Title:
Immunolocalisation of AMPA receptor subunits within the enteric nervous system of the mouse colon and the effect of their activation on spontaneous colonic contractions

Short running title: AMPA receptors in the mouse colon

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Abstract

Background: The appropriate expression of specific neurotransmitter receptors within the cellular networks which compose the enteric nervous system (ENS) is central to the regulation of gastrointestinal (GI) functions. Whilst the ENS expression patterns of the neurotransmitter glutamate have been well documented, the localisation of its receptors on ENS neurons remains to be fully characterised. We investigated the expression patterns of glutamate receptor AMPA subunits within ENS neurons of the mouse colon and the consequences of their pharmacological activation on spontaneous colonic contractility.

Methods: RT-PCR was used to detect individual AMPA receptor (GluR 1-4) subunit expression at the mRNA level in mouse colon tissue. Immunohistochemistry and confocal microscopy was used to localise the expression of the GluR1, 4 subunits in colon tissue. Brain tissue was used as a positive control. Organ bath preparations were used to determine the effect of AMPA receptors activation on the force and frequency of colonic longitudinal smooth muscle spontaneous contractions.

Key Results: GluR1, 3, 4 mRNA was detected in the mouse colon. Immunoreactivity for GluR1 and 4 subunits was detected on the somatic and dendritic surfaces of sub-populations of neurochemically defined ENS neurons. The pharmacological activation of AMPA receptors increased the force but not frequency of spontaneous colonic contractions.

Conclusions & Inferences: Molecularly distinct AMPA receptor subtypes are differentially expressed within the neural networks of the mouse colon and have a direct role in motility. These data provide the rationale for the development of AMPA-selective ligands for the therapeutic delivery to the GIT in motility disorders.
Key words
intestine; CPW399; immunohistochemistry; motility; GluR

Key Messages

- The excitatory neurotransmitter glutamate is widely expressed within the cellular networks of the mammalian enteric nervous system (ENS), yet the expression and function of its receptor subtypes is poorly defined.
- We investigated the expression of AMPA receptor subunits (GluR1-4) within the mouse colon and the effect of their pharmacological activation on spontaneous colonic contractions.
- RT-PCR analyses demonstrated that the GluR1, 3, 4 subunits were expressed in the colon at the mRNA levels, immunohistochemistry and confocal microscopy revealed that the GluR1 and 4 subunits are expressed on neurochemically-defined ENS neurons and organ bath studies indicated that the pan-AMPA receptor activation increased the force but not frequency of spontaneous colonic contractions.
- The data suggest a central role for AMPA receptors in ENS neuronal excitability as well as glutamate-mediated GI contractility.
Introduction

The enteric nervous system (ENS) is principally occupied with the regulation of gastrointestinal (GI) function (1). Located within the muscle layers of the GI tract (GIT), the ENS provides the local neural control of virtually all GI functions such as peristalsis (2), secretion (3), barrier function (4, 5) and local immune function (1, 6, 7), whilst cooperating with CNS reflex and command centres to control digestive functions (8). These diverse, ENS-mediated GI functions are brought about by the exquisite communication between a diverse array of ENS neuronal networks and GI cellular components, allowing for GI homeostasis. The importance of ENS function to GI health is underscored by the plethora of debilitating GI disorders associated with ENS dysfunction such as irritable bowel syndrome (IBS) and inflammatory bowel diseases (IBD) (9). Therefore, understanding the vast repertoire of cells and molecules which underpin coordinated activity between the cellular elements of the ENS and the GIT is central to designing targeted therapies for such disorders implicating ENS dysfunction.

The ENS is composed of morphologically, molecularly and functionally distinct cell-types (10) which communicate using multiple neurotransmitter-receptor systems (11), including glutamate (12). Glutamate is the major excitatory neurotransmitter within the nervous system (CNS) (13, 14). Its importance to overall neural function is exemplified within the CNS, where dysregulation in various glutamatergic pathways within the brain has been directly implicated in a number of neurological disorders such as epilepsy (15), amnesia (16), schizophrenia (17) and cerebral ischemia (18).
Within the ENS, glutamate has been shown to be expressed by different enteric neurons and to act as a neurotransmitter (12, 19).

Glutamatergic neurotransmission occurs in a variety of functional phenotypes due to the structurally and functionally diverse classes of glutamatergic receptors expressed within cellular networks (20). Slow, persistent excitatory glutamatergic currents are mediated primarily by different classes of metabotropic glutamate receptors (21). In contrast, rapid excitatory glutamatergic currents are mediated primarily by ionotropic glutamate receptors which include the N-methyl-D-aspartate receptor (NMDA), Kainite and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subclasses (12, 22). Ionotropic glutamate receptor complexes are integral membrane ion channels, composed of four subunit proteins which assemble to form a central ion channel pore permeable to cations (20). The biophysical and pharmacological diversity of different ionotropic glutamate receptors arises from the molecular diversity of the subunits forming the receptor complex (20). Therefore, to fully understand the role of the glutamate receptor system within specific neural pathways, it is essential to determine which particular glutamate receptor subunits are expressed within the specific cellular elements of the system.

Whilst evidence for the expression of the NMDA receptor subunits has been demonstrated in the ENS of rats (23, 24), the expression of specific AMPA receptor subunits on neurochemically defined cell-types of the ENS remain to be revealed. Since pharmacological data suggest evidence of their expression within the ENS (25-28), the aims of this study were to molecularly and functionally characterise the AMPA receptor system within the colon of the mouse.
Methods and materials

All procedures involving experimental animals were approved by the Animal Welfare and Ethics Committee of the University of Portsmouth and were performed under a Home Office (HO) project licence, by a HO personal licence holder, and in accordance with the Animals (Scientific Procedures) Act 1986 (UK) and associated procedures.

Animals and tissue

Adult C57/BL6 male mice, aged 6-8 weeks, were used throughout the study, apart from a subset of experiments for which we used transgenic reporter mice which expressed green fluorescent protein under the promoters of either the astroglial cytoskeletal protein, glial fibrillary acidic protein (GFP-GFAP) (29), or the transcription factor Sox10 (GFP-Sox10) (30). The transgenic animals were generously provided by Arthur Butt, the University of Portsmouth.

Preparation of tissue from the CNS and ENS

Mice were anaesthetised with isoflurane and pentobarbitone (1.25 mg/kg of bodyweight; i.p.). The animals were transcardially perfused using a fixative containing 1% w/v paraformaldehyde and 15% v/v saturated picric acid in 0.1M phosphate buffer (pH 7.4) according to previously described protocols (31). After perfusion, the brains and colons were removed and post-fixed in the same fixative over night at 4°C. The next day, tissue was washed in 0.1M phosphate buffer until it was clear of the fixative. For CNS reactions, 60 μm-thick coronal and sagittal sections were produced from the hippocampus and cerebellum, respectively, using a
Vibratome. For ENS reactions, whole-mount preparations of the longitudinal muscle-myenteric plexus and circular muscle-submucosal plexus were obtained from the colon, using a dissecting microscope and fine forceps. All sections were stored in 0.1M phosphate buffer containing 0.05% sodium azide.

**Immunohistochemistry and confocal microscopy**

Non-specific binding of secondary antibodies was minimised by incubating the tissue in 20% normal horse serum (NHS), diluted in Tris-buffered saline containing 0.3% Triton-X100 (TBS-Tx), for 2 hours at room temperature. The tissue was then incubated overnight at 4°C with a cocktail of primary antibodies (see Supplementary Information (SI) Table 1) diluted in either 20% NHS-TBS-TX for whole-mount sections of the colon, or TBS-Tx for brain sections. The following day, the tissue was washed three times in TBS-Tx and incubated in a mixture of appropriate secondary antibodies conjugated with either Alexa Fluor 488 (Invitrogen, Eugene, OR), indocarbocyanine (Cy3; Jackson ImmunoResearch), and indodicarbocyanine (Cy5; Jackson ImmunoResearch) for 2 hours at room temperature. The tissue was washed three times with TBS-Tx for 10 minutes and mounted on glass slides in Mowiol mounting medium (Polysciences) and cover slipped.

**Antibody specificity**

The specificity of all the antisera against the glutamate receptor subunits used in this study have been reported upon extensively in other studies concerning the CNS (see SI Table 1). Method specificity was also tested by omitting the primary antibodies in the incubation sequence. To confirm the absence of cross reactivity between IgGs in double and triple immunolabelling experiments, some sections were processed
through the same immunohistochemical sequence, except that only an individual primary antibody was applied with the full complement of secondary antibodies.

*Image acquisition*

Sections were examined with a confocal laser-scanning microscope (LSM710; Zeiss, Oberkochen, Germany) using either a Plan Apochromatic 20x DIC oil objective (NA0.8) (pixel size 0.42 μm), a Plan Apochromatic 40x DIC oil objective (NA1.3) (pixel size 0.29 μm) or a Plan Apochromatic 63x DIC oil objective (NA1.4) (pixel size 0.13 μm). Z-stacks were used for routine evaluation of the labelling. All images presented represent a single optical section. These images were acquired using sequential acquisition of the different channels to avoid cross-talk between fluorophores, with the pinholes adjusted to one airy unit. Images were processed with the software Zen2009 Light Edition (Zeiss, Oberkochen, Germany) and exported into Adobe Photoshop. Only brightness and contrast were adjusted for the whole frame, and no part of a frame was enhanced or modified in any way.

*Reverse transcription-polymerase chain reaction (RT-PCR)*

Small segments of freshly isolated colon and whole brain from three different male C57/BL6 mice were obtained and the tissue was rapidly broken down using a pestle and mortar. The samples were then subjected to RNA extraction using RNeasy mini kit (Qiagen) as per the manufacturer’s protocol. The total concentration and the purity of prepared RNA was determined using a NanoDrop spectrophotometer (Thermo Scientific). After extraction, the RNA was reverse transcribed into complementary DNA (cDNA) using a SuperScript™ First-Strand Synthesis System for RT-PCR kit (Invitrogen) in 20 ul reactions. Subsequently, equal amounts of cDNA (2 μl) were
used for polymerase chain reaction (PCR) together with GoTaq green master mix (Promega), PCR grade water and primers specific for each of the AMPA receptor subunits (SI Table 2). All the AMPA receptor subunit-specific PCR primers used in this study have been previously published (32). Whole brains of the same animals were used as a positive control. Finally, the products of RT-PCR for the transcripts of the AMPA receptor subunits in the mouse brain and colon were run on a 2% agarose gel and the DNA was visualised by SYBR green staining.

Isometric tension recordings of the effects of AMPA receptor activation on the force and frequency of spontaneous contractions of the isolated mouse colon

This preparation was used to investigate the effect of AMPA receptor activation on the force and frequency of spontaneous colonic contractions in vitro, according to our previously published protocols (31). Six to eight weeks old male mice were killed by cervical dislocation, the distal colon was removed and immediately placed in physiological solution containing (in mM): NaCl 140, NaHCO3 11.9, D+ glucose 5.6, KCl 2.7, MgCl2.6H2O 1.05, NaH2PO4.2H2O 0.5, CaCl2 1.8, warmed to 32°C. The intraluminal contents were removed by gently flushing the colon with the physiological solution. Two cm-long segments were mounted in a Harvard organ bath (10 ml chamber) filled with the physiological solution (32°C) and bubbled with gas containing 95% O2 and 5% CO2. Contractile activity for each colon tissue strip was recorded using an isometric force transducer (range 0-25 g) connected to a bridge amplifier, which was in turn connected to a dedicated data acquisition system (Power Lab 2.20 AD Instruments). The sampling frequency was set to 40 Hz and the sensitivity of recording was set to 500 mV. The apparatus was then calibrated using a one gram weight in order to express the changes in the amplitude detected by the
transducer into grams of force. The tissue was then placed under 1 gram of resting tension and allowed to equilibrate for 30 minutes. The AD instrument lab chart 7 program installed on a PC was used to monitor, record and analyse the spontaneous activity. After a stable baseline was established, 10 µM of CPW399, a pan AMPA receptors agonist, was added to the bath and the tissue was allowed to reach maximum response. Ten minute epochs before and after the drug additions were used for quantification of the drug-induced changes in the force and frequency of colonic spontaneous contractions. One piece of tissue was used per animal. The frequency and amplitude of individual spontaneous contractions was manually counted before and after the drug and the average for that animal determined. A mean value for the individual averages was obtained for a particular drug. An N value thus represents one animal and the data are presented as the mean ± SD.

Statistical analysis
All data are presented as the arithmetic mean ± SD unless stated otherwise. Statistical comparisons were made using Student’s t test with N representing the number of animals used.
Results

*Immunolocalisation of glutamate transporter proteins in the ENS of the mouse colon*

As a prelude to determining the location of specific AMPA receptor subunits within the ENS of the mouse colon, we investigated the expression of different classes of glutamate transporter proteins in order to assess the glutamatergic regulatory system within this region of the mouse ENS. Evaluation of immunoreactivity for the three vesicular glutamate transporter (VGLUT) proteins (VGLUT 1-3) revealed VGLUT2 to be the predominant subtype expressed within ENS neurons of the mouse colon (Fig. 1). Indeed, VGLUT2 immunoreactivity presented as varicosities decorating neurons of ganglia both within the myenteric (Fig. 1 A) and submucosal (Fig. 1 B) plexuses, visualised by immunoreactivity for the acetylcholine synthesising enzyme choline acetyltransferase (ChAT). Within the CNS, VGLUT2 is enriched within presynaptic terminals and is indicative of synaptic glutamate release sites. If this sub-cellular localisation pattern is mirrored in the ENS, then this distribution of VGLUT2 immunoreactivity suggest widespread *synaptic* release of glutamate within the cellular networks of the ENS of the mouse colon. No specific signal was detectable for VGLUT1 and VGLUT3.

In light of the strong association of glutamate transporter immunoreactivity within ENS neuronal elements demonstrated above, we then assessed whether, the other major cell-type within the ENS, namely enteric glia also exhibit an association with the molecular machinery which regulates glutamate levels. We therefore performed immunohistochemistry for the glutamate transporter subtype, which at least within the CNS, is principally expressed by glia, namely the excitatory amino acid transporter 1
(EAAT1), using tissue from reporter mice which express green fluorescent protein (GFP) under the promoters of either the scaffolding protein glial fibrillary acid protein (GFAP-GFP) (29) or the transcription factor Sox10 (Sox10-GFP) (30). GFAP and Sox10 have been shown to be exclusively expressed in enteric glia (33). In tissue from GFAP-GFP mice, immunoreactivity for EAAT1 within the ENS of the colon presented as individual clusters which decorated the periphery of neuronal profiles, identified by immunoreactivity for the calcium binding protein calretinin (Fig. 2 A1). Examination of the association between EAAT1 and GFP (Fig. 2 A2) revealed extensive areas of co-localisation between these signals suggesting that EAAT1 signal is predominantly located on glial, rather than neuronal surfaces (Fig. 2 A3). This strong association of EAAT1 expression with enteric glia was reproduced in tissue from Sox10-GFP mice. Immunoreactivity for EAAT1 was once again enriched on the periphery of calretinin-immunopositive cell surfaces (Fig. 2 B1) and appeared to co-localise predominantly with Sox10-GFP immunopositive profiles (Fig. 2 B2, 3). This distribution pattern of EAAT1 was evident in both myenteric and submucosal plexuses. Collectively, these data suggest that enteric glia possess the molecular machinery to regulate extracellular glutamate levels within the ENS of the mouse colon thereby contributing to the glutamatergic neurotransmission within this region of the GIT.

**AMPA receptor subunit mRNA expression within the mouse colon**

We then investigated which particular subunits are expressed at the mRNA levels within this region of the GIT, using RT-PCR and homogenate of whole mouse colon. Homogenates of mouse brain were used as a positive control. RT-PCR revealed
mRNA expression within the colon for all AMPA receptor subunits except the GluR2 subunit (Fig. 3, N = 3 animals).

**Confirmation of AMPA receptor subunit immunoreactivity patterns in brain tissue**

Allied to the mRNA analysis above where we used brain as a positive control, we performed immunohistochemistry for the AMPA receptor subunits on brain tissue in order to confirm that the antibodies and our reaction conditions reproduce previous reports within the CNS as well as allowing for the comparison of CNS localisation patterns with that of the ENS. In our preliminary investigations, we were not able to detect any specific immunoreactive signal within the ENS using the available antibodies against the GluR2 and 3 subunits, even though these antibodies exhibited specific staining within the CNS. Therefore, we report the immunohistochemical staining for only the GluR1 and 4 subunits.

In the cerebellum, labelling for the GluR1 subunit was restricted to the molecular layer (Fig. 4 A1, A2), in agreement with previous reports (34), and was clustered on Purkinje cell dendritic spines, closely apposed to immunoreactivity of postsynaptic density 95 protein (PSD-95), which is an anchoring protein enriched in excitatory synapses (Fig. 4 A3). In the forebrain, labelling for the GluR1 subunit was also enriched within the hippocampus (Fig. 4 A4, A5), in agreement with previous reports (35). Once again, GluR1-immunoreactivity was clustered on dendrites closely apposed to PSD-95 immunoreactive clusters (Fig. 4 A6).

The immunoreactivity pattern of the GluR4 subunit closely mirrored that of the GluR1 subunit and was in accordance with previously published evidence (36), with signal
within the cerebellum restricted to the molecular layer (Fig. 4 B1, 2) and closely associated with that of PSD-95 (Fig. 4 B3). In agreement with previous evidence (36), we did not observe any specific signal for the GluR4 subunit in the hippocampus (Fig. 4 B4, B5). Collectively these data indicate that the antibodies, which used under our reaction conditions, provide immunoreactivity patterns which are consistent with previously published reports.

**Immunolocalisation of PSD-95 in the ENS of the mouse colon**

To identify the putative locations of excitatory synapses within the ENS of the mouse colon, we used immunoreactivity for the anchoring protein PSD-95, which has been shown to be enriched within the postsynaptic compartments of excitatory synapses in both the CNS (37) and ENS (38). Immunoreactivity for PSD-95 signal presented as individual clusters on somatic and dendritic surfaces of ENS neurons (Fig. 5 A1) which were closely apposed to glial processes (Fig. 5 A2, 3).

**Immunolocalisation of the AMPA subunits within the ENS of the mouse colon**

Immunoreactivity for the GluR1 subunit was widely distributed on neurons composing the ganglia of the myenteric and submucosal plexuses, identified by immunoreactivity for nitric oxide synthase (NOS) (Fig. 6 A). However, it was noticeable that some neurons were devoid of immunoreactivity for GluR1 (Fig. 6 A, arrowheads), whilst some NOS-immunoreactive neurons displayed robust GluR1 signal (arrows), suggesting a degree of cell specific expression for this subunit. We used a range of neurochemical markers and high-resolution imaging to confirm which particular types of ENS neurons express the GluR1 subunit. GluR1 immunoreactivity appeared as individual clusters on somato-dendritic cells surfaces of calretinin-immunopositive
(Fig. 6 B1), ChAT-immunopositive (Fig. 6 B2) and on a sub-population of NOS-positive (Fig. 6 B3) neurons. However, we did not detect any GluR1 signal associated with somatostatin-immunopositive neurons (Fig. 6 B4).

The labelling pattern of the GluR4 subunit mirrored that of the GluR1 subunit, with a widespread distribution evident throughout ENS ganglia (Fig. 7 A), although it was evident that the intensity of the signal varied across collections of neurons (Fig. 7A, arrowheads). Using a range of neurochemical markers, we detected immunoreactivity for the GluR4 subunit on the somato-dendritic surfaces of NOS-expressing neurons (Fig. 7 B1) and ChAT-positive enteric neurons (Fig. 7 B2). Unlike the GluR1 subunit, GluR4 subunit was not expressed on calretinin-expressing neurons (Fig. 7 B3). We also did not detect specific GluR4 labelling on somatostatin-immunoreactive enteric neurons (Fig. 7 B4).

A striking observation was the location of GluR4 immunoreactivity on axonal profiles, particularly on varicose plexes, identified by immunoreactivity for NOS (Fig. 7 C), suggesting the expression of this subunit on both pre- and postsynaptic compartments.

Collectively, these data indicate that the GluR1 and 4 subunits are divergently expressed within the cellular networks of the ENS of the mouse colon being targeted to not only different sub-populations of ENS neurons but to different sub-cellular domains.
The effect of AMPA receptor activation on spontaneous colonic contractions

In order to explore the functional consequences of AMPA receptor activation on one aspect of colonic function, namely contractility, we applied the AMPA receptor agonist CPW399 to isolated mouse colon segments and measured the changes in the force and frequency of spontaneous longitudinal muscle contractions. CPW399 has been shown to be a potent agonist at GluR1 and GluR2 subunit-containing AMPA receptors and exhibits minimal desensitisation of these receptors (39), thus allowing for examination of the effects of prolonged AMPA receptor activation. CPW399 at bath concentration of 10 μM significantly increased the force of spontaneous contractions (mean ± SD; baseline, 0.1 ± 0.05 grams versus CPW399, 0.18 ± 0.07 grams, N = 4 animals; \( P = 0.008 \), paired Student’s \( t \) test) (Fig. 8A, B). However, CPW399 at this concentration had no significant effect on the frequency of spontaneous contractions (mean ± SD; baseline, 0.067 ± 0.003 Hz versus CPW399, 0.068 ± 0.005 Hz, N = 4 animals; \( P = 0.565 \), paired Student’s \( t \) test) (Fig. 8C).
Discussion

In the current study, we demonstrate that the GluR1 and 4 receptor subunits are divergently expressed within the neuronal networks of the ENS of the mouse colon and that the activation of AMPA receptors directly influences colonic spontaneous contractility. In addition, we provide evidence for the expression of the molecular machinery which regulates glutamate levels within this region of the GIT. The implication is that the glutamate-AMPA receptor system is an integral component of the neural networks which underlies the modulation of colonic function within the mouse.

Technical considerations

Whilst the immunohistochemical expression of various AMPA receptor subunits within the CNS has been comprehensively described, relatively less is known about the precise cellular localisation patterns in the peripheral nervous system, including the ENS of the mouse colon. The specificity of the CNS labelling pattern of the GluR antibodies used in this study has been verified in knockout mice (data from manufacturer). Unfortunately, the lack of available tissue from such knockout mice precludes similar ENS analyses in this study. We therefore performed parallel studies in tissue from both the CNS and ENS in order to confirm the consistency of the antisera under our reaction conditions. The staining patterns obtained in the mouse cerebellum and hippocampus with antibodies against the GluR1 and 4 subunits were indistinguishable to those previously reported confirming their specificity (Fig. 4). Further evidence of the specificity of the GluR subunit antibodies used is that the cellular immunoreactivity patterns within the ENS not only mirrored the CNS
phenotype but also fulfilled the expectations for neurotransmitter receptor proteins which are predominantly expressed in synaptic junctions. Indeed, the immunoreactivity presented as membrane-bound clusters with relatively less signal evident in the cytoplasm (Fig. 6, 7). An additional level of evidence for the expression of these AMPA subunits within the mouse colon was obtained by confirming their expression at the mRNA level (Fig. 3), with the obvious caveat that expression at the mRNA level may not necessarily mean expression at the protein level. Allied to our mRNA data, we did encounter technical problems in terms of obtaining specific ENS immunolabelling with antibodies against the GluR2 and 3 subunits even though these antisera reproduced previously reported CNS labelling patterns. Nevertheless, our combined data in the CNS and ENS on the localisation of the GluR1 and 4 subunits provides novel insights into glutamate receptor expression within this region of the mouse colon.

*Glutamate receptor expression in the ENS*

The expression of glutamate within the cellular networks of the ENS is well documented (12) as well as its evidence as a neurotransmitter in this branch of the nervous system (19, 40). Although several studies have demonstrated, at a functional level, that application of glutamate has an excitatory impact on enteric neurons; there is contradictory evidence with respect to which glutamate receptors are involved in mediating these effects. For instance, one study suggests that the excitatory effect of glutamate is mediated via metabotropic glutamate receptors and not the ionotropic receptors (41), while others show that such effects are mediated via ionotropic receptors such as AMPA receptors (12, 22). Furthermore, despite functional evidence for the presence of different glutamate receptor subtypes (41),
relatively less is known about the distribution of particular receptor subtypes amongst the various cell-types which compose the ENS. This is lamentable since the elaboration of the rich expression profiles of various glutamate receptor subtypes throughout the cell-types and connected pathways of the CNS has been instrumental in sketching the landscape of the glutamate system and its contribution to various facets of neural function. Therefore, identifying not only the molecular substrates of ENS glutamatergic neurotransmission, but also their positioning within connected circuitry will inform the future functional exploration of the ENS glutamatergic system and its role in GI health and disease.

The majority of evidence for glutamate receptor expression and function within the ENS and GIT is related to the metabotropic (mGluR) sub-class. Indeed, immunolabelling for different sub-classes of mGluR receptors has been reported to be localised to various populations of ENS neurons (42, 43). Less is known about the location of ionotropic glutamate receptor subtypes within ENS circuitry, in particular the NMDA and AMPA subtype. Expression of the NR1 subunit within the ENS has been demonstrated at both the mRNA level (24, 44) and protein levels (12, 45). In contrast, the native location of different AMPA receptor subunits has been reported only in the guinea pig small intestine (12). This study reported GluR1-4 subunits to be localised within the cytoplasm of ENS neurons. In contrast, our data, obtained from the mouse large intestine revealed that immunoreactivity for the GluR1/4 subunits was predominantly localised to somato-dendritic plasma membranes (Figs. 6, 7), the site of expression expected from proteins which primarily function in synaptic junctions. Furthermore, these somato-dendritic expression patterns of the GluR1/4 subunits was closely aligned with the molecular machinery which regulates either
synaptic or extrasynaptic glutamate levels, namely vesicular glutamate transporter 2 (Fig. 1) or the glial glutamate transporter (Fig. 2) respectively. In agreement with data from (12), we confirmed the neurochemical identity of some of the GluR-expressing ENS neurons to be immunoreactive for Chat, NOS and calretinin, although not somatostatin, which is predominantly expressed within enteric interneurons (46) (Figs. 6, 7). Collectively, these data in the mouse colon build on what has been previously reported in the small intestine of the guinea pig by providing a high-resolution image of the complexity of the glutamate-AMPA receptor system within the cellular networks of the ENS.

Glutamate receptor activation and GI motility

In light of the complex expression patterns demonstrated above, we chose to examine the possible functional role of such receptors in only one aspect of GI function, namely motility, using spontaneous colonic contractions as a surrogate marker. The role of glutamate in influencing intestinal contractility has been widely reported with evidence indicating a pro-contractile effect (40). Various classes of both ionotropic and metabotropic glutamate receptors have been implicated in such actions (25, 47). Indeed, in guinea pig, AMPA receptor activation has been shown to enhance neurotransmitter release which enhanced peristalsis (26). Our data using the mouse colon corroborate a role for AMPA receptors in enhancing GI contractility. Intestinal motility or peristalsis arises from the coordinated contraction and relaxation of circular and longitudinal smooth muscles (48). Even though we used a preparation which detects the spontaneous contractions of only the longitudinal smooth muscles of the colon, we were able to demonstrate that the pharmacological activation of all AMPA receptor subtypes enhanced the force of spontaneous contractions (Fig. 8).
Notably, the frequency of such contractions remained unaltered. The balance of neurotransmitter release from acetylcholine (1) and NOS-containing (49) ENS neurons is thought to determine the tone of the contraction and relaxation of intestinal smooth muscle contractions. A distinct cell-type, namely the interstitial cells of Cajal (ICC), is primarily responsible for setting the frequency or rhythm of such smooth muscle contractions. Collectively, this rhythmic contraction of varying degrees of force by smooth muscles allows for the optimal prolusion of intestinal contents. Our study revealed that both cholinergic and NOS-containing neurons expressed the GluR1/4 subunits. The simplistic extrapolation would be that activation of these subunits would results in the excitation of both cholinergic and NOS-containing neurons, resulting in opposing actions on smooth muscle contraction. A possible explanation could be different levels of GluR 1 and 4 subunits expressed on either cholinergic and NOS-immunopositive neurons, resulting in different levels of activation when exposed to a pan-AMPA receptor agonist. It is not possible to explore this theory using immunohistochemistry since the antibodies against the GluR1 and 4 subunits are likely to have inherently different binding affinities. Therefore, in order to dissect the respective function of GluR1 or 4 subunit-containing AMPA receptors on cholinergic and NOS-containing ENS neurons, it would be essential to measure the amplitude of AMPA-mediated excitatory postsynaptic currents at the single cell level of such identified neurons. Nevertheless, it was notable that we were not able to detect any specific signal for the GluR1/4 subunits on ICC (data not shown), which concurs with the lack of an effect of the AMPA agonist on the frequency of spontaneous contractions.
Glutamate receptors in GI disorders

The glutamate-receptor system impacts on a range of GI functions such as motility, (25, 40, 50) as well as secretory and electrolyte balance (27, 28, 51). However, apart from their roles in GI homeostasis, there is growing evidence implicating the dysregulation of different glutamate receptors, with the ensuing excitotoxicity (22) contributing to various GI disorders such as GI inflammation (52), irritable bowel syndrome (IBS) (53), visceral pain (54) and intestinal cancer (55). Whilst most of the current evidence implicates NMDA and mGluR receptors subtypes, roles of AMPA receptors in such disorders are relatively poorly explored. The current study provides the anatomical foundation for exploring potential changes in the AMPA receptor system of the ENS of the mouse colon, using various models of GI disorders.

In summary, the study provides a high resolution map of the location of GluR subunits within the major cell-types of the ENS of the mouse colon, alongside the molecular machinery which regulates glutamate neurotransmission. In addition, we provide evidence that the pharmacological activation of such receptors directly influences colonic contractility. The data provide a platform for future molecular and functional analyses of the glutamate-AMPA receptor system in GI health and disease.
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Author contributions:

MS and JDS designed the research study
MS performed the research
MS, and JDS analysed the data
MS and JDS wrote the paper

Disclosures

The authors declare no conflict of interests
References


**Figure legends**

Figure 1

Immunolocalisation of the vesicular glutamate 2 transporter (VGLUT2) in the ENS of the mouse colon

(A1 and B1) show immunoreactivity for the choline acetyltransferase (ChAT) demonstrating the location of putative cholinergic neurons within ganglia of the myenteric and submucosal plexus respectively. (A2 and B2) show immunoreactivity for the VGLUT2 within the same fields of view. (A3 and B3) are overlays of (A1, A2) and (B1, B2) respectively, showing the widespread association of VGLUT2 immunoreactive varicosities within the myenteric and submucosal plexus neurons of the mouse colon. Note that (A3) is a magnified version of the boxed area in (A1) and (A2). Scale bars: (A) 50 µm; (B) 20 µm

Figure 2

Association of immunoreactivity for the excitatory amino acid transporter 1 (EAAT1) with enteric glia of the ENS of the mouse colon

(A1 and B1) show EAAT1 immunoreactive clusters decorating enteric neurons visualised by immunoreactivity for the calcium binding protein and ENS neuronal marker, calretinin. (A2 and B2) show the widespread association of the EAAT1 immunoreactive clusters with enteric glia which in (A2) are identified by the expression of green fluorescent protein (GFP) which is driven by the promotor for the glial cytoskeletal protein, glial fibrillary acid protein (GFAP), and in (B2) by GFP expression which is driven by the promotor for the transcription factor Sox10. (A3 and
B3) are overlays of (A1, B1) and (A2, B2) respectively, showing the close association between immunoreactivity of EAAT1 with enteric glial profiles.

Scale bars: 10 µm

Figure 3
Expression of AMPA receptor subunits in the mouse colon at the mRNA level

The image is a photomicrograph of a representative electrophoresis gel showing mRNA transcripts for the AMPA (GluR) 1-4 subunits using RT-PCR performed on homogenates from whole mouse brain and colon. Corresponding amplicons of the same size to those obtained from brain samples were consistently detected for the GluR1, 3, 4 subunits but not the GluR2 subunit in colon samples (N = 3 animals). A negative control, no RT reaction was performed with every experiment and brain samples were used as positive control.

Figure 4
Immunolocalisation of the GluR1 and 4 subunits in the mouse CNS

(A1) shows immunoreactivity for the calcium-binding protein, calbindin, in a sagittal section of the mouse cerebellum. The signal is restricted to the somata and dendrites of Purkinje neurons. In the corresponding field of view, immunoreactivity for (A2) the GluR1 subunit mirrors that of calbindin and is enriched in the molecular layer. (A3) is a high resolution image of the molecular layer showing clustered GluR1 immunoreactivity (green) closely associated with immunoreactivity for the excitatory synaptic marker protein, postsynaptic density 95 (PSD95) (red), on calbindin-immunopositive dendrites. (A4) shows immunoreactivity for the neuronal cytoskeletal protein marker, microtubule associated protein 2 (MAP2), within the mouse
neocortex and hippocampus. (A5) in the corresponding field of view, immunoreactivity for the GluR1 subunit is restricted to the hippocampus. (A6) is a high resolution image taken from *stratum radiatum* of the mouse hippocampus, showing clustered GluR1 immunoreactivity (green) closely associated with immunoreactivity for PSD95 (red), on MAP2-immunopositive dendrites. (B1) shows immunoreactivity for calbindin, in a sagittal section of the mouse cerebellum. In the corresponding field of view, immunoreactivity for (B2) the GluR4 subunit is restricted to the molecular layer. (B3) is a high resolution image of the molecular layer showing clustered GluR4 immunoreactivity (green) closely associated with immunoreactivity for PSD95 (red), on calbindin-immunopositive dendrites. (B4) shows immunoreactivity for MAP2, within the mouse neocortex and hippocampus. (B5) in the corresponding field of view, no specific signal the GluR4 subunit was detected. Scale bars: (A, B, 1-2, 4-5) 500 µm; (A 3, 6, B 3) 3 µm

Figure 5

Immunolocalisation of the postsynaptic marker protein postsynaptic density 95 (PSD95) in the ENS of the mouse colon

(A1) shows immunoreactivity for (PSD95) (red) is targeted to the somato-dendritic regions of enteric neurons, identified by immunoreactivity for nitric oxide synthase (NOS) (blue). (A2) shows that in the corresponding field, PSD95 immunoreactive clusters are also closely associated with putative enteric glial profiles, identified by the expression of GFP driven by the transcription factor Sox10. (A3) is an overlay of A1 and A2. Scale bar: 10 µm
Figure 6

Immunolocalisation of the GluR1 subunit in the ENS of the mouse colon (A1) is an overview of a region of the myenteric plexus showing numerous ganglia, identified by immunoreactivity for NOS. (A2) shows that in the corresponding field of view, immunoreactivity for the GluR1 subunit is widely distributed across ganglia. However, it is noticeable that some NOS immunoreactive neurons appear to be devoid of GluR1 immunoreactivity (arrowheads) whilst GLUR1 signal is evident on putative neurons which are devoid of NOS immunoreactivity (arrows). (A3) is an overlay of (A1) and (A2). GluR1 subunit immunopositive puncta (red) were detectable on the somato-dendritic surfaces of (B1) calretinin-immunopositive, (B2) ChAT-immunopositive, and (B3) a sub-population of NOS-immunopositive but not the (B4) somatostatin-immunopositive enteric neurons. Scale bars: (A) 50 µm; (B) 10 µm

Figure 7

Immunolocalisation of the GluR4 subunit in the ENS of the mouse colon (A1) is an overview of a region of the myenteric plexus showing numerous ganglia, identified by immunoreactivity for NOS. (A2) shows that in the corresponding field of view, immunoreactivity for the GluR4 subunit is widely distributed across ganglia. However, it is noticeable that there are gradients for GluR4 immunoreactivity within ganglia (arrowheads). (A3) is an overlay of (A1) and (A2). GluR4 immunoreactive clusters were located on the somato-dendritic surfaces of (B1) NOS-immunopositive and (B2) ChAT-immunopositive enteric neurons. However, no specific immunoreactivity for GluR4 subunit (red) was detected on either (B3) calretinin-immunopositive or (B4) somatostatin-immunopositive enteric neurons. (C1) shows ENS axonal profiles, identified by NOS-immunoreactivity. (C2) in the same field of
view, GluR4 immunoreactivity closely follows the pattern of NOS labelling. (C3) an overlay of the boxed areas in (C1) and (C2) showing the extensive degree of co-localisation of GluR4 immunoreactivity with that of NOS, particularly within the axonal varicosities (arrowheads).

Scale bars: (A) 50 µm; (B) 10 µm; (C) 20 µm

Figure 8

The pharmacological effect of activating AMPA receptors on spontaneous colonic contractions

(A) shows a representative trace demonstrating the effect of the AMPA receptor agonist CPW399 on the spontaneous longitudinal smooth muscle contractions in an isolated segment of the mouse colon. Note the significant increase in the size of spontaneous contractions after administration of CPW399. Quantification of the effect of CPW399 10 µM on (B) the force and (C) the frequency of spontaneous colonic contractions. Bars represent means and the lines represent the SD. N = 4 animals, * P< 0.05. Scale bars: (A) vertical 0.25 grams, horizontal 2.5 minutes.
## Supplementary Tables

### Supplementary Table 1

Details of primary antibodies used in the study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Dilution</th>
<th>Source</th>
<th>References</th>
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<tr>
<td>Calretinin</td>
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<td>Swant (7699/3H)</td>
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<td>PSD 95</td>
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### Supplementary Table 2

**Table of RT-PCR primer sequences**

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