestine (MP, SM)	mouse, rat, guinea pig, human	IHC, RT-PCR, WB	Tong and Kirchgessner, 2003
EAAT1 (GLAST)	stomach (EGC, MP)	mouse	IHC	Iwanaga et al., 2005
	colon (MP, SP)	mouse	IHC	Seifi and Swinnyy, 2016
EAAT2 (GLT-1)	small intestine (EC, M, MP)	mouse, rat	IHC, RT-PCR	Schmitt et al., 2002; Iwanaga et al., 2005
	colon (EC, MP)	mouse	IHC	Iwanaga et al., 2005
EAAT3 (EAAC1)	small intestine (M, MP, SP)	guinea pig, mouse	IH, IHC	Iwanaga et al., 2005; Liu et al., 1997
EAAT4	stomach (SM, MP, SP)	mouse	IHC	Iwanaga et al., 2005
	small intestine (SM, MP, SP)	mouse	IHC	Iwanaga et al., 2005
VGluT1	PNS (DRG)	mouse, guinea pig	IHC	Brumovsky et al., 2011; Chen et al., 2015
	small intestine (MP)	human	IHC	Linke et al., 2008
	colon, rectum (IGLE, MP)	guinea pig, mouse	IHC	Olsson et al., 2004; Chen et al., 2015;
VGluT2	CNS/PNS (NGV, NTS/DRG)	mouse, rat	IHC	Tong et al., 2001; Brumovsky et al., 2011
	esophagus (IGLE)	mouse, rat	IHC	Raab and Neuhuber, 2004
	small intestine (MP, SP)	guinea pig, human, mouse, rat	IHC	Tong et al., 2001; Linke et al., 2008
	colon, rectum (IGLE, MM, MP, SP)	guinea pig, mouse	IHC, RT-PCR, WB	Olsson et al., 2004; Brumovsky et al., 2011; Chen et al., 2015; Seifi and Swinnyy, 2016
VGluT3	small intestine (MP)	human	IHC	Linke et al., 2008

Method applied: ICC: immunocytochemistry; IH: in situ hybridization; IHC: immunohistochemistry; NB: northern blot; RT-PCR: real time-polymerase chain reaction; WB: western blotting.
Anatomic sites: **CNS:** NTS: nucleus of the solitary tract, NVG: nodose vagal ganglion, NA: nucleus accumbens; DMV: dorsal motor vagal ganglion. **PNS:** DRG: dorsal root ganglion . **ENS:** CC: chief cells; EC: endocrine cells; EGC: enteric glial cell; IGLE: intraganglionic laminar endings; M: mucosa, MM: muscolaris mucosae; SP: submucosal plexus, MP: myenteric plexus, PC: parietal cells; SM: smooth muscle; S: serosal membrane; WW: whole wall.

potentiate afferent responses after either tension or mucosal receptors stimulation in a murine *in vitro* gastro-esophageal vagal preparation suggested that both receptors are peripherally located and participate to the excitatory modulation of vagal afferent mechanosensitivity (Slattery et al., 2006). This excitatory effect was attenuated by the antagonists of AMPA and NMDA receptors, NBQX and (−)-AP5, respectively, indicating a peripheral tonic modulation by endogenous glutamate. The presence of different AMPA and NMDA receptor subunits in the NVG and on peripheral afferent terminals, has been proposed by several groups by means of RT-PCR, in situ hybridization and immunohistochemistry approaches (Shigemoto et al., 1992; Chang et al., 2003; Slattery et al., 2006; Banerjee et al., 2009). Glutamate participates to the modulation of central and peripheral neuronal sites controlling also LES pressure. Microinjections of the amino acid into the caudal and rostral area of the cat DMV exerted an excitatory and inhibitory control of LES, respectively, suggesting the occurrence of a dual modulation depending on the DMV site involved (Rossiter et al., 1990). Peripheral NMDA receptors located in the myenteric plexus of the rabbit LES mediate NANC relaxations, by modulating the NO-cGMP pathway (Kohjitani et al., 2005).

NMDA receptors are considered to play also a central role in the regulation of extrinsic primary neurons conveying visceral sensitivity from the gastrointestinal tract to the CNS (McRoberts et al., 2001). Accordingly, GluN1 and GluN2A subunits located on dorsal root ganglions (DRGs), on the NVG and on terminals impinging on the cat esophagus have been described to contribute to visceral pain perception from this gut region (Banerjee et al., 2009).

mGlu receptors may also participate to transmit vagal afferent signals from the upper part of the gastrointestinal tract to the CNS. Application of standard RT-PCR and retrograde fluorescent immunolabelling techniques showed the presence of all mGluRs mRNAs and protein in the NVG and NTS of several species including rat, human, dog, rodent and ferret (Hoang and Lay, 2001; Page et al., 2005; Slattery et al., 2006). In the rat and human esophagus, mGlu1 and mGlu4 receptors are also located at a postjunctional level in the mucosal and smooth muscle layer (Akiba et al., 2009; Chang et al., 2005a).

4.3.2. Glutamate receptor modulation of the gastric function

Similarly to the esophagus, glutamatergic pathways in the stomach are mainly of extrinsic origin and may participate to either inhibitory or excitatory motor responses depending on the nature of the stimulus and the region involved. Vago-vagal reflexes play an important role in the fine tuning of the gastric contractions and acid secretion, while the relevance of the ENS in the coordination of gastric muscle activity is less certain (Furness, 2012). In an early study carried out in the rat, the amino acid has been shown to exert an inhibitory effect on both tonic and phasic contractile gastric responses, via activation of the dorsal vagal complex (Raybould et al., 1989). Several studies have been successively carried out to dissect the mechanism/s as well as specific glutamate receptors

Table 2
Pharmacological properties of compounds acting on iGluRs, mGluRs and reuptake transporters mentioned in the text.

Target	Compound	Action	Species	References
NMDA receptor	(−)-AP7	*competitive antagonist*	cat, rat	Davies et al., 1986
	(−)-AP5	*competitive antagonist*	cat, rat	Davies et al., 1986
	CGS19755	*competitive antagonist*	mouse, rat	Lehmann et al., 1988
	ketamine	*non competitive antagonist*	cat, rat	Anis et al., 1983
	MK-801	*non competitive antagonist*	rat	Wong et al., 1986
GLUN2B subtype	ifenprodil	*non competitive antagonist*	human	Hess et al., 1996
glycine site associated with NMDA receptor	kynurenic acid	*competitive antagonist*	rat	Birch et al., 1988
	7ClKynA	*competitive antagonist*	rat	Kemp et al., 1988
	5,7diClKynA	*competitive antagonist*	rat	McNamara et al., 1990
AMPA/kainate receptor	CNQX	*competitive antagonist*	rat	Honoré et al., 1988
	DNQX	*competitive antagonist*	rat	Honoré et al., 1988
AMPA receptor	NBQX	*competitive antagonist*	rat	Wilding and Huettner, 1996
	IEM-1460	*selective GluA2 (Ca^{++} permeable)-lacking AMPA receptor antagonist*	rat	Buldakova et al., 1999
GLUA1/2 subtype	(S)-CPW399	*agonist*	rat	Campiani et al., 2001
mGlu1/5 receptor	(S)-3,5-DHPG	*agonist*	rat	Schoepp et al., 1994
mGlu1α receptor	AIDA	*competitive antagonist*	human	Moroni et al., 1997
mGlu5 receptor	(*RS*)-CHPG	*agonist*	rat	Doherty et al., 1997
	MPEP	*non competitive antagonist*	human	Gasparini et al., 1999
	MTEP	*non competitive antagonist*	mouse	Brodkin et al., 2002
	ADX10059	*negative allosteric modulator*	human	Keywood et al., 2009
Group III mGlu receptor	MAP4	*competitive antagonist*	rat	Jane et al., 1994
mGlu7 receptor	AMN082	*agonist*	mice	Mitsukawa et al., 2005.
EAAT	riluzole	*activator*	rat	Azbill et al., 2000

involved. Single cell recording techniques showed that distension of the rat stomach and duodenum stimulates vagal afferents innervating the NTS leading to glutamate release. The amino acid, mainly via AMPA/kainate receptors, activates inhibitory neurons in the NTS that project to the vagal DMV, thus inducing an inhibitory effect on gastric motility and emptying (Zhang and Fogel, 2003). In functional studies carried out in the dog and rat, NMDA and AMPA/kainate receptor antagonists were able to attenuate the mechano-transduction properties of vagal sensory afferents after antrum distension (Furukawa et al., 2001; Sengupta et al., 2004). Electrophysiological measurement of the ferret vagal preganglionic neurons projecting to the periphery, showed that AMPA/kainate, more than NMDA, receptors are required for synaptic neurotransmission of mechano- and chemosensitive vagal inputs from the esophagus and stomach onto vagal efferents (Partosoedarso and Blackshaw, 2000). A small proportion of rat DMV neurons may, however, be excited by gastrointestinal distension, possibly as a consequence of a direct effect of glutamate released from vagal afferents departing from the NVG and directly contacting DMV neurons (Zhang and Fogel, 2003). Analogously, microinjections of kainic acid and NMDA in the dorsal vagal complex of the rat increased intragastric pressure and motility. Both responses were selectively abolished by prior administration of the respective antagonists, DNQX and (−)-AP5 (Table 2), suggesting a role for iGluRs in vagally-mediated excitation of gastric motility (Sivarao et al., 1999). In the rat, retrograde tracing immunohistochemistry showed that all vagal afferent neurons projecting from the stomach to the NVG express NMDA receptor subunits. The great majority of gastric afferents exhibited GluN1 immunoreactivity, whereas GluN2C and GluN2D subunits were expressed by more restricted neuronal populations (Czaja et al., 2006). At a peripheral level, RT-PCR analysis showed that the mRNAs of all NMDA subunits are present in the rat stomach, although expression of GluN1 and GluN2D seems to be predominant (Watanabe et al., 2008). Interestingly, expression of GluN2D mRNA and protein increased in mucosal cells and in the myenteric plexus after vagotomy during dilatation, suggesting that GluN2D subunit transcription, translation and trafficking is under control of the vagus nerve and may play a role in the regulation of gastric relaxations (Watanabe et al., 2008). Pharmacological evidences of a potent local excitatory effect of NMDA and kainate receptors located on intrinsic myenteric neurons on the rat gastric fundus smooth muscle have also been given (Janković et al., 1999). GluN1, GluN2A and GluN2B postjunctionally located in the mucosal layer, submucosal, and myenteric neurons of the rat stomach may influence histamine-induced acid secretion and blood flow (Tsai et al., 2004). mGlu receptors expressed by non-neuronal cells in the gastric mucosa, play a role in conveying sensory information to vagal afferent fibers and participate to digestion of food (Julio-Pieper et al., 2011). RT-PCR, western blotting and immunohistochemistry investigations showed that mGlu1 receptors are present on the apical membrane of chief and parietal cells of the rat stomach and may participate to the gastric phase of protein digestion (San Gabriel et al., 2007). In the same experimental model, parietal cells displayed immunoreactivity for mGluR2/3 receptors, which may take part to gastrin secretion and gastric acid homeostasis regulation (Gill and Pulido, 2001). In a more recent study carried out in the rat stomach, mGlu1-8 mRNAs were detected in different isolated cell fractions, comprising parietal and chief cells, large and small endocrine cells, like D cells of the rat stomach, which contribute to luminal glutamate sensing as well as to regulation of somatostatin secretion (Nakamura et al., 2010).

4.3.3. Glutamate receptor modulation of the small and large intestine function

In contrast to the esophagus and stomach, intrinsic neuronal circuitries of the ENS, which are composed of sensory neurons, interneurons and several classes of motor neurons, play a major role in the control of small intestine and colon functions, including muscle activity, transmucosal fluid fluxes and local blood flow (Furness et al., 2014). Both iGlu and mGlu receptors have been localized and are abundantly expressed in the ENS innervating the small and large intestine of different species (Kirchgessner, 2001) (Fig. 3). Immunohistochemistry and in situ hybridization studies suggest that, among the different iGlu receptor subunits, GluN1 is expressed at a somato-dendritic level and along neurites of the vast majority of myenteric neurons in the human colon and in submucosal and myenteric neurons of the guinea pig ileum and rat colon (Liu et al., 1997; Giaroni et al., 2003; Burns et al., 1994, 1995). The

abundance of GluN1 receptors in the ENS has been recently observed also in primary cultures of rat myenteric neuron (Carpanese et al., 2014) and may reflect the functional relevance of this receptor pathway in the intestine. Two of the four variants of the GluN2 subunit, GluN2A and GluN2B, have been detected in guinea pig ileum myenteric neurons and in enteric glial cells of the newborn rat small intestine (Liu et al., 1997; Von Boyen et al., 2006). The predominance of one of these two subunits has important consequences on NMDA receptor function, since GluN2A containing receptors are classically considered to endorse neuroprotective actions, while GluN2B subunits are coupled to several signaling pathways that lead to neurotoxicity (Zhang and Luo, 2013).

The coordination of excitatory and inhibitory enteric reflexes within myenteric plexus circuitries underlies regulation of intestinal motility. Glutamate receptors may influence motor responses in the intestine, although the exact pathways and neurotransmitter involved have not yet been fully elucidated. In guinea pig ileum LMMP preparations, glutamate induced contractions that were mimicked by NMDA receptor agonists and blocked by the selective antagonists such as (−)-AP5, MK-801 and by the pore blocker, Mg^{++} (Shannon and Sawyer, 1989; Campbell et al., 1991). The excitatory action of glutamate on intestinal smooth muscle cells is indirectly induced by activation of cholinergic neurons (Wiley et al., 1991). Accordingly, NMDA receptors have been shown to facilitate both spontaneous and stimulated ACh release from myenteric neurons of the guinea pig ileum and human colon (Giaroni et al., 2003; Wiley et al., 1991; Sinský and Donnerer, 1998). There are evidences suggesting that the excitatory control of NMDA receptors on enteric cholinergic neurons is not direct in nature, but mediated by the intermediate release of NO (Milusheva et al., 2005). The existence of an interplay between enteric nitrergic and glutamatergic pathways has been recently demonstrated by the ability of NMDA to concentration-dependently enhance NO release from guinea pig ileum isolated segments (Filpa et al., 2015). In analogy with the CNS, enteric NMDA receptors seem to promote NO production mainly by activating Ca^{++}-dependent neuronal NO synthase (nNOS), although participation of Ca^{++}-independent NOS, such as inducible nitric oxide synthase (iNOS) was not excluded (Bagyánski et al., 2011; Giaroni et al., 2013; Frade et al., 2009). Conversely, NO generated mainly via nNOS, has been shown to promote glutamate release from guinea pig ileum myenteric plexus (Filpa et al., 2015). Glutamate actions on intestinal smooth muscle may also depend on the ability of NMDA receptors to modulate opioid-mediated inhibition of cholinergic twitch response (Donnerer and Liebmann, 2007). Furthermore, both morphine and endogenous opioids induce glutamate release and modulate NMDA-mediated excitatory effect in guinea pig ileum LMMP, suggesting that a cross-talk between excitatory NMDA and inhibitory opioid pathways occurs also in this model (Donnerer and Liebmann, 2009). Finally, another mechanism proposed for the modulation of glutamate release from enteric neurons is represented by NMDA autoreceptor-mediated positive feedback (Sinský and Donnerer, 1998; Giuliani et al., 2006).

According to the hypothesis that NMDA receptors participate to visceral sensitivity neurotransmission, GluN1 immunoreactivity was found in the neuronal soma of extrinsic primary afferent thoracolumbar DRGs, as well as in their peripheral terminals innervating the rat colonic mucosa (McRoberts et al., 2001). GluN1 subunit was largely co-expressed with capsaicin-sensitive transient potential vanilloid receptor, TRPV-1, which are involved in the neurotransmission of visceral pain (McRoberts et al., 2001). Peripherally and centrally located NMDA receptors may contribute to development of visceral hypersensitivity in non-pathological conditions, since after both intrathecal and intraperitoneal administration of the non competitive NMDA antagonist, MK-801 (Table 2) hypersensitivity responses to both innocuous (low

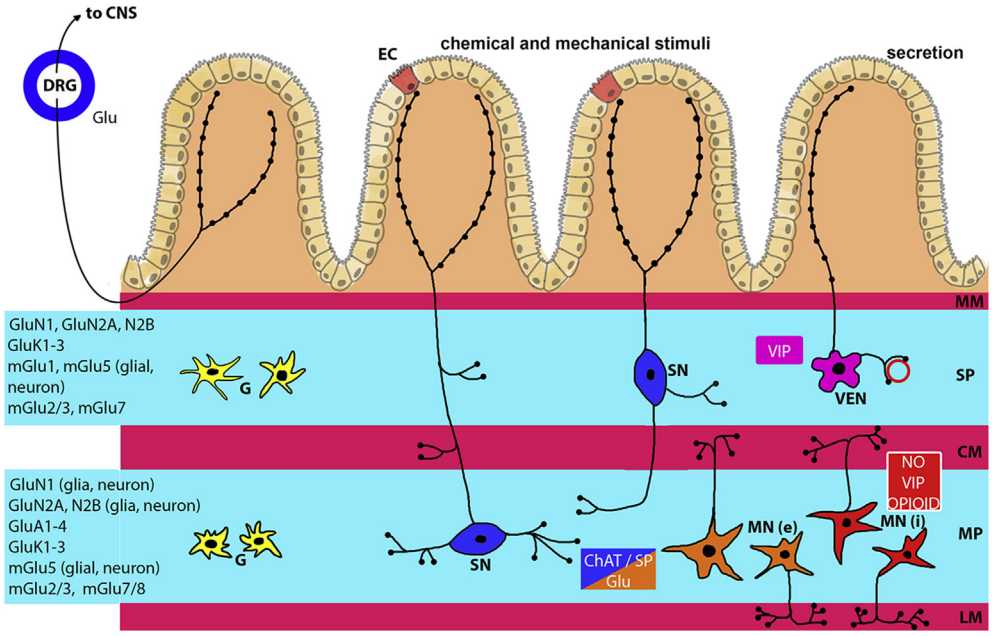

Fig. 3. Schematic representation of glutamatergic neurotransmission in the small intestine. Intrinsic primary afferent neurons (SN) in the submucosal (SP) and myenteric plexus (MP) and excitatory (e) motor neurons (MN) in the myenteric plexus express glutamate (Glu), ChAT and SP immunoreactivity. Glu is also found in dorsal root ganglia containing the soma of extrinsic primary afferent neurons projecting to the CNS. Some of the major ionotropic and metabotropic glutamate receptors present within the relevant gut layers are listed in the left side of the schema. In the SP glutamate receptors are expressed by intrinsic primary afferent neurons that project to the mucosa and detect chemical and mechanical stimuli and by visceroeffector neurons (VEN), which contain VIP and participate to secretomotor reflexes. In the MP, glutamate receptors exert a modulatory role on both excitatory and inhibitory (i, containing NO, opioid and VIP) motor neurons involved in the ascending excitation and descending inhibition of the peristaltic reflex. Glutamate-expressing enteric glial cells (G) have been found both in the SP and MP. Abbreviations: ChAT, cholineacetyltransferase; SP, substance P; CNS, central nervous system; NO, nitric oxide; VIP, vasoactive intestinal peptide, EC, enterochromaffin cell; MM, muscolaris mucosae; CM, circular muscle; LM, longitudinal muscle.

pressure) and noxious (high pressure) stimuli induced by colorectal distension in rats were completely abolished (Gaudreau and Plourde, 2004). In isolated rat DRGs innervating the distal colon, patch-clamp techniques and intracellular Ca^{++} imaging showed that a functional NMDA receptor is predominantly composed of GluN2B subunits (Li et al., 2004).

Although the majority of studies on iGluR-mediated glutamatergic neurotransmission in the small and large intestine have focused on the role of NMDA receptors, there are reports indicating that also AMPA and kainate may participate in the regulation of the intestinal function. In the guinea pig ileum, GluA1 subunits are co-expressed with calbindin, a Ca^{++} binding protein that has been considered a marker for intrinsic primary afferent neurons (Liu et al., 1997; Kirchgessner, 2001). GluA2/3 and GluA4 subunits have been detected in cholinergic and non-cholinergic interneurons and motor neurons, suggesting that AMPA receptors may be involved in the modulation intestinal motility (Liu et al., 1997; Kirchgessner, 2001). In a more recent paper, a pan-GluA1-4 antibody showed immunoreactivity on rat small intestine isolated primary cultures of myenteric neurons (Carpanese et al., 2014). RT-PCR studies revealed the presence of GluA1, 3 and 5 in isolated cultures of enteric glial cells (Von Boyen et al., 2006). In the guinea-pig distal colon, AMPA dose-dependently increased electrically-stimulated longitudinal muscle contractions and peristalsis, while CNQX, an AMPA/kainate antagonist (Table 2), virtually abolished peristalsis (Giaroni et al., 2000). In a recent study, GluA 1, 3 and 4 subunits were shown to divergently immunolocalize within different subpopulation of the mouse colon ENS. In this model activation of AMPA receptors directly influenced colonic spontaneous contractility. In particular, application of (S)-CPW399, a potent GluA1/2 agonist (Table 2), caused a significant dose-dependent increase of the force, but not of the frequency of longitudinal muscle spontaneous contractions (Seifi and Swinnyy, 2016). Both in the rat and guinea pig ileum, GluK1-3 subunits have been localized to nerve terminals and on the soma of myenteric and submucosal neurons and in enteric glial cells using pan non-selective antibodies against GluK1-3 (Liu et al., 1997; Carpanese et al., 2014). Kainate receptors, however, do not seem to have a role in the modulation of intestinal motility, as suggested for the guinea pig distal colon, where *in vitro* addition of kainic acid did not influence either ACh release or peristalsis (Giaroni et al., 2000). Accordingly, in the guinea pig ileum, kainic acid was unable to affect electrically-evoked contractions (Luzzi et al., 1988). GluK1 subunit may play an ontogenetic role since it is present in the early stage of the rat gut postnatal development (Bettler et al., 1990).

In the guinea pig small intestine, immunoreactivity for mGlu5 and mGlu1 receptors was observed in the plasma membrane of submucosal and myenteric neurons (Hu et al., 1999; Liu and Kirchgessner, 2000). In the submucousal plexus, mGlu5 containing cells co-expressed vasoactive intestinal peptide (VIP), a marker for secretomotor neurons (Liu and Kirchgessner, 2000). Functionally active mGlu5 have been described to participate to enteric reflexes since the Group I mGlu agonist, (RS)-3,5-DHPG (Table 2), induced mGlu5 internalization in isolated enteric neurons, an effect mimicked in guinea pig ileum mucosal preparations by mechanical stimulation (Liu and Kirchgessner, 2000). Blockade of mGlu5 with Group I mGlu receptor antagonists, such as AIDA and MPEP (Table 2), reduced stimulus-evoked slow excitatory postsynaptic potentials (EPSPs) in submucosal S type neurons, suggesting that mucosal stimulation activates mechanosensitive pathways that use mGlu5 receptors to excite neurons in the submucosal ganglia (Liu and Kirchgessner, 2000). These data contrast with those obtained by another group showing that group I mGlu receptors inhibit slow EPSPs and potentiate slow inhibitory postsynaptic potentials (IPSPs) in S-type submucosal neurons (Ren et al., 1999). In the ileum and colon of guinea pig, mouse and rat, mGlu5 receptors are expressed by enteric glial cells where they may participate to adaptive changes as suggested by the ability of the selective agonist, (RS)-CHPG (Table 2), to induce a concentration-dependent increase of c-FOS and of ERK 1/2 phosphorylation (Nasser et al., 2007). The presence of Group II and Group III receptors in the ENS has also been demonstrated by immunohistochemical, biomolecular and electrophysiological studies. Immunoreactivity for mGlu 2/3 receptors was distributed in the submucosal and myenteric plexus and in nerve fibers of the rat ileum and jejunum (Larzabal et al., 1999). In guinea pig isolated submucosal and myenteric plexus primary neuron culture, functional mGlu 2/3 were found in tight contact with VGLUT2 immunoreactive nerve terminals and their activation by selective agonists was associated to $G_{\alpha i}$ protein-mediated inhibition of N type Ca^{++} channels (Chen and Kirchgessner, 2002). In rat small intestine myenteric plexus, RT-PCR investigations showed high levels of mGlu7 and mGlu8 mRNAs, while immunoreactivity for mGlu8 receptors was observed in the ENS of human, rat, mouse and guinea pig specimens and colocalized with nNOS (Tong and Kirchgessner, 2003). In the guinea pig, mGlu8 agonists induced a facilitatory effect on motility and on longitudinal muscle contraction, while mGlu8 antagonists induced an inhibitory effect on both parameters, suggesting the occurrence of a tonic glutamatergic control of colonic motor responses via mGlu8 receptors (Tong and Kirchgessner, 2003). Interestingly, in the rat rectum activation of mGlu receptors by non selective agonists induced stronger contractions than those obtained in the presence of kainic acid and NMDA, respectively (Janković et al., 1999). Colonic mGlu receptors may participate to glutamate-mediated modulation of the intestinal mucosal function by acting either on enteric neurons or on non-neuronal epithelial cells. In normal human colon epithelium mGlu4 receptors have been detected by immunohistochemistry (Chang et al., 2005a). More recently, in situ hybridization and western blotting approaches showed that mGlu7 mRNA and protein are highly expressed in the mouse colon (Julio-Pieper et al., 2010). Exposure of mucosa/submucosa preparations to a novel selective mGlu7 agonist, AMN082 (Table 2), potentiated stress-induced secretory responses, probably through the activation of submucosal neuronal circuitries, suggesting a role for mGlu7 receptors in the development of stress-associated gastrointestinal secretory disorders such as diarrhea or constipation.

5. Glutamatergic neurotransmission in gastrointestinal diseases

Glutamate release, uptake, metabolism and signaling disorders are described to contribute to the etiology of several CNS pathologies, including neurodevelopment, neurodegenerative and psychiatric diseases (Miladinovic et al., 2015). There is increasing evidence suggesting that dysregulation of the enteric glutamatergic neurotransmitter machinery may participate to development of symptoms in several pathologies also in the gastrointestinal tract. Such disarray principally involves changes in receptor expression and function, although severe insults to the gut, such as those occurring during an inflammatory or I/R injury, may be associated with neurotoxicity (excitotoxicity), caused by the excessive increase of extracellular glutamate concentrations. Excitotoxicity involves excessive glutamate-mediated postsynaptic excitation, resulting from enhanced pre-synaptic release superimposed on deficient uptake and/or cytosolic efflux (Obrenovitch and Urenjak, 1997). A simplified overview of intracellular events consequent to the glutamate-mediated excitotoxic damage comprises the breakdown of ionic homeostasis, leading to an excessive raise in

intracytoplasmic Ca^{++} concentrations, mediated by extrasynaptically located NMDA receptors and by non-NMDA receptors, and ultimately to neuronal necrosis and apoptosis (Pellegrini-Giampietro et al., 1997; Lau and Tymianski, 2010). Direct evidence for excitotoxic-induced necrosis and apoptosis of enteric neurons has been given by exposing guinea pig ileum isolated enteric ganglia to high extracellular glutamate concentrations (Kirchgessner et al., 1997). These effects were mimicked by NMDA and blocked by the competitive antagonist (−)-AP5 (Table 2), suggesting that glutamate neurotoxicity in the ENS is mostly mediated via overactivation of NMDA receptors, although also kainate receptors seem to be involved, as observed in the CNS (Kirchgessner et al., 1997; Lau and Tymianski, 2010).

The possibility to shed more light on the occurrence of neuronal dysfunction/damage involving the enteric glutamatergic neurotransmission following various acute or chronic challenges to the gastrointestinal tract may allow to discover neuroprotective strategies aimed to slow the progression of gut diseases. In the following chapters, we will consider different gastrointestinal pathologies, all characterized by alterations of enteric neuronal circuitries, including enteric glutamatergic pathways, such as gastroesophageal reflux, gastric acid hypersecretory diseases, IBD, IBS and intestinal I/R damage.

5.1. Gastroesophageal reflux and gastric acid hypersecretory diseases

The ability of glutamate to regulate LES pressure led several investigators to evaluate whether glutamate receptors could represent targets for the management of transient lower esophageal sphincter relaxations (TLESRs) (Mittal et al., 1995; Hirsch et al., 2002; Iwakiri et al., 2005). TLESRs are vago-vagal reflexes, represented by a prolonged distension of the LES in the absence of a swallow and are initiated by gastric distention (Boeckxstaens, 2010). TLESRs represent the major determinant of reflux in healthy subjects and in most patients with gastroesophageal reflux disease (GERD) (Boeckxstaens, 2010). In human healthy subjects riluzole, an approved neuroprotective agent activating glutamate reuptake (Table 2), decreased the rate of TLESRs triggered by gastric distension suggesting that glutamate participates to the modulation of neuronal circuitries underlying TLESRs (Hirsch et al., 2002). Lehmann and Brändén (2001) observed that in vivo administration of CGS19755, an antagonist at NMDA receptors (Table 2), induced a dual effect on TLESRs in awake dogs, which could be either excitatory or inhibitory depending on the individual animal studied. More consistent and promising results have been successively obtained on the ability of glutamate to modulate TLESRs via mGlu receptor modulation (Julio-Pieper et al., 2011). In the ferret, retrograde tracing revealed that vagal afferents express mGlu protein involved in the modulation of the TLESR (Page et al., 2005). Pharmacological studies aimed to screen the ability of selective Group I, II and III agonists and antagonists to modulate TLESR, allowed to identify mGlu5 as a major player. Both in ferrets and in dogs, MPEP, a selective mGlu5 antagonist, displayed the highest inhibitory effect on TLESR responses (Frisby et al., 2005; Jensen et al., 2005). On the basis of these observations, small negative allosteric modulators of mGlu5 receptors, such as ADX10059, have been designed in the last few years for the potential management of GERD (Keywood et al., 2009; Zerbib et al., 2011).

iGlu and mGlu receptors, involved in the control of esophageal pain perception may participate to acid-induced esophageal hypersensitivity. Down-regulation of GluN2A mRNA and up-regulation of GluN1 mRNA was observed in the cat esophagus, DRG and NVG after repetitive acid exposure (Banerjee et al., 2009). In the proximal esophagus of human healthy subjects, ketamine (Table 2) was able to prevent and reverse hypersensitivity caused by acid infusion in the distal esophagus, possibly by blocking NMDA receptor-mediated central sensitization (Willert et al., 2004). Acute acid infusion determined up-regulation and calmodulin-kinase II mediated phosphorylation of the GluA1 subunits in the rat anterior and midcingulate cortices, indicating that activation of AMPA receptors may represent another central mechanism underlying acid-induced esophageal sensitization (Banerjee et al., 2013). At this level, microinjection of the AMPA receptor antagonist, IEM-1460 (Table 2), completely blocked the increase of neuronal firing consequent to repeated esophageal acid exposure (Banerjee et al., 2013). On the whole, these data suggest that compounds acting at NMDA, AMPA or mGlu receptors, particularly mGlu5, may have a potential role in the treatment of patients with GERD, who display a low threshold of pain perception.

The possibility to pharmacologically influence intrinsic and extrinsic glutamatergic pathways impinging on the stomach may bear a potential role in the treatment of certain gastric acid hypersecretory disorders. Administration of glutamate into the rat femoral vein reduced histamine- and oxotremorine-stimulated gastric acid secretion, via activation of peripheral non-NMDA, possibly AMPA receptors (Tsai et al., 1999). Activation of glutamate receptors in brain regions controlling the gastric function may result either in stimulation or inhibition of gastric secretion, depending on the nature of the stimulus, the brain area involved and the dose of glutamate receptor agonists used. Activation of central NMDA and AMPA/kainate receptor pathways may underlay the inhibitory effect on gastric acid secretion exerted by peripherally administered endotoxin. This central pathway involves the NO/cyclic GMP cascade (García-Zaragozá et al., 2000). Microinjection of kainate into the ventromedial arcuate nucleus of the hypothalamus induced a significant inhibition of pentagastrin and gastric acid secretion in urethane anesthetized rats (Tebbe et al., 2001). Lateral ventricular administration of NMDA and kainate stimulated gastric acid secretion in rats, this effect was blocked by selective antagonists as well as by vagotomy, suggesting the involvement of glutamate receptors possibly located in the lateral hypothalamus (Tsuchiya et al., 2001). The effect of centrally administered kainate on gastric acid secretion was dose-dependent, since low doses injected into the raphe pallidus acted in a gastric protective manner and reduced ethanol-induced gastric acid secretion, while, higher doses increased this parameter and produced gastric lesions (Kaneko and Tachè, 1995; Kaneko et al., 1995). Glutamate, via mGlu receptors, may influence mucosal defense mechanisms to prevent subsequent injury attributable to excessive acid exposure in the duodenum. In the rat duodenum, luminal glutamate was shown to increase mucus alkalinization and mucus gel thickness and this effect was mimicked by L-AP4, an agonist at mGlu4 receptors and abolished by the mGluR4 antagonist, MAP4 (Table 2). On opposite (S)-3,5-DHPG, an mGluR1/5 agonist increased intracellular pH, whereas AIDA, an antagonist at mGluR1/5, partially inhibited glutamate-mediated alkalinization (Akiba et al., 2009). On the basis of these observations, development of molecules selectively targeting specific iGlu and mGlu receptors increasing gastric mucosal defenses, may be of interest in the treatment of gastric acid hypersecretory diseases.

5.2. Inflammatory bowel disease (IBD)

IBD primarily comprises two diagnostically distinct, but pathologically similar disorders: Crohn's disease (CD) and ulcerative colitis (UC). Immune, genetic and environmental factors are thought to contribute to IBD, although the exact etiology remains to be unveiled. Patients with IBD commonly manifest symptoms suggestive of disturbed gastrointestinal function, characterized by

sensory, motor and secretory alterations (Lomax et al., 2005). The hallmark of IBD is chronic, uncontrolled inflammation of the intestinal mucosa which can manifest itself along the entire lenght of the gastrointestinal tract, as in CD, or that may be confined to the rectum and colon, as in UC (Hanauer, 2006; Lakatos et al., 2006). Mucosal damage, abnormal secretion and visceral sensation may represent transient symptoms following the inflammatory challenge, which may lead to more persistent changes in enteric neuronal circuitries as well as in the smooth muscle layer, resulting in dismotility. Inflammation leads to derangements of enteric neuronal circuitries, neuronal hyperexcitability of afferent neurons, synaptic facilitation and attenuated inhibitory neuromuscular neurotransmission (Lomax et al., 2005; Sharkey and Kroese, 2001). The cross-talk occurring among different cell populations, constituting the enteric microenvironment, and infiltrating inflammatory cells may account for the structural and functional changes occurring in enteric circuitries in response to an inflammatory stimulus (Giaroni et al., 1999; Vasina et al., 2006). Neuronal cells in the ENS are located in close proximity to mucosal immunocytes and may regulate one another's functions by releasing a complex set of cytokines, neurotransmitters and hormones. Neuronal activation can lead to degranulation of mast cells and recruitment of neutrophils to the area. Receptors for neuropeptides released by enteric nerves are present on lymphocytes, whose stimulation by SP or VIP, can induce their differentiation and alter their production of immunoglobulins (Lakhan and Kirchgessner, 2010). Neuro-immune interactions may also help explain the occurrence of damage at gastrointestinal sites distant from the inflammatory processes (De Schepper et al., 2008). In rats, trinitrobenzensulphonic acid (TNBS)-induced colitis produced a decrease of NA and ACh release, which was observed not only in the inflamed colon but also in the normal ileum (Blandizzi et al., 2003). Interestingly, increased levels of IL-6 and histamine during inflammation may exert a presynaptic inhibitory effect on the release of different neurotransmitters even at sites distant from the lesion (Xia et al., 1999; Liu et al., 2000). These data strengthen the hypothesis for the existence of specific functional interactions between different subpopulation of enteric neurons and immunocytes (Bueno, 2000).

In this scenario it is particularly important to unveil possible modulators of pro-inflammatory states in the gut, in order to prevent the occurrence of more obvious inflammatory conditions. Several reports are available to demonstrate that glutamatergic transmission participates to the development of neuroinflammatory responses. The cascade of molecular events activated by glutamatergic pathways in response to inflammation is complex and may involve the modulation of oxidative and nitrosative stress pathways (Kaszaki et al., 2012). Glutamate, via NMDA receptor activation and subsequent elevation of intracellular free [Ca^{2+}] may lead to reactive-oxygen species (ROS) formation, including superoxide, via xanthine oxidoreductase (XOR), as well as to NO generation, by NOS activation (Kaszaki et al., 2012). The reaction between ROS and NO leads to the generation of peroxynitrite which have been implicated in the pathogenesis of a variety of acute and chronic inflammatory stases, also in the gastrointestinal tract (Krieglstein et al., 2001). In addition, radicals generated during NMDA-stimulated metabolism of arachidonic acid may add to oxidative injury (Chang et al., 2008). Interestingly, human data have shown that both NO and prostaglandin may represent important factors reducing gastrointestinal motility in pro-inflammatory conditions (Kalff et al., 2003). Another possibility is that enteric glutamatergic pathways may be involved in neuroimmune responses developing during inflammation. In this condition, enteric neurons are able to produce various cytokines and chemokines through IL-1β- and TNFα-dependent pathways, which on their own support the development of immune cell infiltrates within the ENS (Tixier et al., 2006). In the CNS, these pro-inflammatory cytokines may contribute to neuronal injury through the activation of microglia, the induction of NO and arachidonic acid release, the generation of prostaglandins and other inflammatory eicosanoids (Montgomery and Bowery, 2012). More importantly, IL-1β- and TNFα contribute to glutamate-induced excitotoxicity by increasing its release and reducing its uptake at a glial level, as well as by modifying expression and localization of NMDA and AMPA receptors (Viviani et al., 2014). On its own, glutamate, via NMDA receptors, has been shown to induce IL-1β and TNFα expression in neuronal cells (Kitaoka et al., 2007; Viviani et al., 2014) and to modulate IL-1β-induced neural cell injury *in vitro* (Radesäter et al., 2003).

In this perspective glutamate, as an enteric excitatory neurotransmitter, may play an important role in the transduction of local inflammatory signals influencing gastrointestinal motility. Indeed in the last few years several groups have demonstrated that blockade of NMDA receptors may provide gastrointestinal neuroprotection against inflammation and glutamate-induced neurotoxicity, suggesting a possible role of endogenous glutamate in both acute and chronic inflammatory conditions. In particular, evidences obtained in animal and human studies have recently pointed to the possible efficacy of kynurenic acid (KA), an endogenous competitive antagonist at the glycine site associated with NMDA receptors (Table 2), in IBD management (Kaszaki et al., 2012). In the gut, KA is endogenously produced by enterocytes, along the side branch of tryptophan-L-kynurenine pathway as well as by the enteric microbiota (Kuc et al., 2008; Turski et al., 2013). There are reports from clinical studies suggesting that dysregulation of KA production may influence a number of pathologies in the gastrointestinal tract, including gastroduodenal ulcers, IBS, coeliac disease and IBD (Turski et al., 2013). Interestingly, KA serum levels were found to be higher in patients with either IBD or coeliac disease (Forrest et al., 2002; Christmas et al., 2010) further strengthening the concept that modulation of the glycine site associated with NMDA receptors may represent a promising therapeutical target to treat neuromuscolar dysfunctions associated with IBD. In the dog and rat colon, administration of KA during the acute phase of an experimentally-induced inflammatory response, decreased motility index, NOS and myeloperoxidase activity and ROS produced by XOR (Kaszaki et al., 2008; Varga et al., 2010). The Authors hypothesize that, during the early inflammatory phase, NMDA receptors may participate to XOR-dependent formation of superoxide radicals, and successively, by interaction with NO, to peroxynitrite. This highly toxic molecule exerts an inhibitory action on nNOS in myenteric neurons, thereby altering smooth muscle relaxation (Kaszaki et al., 2012).

The response of the ENS to inflammation may vary according the type (acute vs chronic), extent and intensity of the inflammatory challenge along the gastrointestinal tract (Vasina et al., 2006). Long lasting and severe inflammation causes dysfunction of the nitrergic neurotransmission, changes of smooth contractility and increased production of different pro-inflammatory cytokines, including IL-1β, IL-6 and TNFα (Sharkey and Kroese, 2001; Gyires et al., 2014). During a chronic inflammatory challenge in the gut, overactivation of NMDA receptors has been shown to induce neuronal excitotoxic death via excessive NO production by activation of different NOS isoforms (Kaszaki et al., 2012). In a chronic model of TNBS-induced colitis in the rat, KA and SZR-72, a centrally-acting analogous of KA, were able to normalize the increased frequency of intestinal movements, to decrease NO-linked nitrosative stress and to reduce IL-6 and TNFα production (Érces et al., 2012). Accordingly, in the rat intestine, glutamate, via NMDA receptors, induced the production of pro-inflammatory cytokines, such as TNF-α, suggesting the existence of a possible glutamate-NMDA-inflammation axis in the

ENS (Xu et al., 2005).

5.3. Irritable bowel syndrome (IBS)

IBS entails a heterogeneous group of functional lower gastrointestinal tract disorders characterized by abdominal pain or discomfort associated with altered bowel habits and disordered defecation (Longstreth et al., 2006). Different psychological and biological factors have been described to contribute to the pathophysiology of IBS, including psychosocial disturbances, abnormal motility, visceral hyperalgesia, disturbance of brain-gut interaction, changes in CNS processing, autonomic nervous system dysfunction, genetic and environmental factors and postinfectious events (Drossman et al., 2002). In this latter regard, in IBS patients, changes in both sensory and motor responses have been suggested to depend, at least in part, upon a subclinical low-grade immune activation, in the absence of overt alarming features of organic inflammatory pathology. IBS patients may show moderate increased levels of proinflammatory cytokines, including IL-6, IL-8, TNFα and IL-1β, and of the neutrophilic marker, fecal calprotectin (Dinan et al., 2006; De Schepper et al., 2008; Brierly and Linden, 2014). Furthermore, an increased number of immunocytes have been identified in the mucosal and muscolar layer of the jejunum, ileum and colon of patients with IBS with respect to healthy controls (Drossman et al., 2002; Guilarte et al., 2006; Brierly and Linden, 2014). Interestingly, symptoms compliant to IBS have been reported in patients with inactive IBD and after an acute bout of infectious gastroenteritis (De Schepper et al., 2008; Brierly and Linden, 2014). Regardless of the inflammatory origin in IBD and IBS patients, long-term neuroplastic changes in the neuronal circuitries of the brain-gut axis may induce a diffuse and poorly localized chronic abdominal pain (Veremulen et al., 2014). Peripheral mechanisms within the enteric microenvironment may contribute to the development of visceral hypersensitivity. In addition, neuronal synaptic changes along with increased neurotransmitter release may occur in the spinal cord and brain, leading to a state of central "wind-up" (Holzer, 2001; Veremulen et al., 2014). Although there is no direct evidence for the participation of glutamate to visceral pain transmission, the ability of NMDA receptor antagonists to reduce pelvic and splanchnic afferent stimulation from the colon to mechanical stimuli is highly indicative of an endogenous glutamatergic modulation of mechanosensitive pathways (Blackshaw and Gebhart, 2002). Intrathecal administration of NMDA to the lumbar rat spinal cord, concentration-dependently enhanced visceromotor responses to noxious colorectal distension and this effect was blocked by the (−)-AP5 and by the antagonist at the glycine site associated with NMDA receptor, 7-chloro-kynurenic acid (Kolhekar and Gebhart, 1994, 1996) (Table 2). In a rat model of zymosan-induced colitis, Coutinho et al. (1996) demonstrated the anti-hyperalgesic effect obtained after spinal administration of the NMDA antagonist, MK-801. In a successive study the same group showed that, administration of either MK-801 or L-NAME, an inhibitor of nNOS, in the rostral ventromedial medulla, reduced zymosan-produced hyperalgesia to colorectal distension, suggesting that both NMDA receptors and NO play a facilitatory role on descending modulatory systems of pain perception (Coutinho et al., 1998). In contrast, non-NMDA receptors may inhibit descending pathways, since the non selective AMPA/kainate receptor antagonist, DNQX, further enhanced zymosan-induced hyperalgesia (Coutinho et al., 1998). GluN1 subunits localized to cell bodies in DRG and in peripheral terminals of primary afferent innervating the rat colon mediate the local release of neuropeptides, such as CGRP and SP, which play an important role in neurogenic inflammation and hyperalgesia (McRoberts et al., 2001; Seybold, 2009; Raddant and Russo, 2011; Onaga, 2014). In rat lumbosacral DRGs, collected 5 days after inducing colitis with TNBS, NMDA currents measured by whole-cell patch recordings were significantly higher with respect to those measured in preparations obtained from control animals. The selective GluN2B antagonist, ifenprodil (Table 2), was able to drastically reduce such enhancement. In addition, in DRGs of TNBS-treated animals, GluN2B subunits expression and phosphorylation selectively increased, suggesting a major role for GluN2B in the development and persistence of colon inflammation and of the severity and duration of peripheral and central sensitization (Li et al., 2006). In a recent study carried out in rats, administration of mustard oil induced visceral hypersensitivity and enhanced expression of GluN2B and GluA2 receptors in the anterior cingulated cortex neurons, a brain region critically involved in the modulation of visceral pain responses (Zhou et al., 2014). In agreement with this study, GluA1, but not GluA2 and GluA3, receptor trafficking increased in synaptosomal membranes obtained from lumbar spinal cord neurons in a mice model of visceral nociception induced by intracolonic capsaicin (Galan et al., 2004). The expression of GluN1, AMPA receptors and of other protein associated with synaptic plasticity, such as postsynaptic density-95 (PSD-95), synaptophysin, calbindin-28 K and glial-derived nerve growth factor, was up-regulated in the rat ileum, caecum and colon, 8 weeks post infection by an oral administration of phosphate buffer saline with *Trichinella spiralis* larvae (Yang et al., 2009).

Persistence of pain perception in IBS depends upon changes in afferent neurons and CNS pain processing leads to chronic visceral hypersensitivity (Veremulen et al., 2014). NMDA receptors in the spinal cord play an important role in the development and maintenance of allodynia and hyperalgesia, by integrating the activity of groups of neurons and amplifying nociceptive signals, thus leading to "wind-up" of central responses to nociceptive stimuli (Davies and Lodge, 1987). Sixteen weeks after cessation of a TNBS treatment, Sprague Dawley rats exhibited chronic visceral pain, with no evident signs of colitis, resembling patients who develop chronic gastrointestinal symptoms following enteric infection (Zhou et al., 2007a, 2007b). In these hypersensitive animals, expression of specific splice variants of GluN1 were up-regulated in laminae I and II of the spinal cord (Zhou et al., 2006a). The same splice variants were overexpressed and phosphorylated in the rat myenteric plexus at a time when visceral hypersensitivity was present (14 days after TNBS treatment), suggesting that up-regulation of colonic myenteric plexus NMDA receptors may represent a peripheral mechanism involved in the development of neuronal plasticity and chronic visceral hypersensitivity (Zhou et al., 2006b, 2009). In the CNS, activation of glutamate receptors are responsible for long-term changes of the neuronal function, such as long-term potentiation (LTP), which is thought to modulate learning and memory processes and to participate to the generation of chronic pain perception (Kannampalli and Sengupta, 2015). Analogously, glutamate has been proposed to participate to activity-dependent adaptive changes of motor and sensory responses occurring in the gut after repetitive stimulation of neuronal circuitries in the ENS (Liu and Kirchgessner, 1999; Furness et al., 2000).

Although the vast majority of studies on glutamate-mediated visceral pain have focused on the role of NMDA and AMPA receptors, also kainate and mGlu receptors may be involved. GluK6 levels in the spinal cord and visceral pain perception increased after administration of formalin in the rat rectum to reproduce an acute model of inflammatory visceral pain. Both parameters returned to basal level after pre-treatment with GluK6 antisense oligodeoxynucleotides, suggesting the involvement of kainate receptors in both responses (Zhang et al., 2009). mGlu5 participation to mechanically-evoked visceral nociception in the gastrointestinal tract has been demonstrated by the ability of the antagonists MPEP

and MTEP (Table 2) to inhibit viscero-motor responses and cardiovascular changes evoked by colorectal distension in conscious rats (Lindström et al., 2008). Interestingly, colonic noxious stimulation increased the number of c-fos positive neurons in the rat DRG of the thoracic and lumbar spinal cord. This increase was significantly reduced by MPEP suggesting the involvement of mGlu5 receptors (Bianchi et al., 2003). In an IL-10 gene deficient mouse model of colitis, mGlu5 receptor expression on enteric glial cells was drastically reduced, possibly as a consequences of IL-1β or protease receptor −1 (PAR-1) stimulation (Nasser et al., 2007). On the basis of their results the Authors suggest that colitis-induced mGlu5 receptor downregulation may represent a protective mechanism to limit glial NMDA receptors stimulation and toxicity by mGlu5 receptors. In comparison with reflux disease, however, the progress of translational studies evaluating mGlu receptors as potential targets in the management of visceral pain is slow, and, to our knowledge, there are no published clinical trials at the moment. Glutamate transport, via EAATs, may also participate to visceral pain perception (Moloney et al., 2015). The systemic administration of riluzole, an activator of glutamate transport via EAAT, counteracted gastrointestinal hypersensitivity in rat and human models of visceral hypersensitivity (Gosselin et al., 2010; Mishra et al., 2014). Positive modulation of EAAT2, the main glial transporter for glutamate re-uptake, has been found to be protective against visceral pain (Moloney et al., 2015). Overexpression of EAAT2 induced by the cephalosporin antibiotic, ceftriaxone, produced a twofold enhancement of glutamate uptake, providing protective effects against colonic distension-induced nociception (Lin et al., 2009). Interestingly the effect of ceftriaxone was blocked by systemic and intrathecal administration of dihydrokainate, a selective blocker of EAAT2 (Lin et al., 2009). In a further study, the same group demonstrated that adeno-associated virus-mediated EAAT2 overexpression efficaciously reduced visceromotor responses to colorectal distension (Lin et al., 2011). The presence of all VGLUTs on colorectal DRG neurons would suggest the involvement of the glutamate storage mechanism in the development of visceral pain. However, the use of transgenic mice, KO for each specific transporter, has not yet given scientific proofs to this hypothesis (Malet and Brumovsky, 2015). Nevertheless, enhancement of VGLUT3 expression in DRGs and in the prefrontal cortex have been observed in rats showing increased visceromotor responses to colorectal distension after *Trichinella spiralis*-induced inflammation (Yang et al., 2012, 2015).

5.4. Intestinal I/R injury

Intestinal ischemia resulting from small bowel transplantation, aneurism of the abdominal aorta, cardiopulmonary bypass, acute mesenteric venous or arterial thrombosis, embolism, intestinal occlusion, necrotising enterocolitis in the human premature newborn and IBD represents a major clinical problem (Haglund and Bergqvist, 1999; Gonzalez et al., 2015). Both acute and chronic interruption of blood supply may lead to functional and structural alterations of the gastrointestinal tract and the subsequent restoration of blood flow plays only a temporary role in rescuing ischemic tissues, since reperfusion may lead to further tissue damage by inducing a cascade of molecular and cellular events which surpass the original ischemic insult (Massberg and Messmer, 1998; Chang et al., 2005b). Epithelial damage primarily, although transiently, occurs and leads to hindrance of nutrient absorption, water and electrolyte transport, and allows bacteria and toxins to enter the gut wall, eventually inducing endotoxic-mediated systemic inflammatory response (Chang et al., 2005b; Matthijsen et al., 2009). The epithelium repairs within a few days of restoration of blood flow, but abnormalities of absorptive function are seen beyond this time and may involve derangements of smooth muscle layers and enteric ganglia leading to altered gut motor function (An et al., 2005; Sileri et al., 2002; Lindeström and Ekblad, 2004). A significant reduction of intestinal motility has been observed after both *in vivo*- and *in vitro*-induced I/R injury, and is mainly caused by impairment of enteric cholinergic pathways (Udassin et al., 1995; Hierholzer et al., 1999; Corbett and Lees, 1997).

In analogy with the CNS, excessive amounts of glutamate released from enteric neurons may participate to functional changes occurring after an I/R injury in the gut (Arundine and Tymianski, 2003; Giuliani et al., 2006). In mucosa deprived segments of the guinea pig ileum, a significant and long lasting increase of glutamate release from myenteric neurons was observed in "*in vitro*" conditions mimicking I/R (Giuliani et al., 2006; Giaroni et al., 2011). Such enhancement was subject to a positive feedback mediated by NMDA receptors since it was sensitive to the NMDA receptor antagonists, (−)-AP5 and 5,7-diChloro-kynurenic acid (5,7-diCl-KYN) (Table 2) (Giuliani et al., 2006; Giaroni et al., 2011).

Excessive activation of enteric glutamatergic pathways may contribute to dysregulation of the cholinergic function during an I/R injury in the gut. In these conditions, spontaneous basal ACh release may increase, reflecting a possible transitory rise in spontaneous synaptic activity which precedes the functional failure of neurotransmission (Larson and Martins, 1981; Bukharaeva et al., 2005; Giuliani et al., 2006). Glutamate, via activation of NMDA receptors has been shown to contribute to the outburst of spontaneous endogenous ACh release after an ischemic damage in the guinea pig ileum (Giuliani et al., 2006). High extracellular neurotoxic concentrations of glutamate may influence the release of other neurotransmitter, such as NO. In the rat small intestine, dysmotility following in vivo I/R was attributed to local release of glutamate, leading to NO production and to adaptive changes in several enteric neurotransmitter pathways, via activation of NMDA receptors (Calcina et al., 2005). The importance of nitrergic pathways in the development of I/R damage in the ENS has been demonstrated by the selective and persisting morphological damage occurring in nNOS-immunoreactive neurons in the guinea pig ileum 24 h after clamping the superior mesenteric artery (Rivera et al., 2012). Recently, the ability of (−)-AP5 and 5,7-diCl-KYN to drastically reduce I/R-mediated NO production from the guinea pig ileum after an *in vitro* I/R damage has provided direct evidence for the involvement of NMDA receptors. Conversely, NPLA and 1400W, nNOS and iNOS blockers, respectively, decreased I/R-induced glutamate release strengthening the concept that a strong interplay exists between glutamate and NO in myenteric neurons in conditions of energy depletion which may contribute to pathophysiology and progression of symptoms of intestinal ischemia (Giaroni et al., 2013; Filpa et al., 2015). One of the major mechanisms underlying I/R-induced overactivation of NMDA receptors in guinea pig ileum myenteric neurons is represented by increased availability of GluN1 subunits which apparently is not dependent upon *de novo* synthesis, but upon PKC-mediated posttransductional changes (Giaroni et al., 2011). Direct evidence for a neurotoxic effect exerted by activation of NMDA and AMPA/kainate receptors on enteric neurons during a metabolic insult has been recently given by the observation that in primary cultures of rat ileum myenteric ganglia exposed to *in vitro* I/R conditions, both neuron count and viability increased in the presence of (−)-AP5 and CNQX, an AMPA/kainate receptor antagonist (Carpanese et al., 2014). Interestingly, the I/R damage did not modify the number of GluN1 and GluA1–4 immunopositive neurons in culture, while the number of GluK1–3 positive neurons increased, suggesting a major role for this receptor in the neurotoxic damage. In line with this hypothesis, exposure of cultured myenteric ganglia to kainic acid, an agonist at kainate receptors, produced swelling and formation of blebs along neurites,

as well as collapse in mitochondrial membrane potential (Kirchgessner et al., 1997). In agreement with the well established concept that glutamate-mediated neurotoxicity after I/R injury leads to production of toxic ROS as a consequence to mithocondrial dysfunction (Lau and Tymianski, 2010), ROS levels significantly and progressively increased in isolated myenteric ganglia of the guinea pig ileum with respect to control values after I/R (Carpanese et al., 2014). Again, all iGlu receptor types contributed to such increase, although AMPA/kainate receptors seemed to retain a more important role. Disruption of Ca^{++} regulatory mechanisms and generation of ROS following and I/R damage may have important functional consequences at the intestinal level. In the mouse proximal jejunum, motility changes occurring during re-oxygenation after hypoxic insults in the gut have been correlated with a disruption of the intracellular redox state (Bielefeldt and Conklin, 1997).

Alterations of enteric glutamatergic pathways may contribute also to mucosal derangement associated with the I/R insult. In the rat ileum, ketamine, a putative NMDA receptor antagonist with anaesthetic and sedative properties, but not pentobarbital sodium, significantly reduced mucosal damage 45 min after ischemia followed by 60 min of reperfusion (Cámara et al., 2008). Pre-emptive administration of ketamine to rats before inducing in vivo I/R injury, has been shown to reduce leukocyte infiltration in the mucosa, serum levels of adhesion molecules such as P-selectin, as well as antithrombin-III depletion (Guzmán-de la Garza et al., 2010a). In a successive study the Authors found that chemical ablation of the myenteric plexus with benzalkonium chloride abolished ketamine protective actions, suggesting that myenteric neuronal circuitries are necessary in order to induce ketamine effects and that NMDA receptors located on myenteric neurons may participate to I/R mucosal injury (Guzmán-de la Garza et al., 2010b). Such modulatory effect may depend upon the ability of NMDA receptors to directly influence epithelial cells integrity as well as to regulate mucosal blood flow and metabolism.

6. Areas of importance for advancing the field

Despite the growing evidence that glutamate, as a neurotransmitter, may play a role in the modulation of secretory, motor and sensory functions in the gut, both in physiological and in pathological conditions, glutamatergic mechanisms influencing the activity of enteric neuronal circuitries in normal and disease states remain largely to be elucidated. Accordingly, the possibility to alleviate symptoms or inhibit the progression of gut diseases via modulation of the enteric glutamatergic neurotransmission is gradually moving ahead. In order to improve the knowledge in this area future investigations would benefit from experimental approaches aiming to better characterize the electrophysiological and functional properties of different glutamate receptor subtypes on specific enteric neuron types and along the brain-gut axis. Additionally, in view of the stringent relationship existing between the effects of the aminoacid and the maintenance of the steep extracellular/intracellular concentration gradient, more effort should be addressed to evaluate the relevance of glutamate synthesis, release and reuptake modulation in enteric synapses. From a biomolecular standpoint it would be helpful to fully elucidate the multiple and complex pathways linking glutamate to neuronal response/damage in the gut, which involve, for instance, the interplay with enteric nitrergic pathways as well as the activation of intracellular molecular cascades, such as ROS production and arachidonic acid pathway activation.

In this perspective it would be of interest to resort to transgenic animal models, carrying deletion for glutamate receptor subtypes and transporter systems, to target the expression/deletion of specific receptor subunits as well as to apply elective single-cell patch clamp techniques to measure the electrophysiological properties of glutamate receptors subtypes in specific enteric neuronal populations. A more detailed characterization of the role of different glutamate receptor subtypes and transporters in the modulation of intrinsic and extrinsic neuronal pathways in the gut functions relies on the availability of selective pharmacological compounds. The majority of preclinical and clinical studies, up to now, have been carried out with iGluR antagonists, whose potential clinical usefulness is, however, limited by the variety of side effects, including cardiovascular, behavioural and potentially cytotoxic activities (Kostandy, 2012). To re-address this issue in the drive to develop better potential pharmacological tools, more selective molecules, such as GluN2B antagonists, may display neuroprotective actions with minimal side effects and better tolerability (Mony et al., 2009; Yuan et al., 2015). Other mechanisms, including modulation of the glycine site of NMDA receptors or glutamate reuptake systems may provide more effective treatments in controlling the enteric glutamatergic neurotransmission (Kaszaki et al., 2012; Miladinovic et al., 2015). In addition, the possibility to relay upon mGlu receptor allosteric modulators of mGlu receptors may be even more promising for the ability of mGlu receptors to fine-tune neurotransmission (Traynelis et al., 2010).

7. Conclusions

A vast number of studies, resorting to both basic and clinical studies, is available to suggest that glutamate may represent a neuromodulator/neurotransmitter in the ENS involved in the modulation of different digestive functions. In normal physiological conditions, glutamate influences both visceral sensitivity and motility through the modulation of extrinsic and intrinsic neuronal pathways innervating the gastrointestinal tract. Dysfunction of enteric glutamatergic pathways may, however, represent a critical factor in the pathogenesis and/or in the clinical presentation of some gastrointestinal diseases, such as gastroesophageal reflux, gastric acid hypersecretory disease, IBD, IBS and intestinal I/R injury. In this view, studies aiming to clarify the role of glutamate in the ENS may eventually lead to the discovery of molecules with a potential therapeutic interest in the treatment of these disease states.

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