Title

Molecular and functional diversity of GABA-A receptors in the enteric nervous system of the mouse colon

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Abstract (250)

The enteric nervous system (ENS) provides the intrinsic neural control of the gastrointestinal tract (GIT) and regulates virtually all GI functions. Altered neuronal activity within the ENS underlies various GI disorders with stress being a key contributing factor. Thus, elucidating the expression and function of the neurotransmitter systems which determine neuronal excitability within the ENS, such as the GABA-$GABA_A$ receptor ($GABA_AR$) system, could reveal novel therapeutic targets for such GI disorders. Molecular and functionally diverse $GABA_ARs$ modulate rapid GABAergic-mediated regulation of neuronal excitability throughout the nervous system. However, the cellular and sub-cellular $GABA_AR$ subunit expression patterns within neurochemically-defined cellular circuits of the mouse ENS, together with the functional contribution of $GABA_AR$ subtypes to GI contractility remains to be determined. Immunohistochemical analyses revealed that immunoreactivity for the $GABA_AR$ gamma (γ) 2 and alphas (α) 1, 2, 3 subunits was located on somato-dendritic surfaces of neurochemically distinct myenteric plexus neurons, whilst being on axonal compartments of submucosal plexus neurons. In contrast, immunoreactivity for the α4-5 subunits was only detected in myenteric plexus neurons. Furthermore, α-γ2 subunit immunoreactivity was located on non-neuronal interstitial cells of Cajal. In organ bath studies, $GABA_AR$ subtype specific ligands had contrasting effects on the force and frequency of spontaneous colonic longitudinal smooth muscle contractions. Finally, enhancement of γ2-$GABA_AR$ function with alprazolam reversed the stress-induced increase in the force of spontaneous colonic contractions. The study demonstrates the molecular and functional diversity of the $GABA_AR$ system within the mouse colon providing a framework for developing $GABA_AR$-based therapeutics in GI disorders.

Introduction

The ENS is a large collection of neurons within the muscle wall of the gastrointestinal tract (GIT) which provides the intrinsic neural control of virtually all GI functions (Goyal and Hirano, 1996; Furness, 2006) with ENS neuropathies being thought to underlie a range of GI disorders (Di Nardo et al., 2008; Furness, 2008). Furthermore, exposure to psychosocial stress adversely affects GI function and is a risk factor for the development of GI disorders such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) (Mawdsley and Rampton, 2005; Larauche et al., 2009; Konturek et al., 2011). Importantly, altered levels of neuronal activity within the ENS are implicated in such GI disorders (Margolis and Gershon, 2009; Ohman and Simren, 2010) with treatment aimed primarily at the alleviation of the symptoms (Di Nardo et al., 2008). Thus, elucidating the expression and function of the neurotransmitter systems which determine neuronal excitability within the ENS, such as the GABA-$GABA_AR$ system (Krantis, 2000) could reveal novel therapeutic targets for such GI disorders.

$GABA_ARs$ are chloride permeable integral membrane ion channels composed of five interacting subunit proteins which mediate the effects of the neurotransmitter GABA (Farrant and Nusser, 2005). While only five subunits are required to form a functional receptor, up to nineteen molecularly distinct $GABA_AR$ subunits have been identified (Olsen and Sieghart, 2009). $GABA_AR$ biology has been pioneered in the central nervous system (CNS) where diverse $GABA_AR$ subunit assembly combinations manifest in functionally (Belelli et al., 2009; Eyre et al., 2012) and pharmacologically (Rudolph and Knoflach, 2011) diverse receptor subtypes within distinct regions

(Wisden et al., 1992; Fritschy and Mohler, 1995; Hortnagl et al., 2013) of the CNS, emphasising the importance of identifying which particular $GABA_AR$ subunits are expressed within a particular neural system. Despite the recognised importance of $GABA_ARs$ to neural function and clinical medicine, relatively less is known about $GABA_AR$ expression and function within the peripheral nervous system and the ENS in particular.

$GABA_AR$ subunit mRNA expression has been demonstrated in the rat small intestine (Zeiter et al., 1996; Poulter et al., 1999). However, the expression of particular $GABA_AR$ subtypes at the cellular and sub-cellular level of neurochemically-defined cells remains to be fully elucidated (Krantis et al., 1995). Furthermore, while pan-$GABA_AR$ ligands have been used to demonstrate the effects of $GABA_AR$ modulation on intestinal contractility (Tonini et al., 1987; Tonini et al., 1989a; Roberts et al., 1993; Hebeiss and Kilbinger, 1999; Bayer et al., 2002; Bayer et al., 2003), the functional contribution of specific $GABA_AR$ subtypes to GI contractility is yet to be determined. Here, we provide high resolution immuno-localisation of the $GABA_AR$ α1-5 and γ2 subunits on neurochemically-defined ENS cells of the mouse colon and use $GABA_AR$ subunit-selective drugs to demonstrate that the pharmacological enhancement of the function of different $GABA_AR$ subtypes has contrasting effects on the amplitude and frequency of spontaneous colonic longitudinal smooth muscle contractions *in vitro*. Finally $GABA_AR$ ligands reversed the stress-induced changes in colonic contractility suggesting a role for these agents in treating stress-induced GI disorders.

Materials and Methods

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

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was used to detect which $GABA_AR$ subunits are expressed in the mouse colon at the mRNA level with matched brain tissue used as the positive control. Adult male C57/BL6J mice (Charles River) (N=3) were killed by cervical dislocation and the segments of the colon and whole brain removed and snap frozen in liquid nitrogen. The frozen tissue was homogenised from which RNA was extracted using an RNeasy^R mini kit (Qiagen) according to the manufacturer's protocol. RNA was reverse transcribed into complementary DNA (cDNA) using SuperScript™ First-Strand Synthesis System for RT-PCR kit (Invitrogen). Equal amounts of cDNA (1-2 µl) were then used for subsequent polymerase chain reaction (PCR) using GoTaq^R green mastermix (Promega), PCR grade water and specific primers. Exon-exon spanning $GABA_AR$ subunit specific PCR primers used in the study (Table 1) have previously been published (Glassmeier G et al., 1998; Gustincich S et al., 1999; Tan S et al., 2011). The RT-PCR transcript products for the $GABA_AR$ subunits and the positive control β-actin from brain and colon tissue were run on a 2% agarose gel and the DNA was visualised under ultraviolet light using a SYBR green-based DNA stain.

Tissue preparation for immunohistochemistry

Adult male C57/BL6J (Charles River) 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% paraformaldehyde and 15% v/v saturated picric acid in 0.1M phosphate buffer (pH 7.4) according to previously described protocols (Corteen et al., 2011). After perfusion, the brains and colons were removed and post-fixed in the same fixative over night at 4^{o}C. The next day, tissue was washed in 0.1M phosphate buffer until it was clear of the fixative. Whole-mount preparations of the longitudinal muscle-myenteric plexus and circular muscle-submucosal plexus were obtained using a dissecting microscope and fine forceps, which were then stored in 0.1M phosphate buffer containing 0.05% sodium azide.

Immunohistochemistry

The native $GABA_AR$ subunit immunoreactivity patterns within the ENS of the mouse colon were confirmed in at least 4 animals. Non-specific binding of secondary antibodies was blocked by incubating the tissue with 20% normal horse serum for 2 hours at room temperature. The tissue was incubated with cocktails of primary antibodies (Table 2), diluted in Tris buffer saline containing 0.3% Triton X-100 (TBS-Tx) and 20% normal horse serum, overnight at 4°C. After washing with TBS-Tx, the tissue was 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 in TBS-Tx and mounted on glass slides in Mowiol mounting medium (Polysciences) and then cover slipped.

Antibody specificity

Although the specificity of all the antisera against the $GABA_AR$ subunits used in this study have been reported upon extensively in other studies concerning the CNS (see Table 2), the specificity of the signal obtained in the ENS in this study was confirmed using perfusion-fixed, matched brain-colon tissue from $GABA_AR$ subunit-specific gene deleted mice. 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 40x DIC oil objective (NA1.3) (pixel size 0.29 µm), a Plan Apochromatic 63x DIC oil objective (NA1.4) (pixel size 0.13 µm) or a Plan Apochromatic 100x DIC oil objective (NA1.46) (pixel size 0.08 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 Zen2008 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.

Isometric tension recordings of the effects of $GABA_AR$ subunit-specific ligands on colonic longitudinal muscle contractions from isolated mouse colon segments

The pharmacological activation of $GABA_A$Rs within the colon was explored with a view to understanding their potential roles in one aspect of colon physiology, namely colonic smooth muscle contractility. Intestinal motility or peristalsis arises from the coordinated contraction and relaxation of circular and longitudinal smooth muscles (Smith and Robertson, 1998). The effect of the GABA-$GABA_A$R system on the contractility of intestinal circular smooth muscles has been widely explored (Tonini et al., 1989b; Tonini et al., 1989a; Bayer et al., 2002; Bayer et al., 2003). Therefore, we focused on the effect of ENS $GABA_A$R activation on longitudinal smooth muscle contraction by measuring the changes in the force and frequency of spontaneous contractions *in vitro*. The activity of the interstitial cells of Cajal (ICC) is thought to underlie such intestinal spontaneous contractions (Sanders and Ward, 2006). Six to eight week old male mice were killed by cervical dislocation and the distal colon was removed and immediately placed in physiological solution containing (mM): NaCl 140, NaHCO3 11.9, D+ glucose 5.6, KCl 2.7, $MgCl_2.6H_2O$ 1.05, $NaH_2PO_4.2H_2O$ 0.5, $CaCl_2$ 1.8, warmed to 32°C. The intraluminal contents were removed by gently flushing the colon with the physiological solution. Approximately 2 cm-long segments were mounted in a Harvard organ bath (10 ml chamber) filled with the physiological solution (32^oC) and bubbled with gas containing 95% O_2 and 5% CO_2. 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 activity. After a stable baseline was established, the drugs were 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.

In a subset of experiments, we investigated the effects of alprazolam on the contractile responses evoked by transmural nerve stimulation (10 Hz, 60 V and 0.2 ms duration) (Bayer et al., 2003). The electric pulses were delivered for 10 seconds and a single contraction was observed as a result. The tissue was then washed several times with the physiological solution and allowed to stabilise for 15 minutes. Alprazolam or TTX were then individually added to the bath for 10 minutes after which the electrical stimulation was repeated.

Acute restraint stress

To probe the possible involvement of $GABA_A$Rs in stress-induced alterations of GI contractile function or provide evidence of their therapeutic potential in associated disorders, we exposed mice to acute restraint stress (Buynitsky and Mostofsky, 2009) and compared the effects of the benzodiazepine alprazolam on the force and

frequency of spontaneous colonic contractions. This model was used since it induces a robust local stress response within the GIT which engages a range of intestinal cellular elements such as neurons, muscle and immune cells (Tache and Perdue, 2004; Zheng et al., 2009). We focused on only one aspect of such a stress-response, the changes in longitudinal smooth muscle contractility. Animals were divided into stress and control experimental groups one week prior to the start of the experiment in order to allow adaptation to the new cage environment before commencing the stress. To deliver restraint, mice were restrained for 60 minutes using a Broome rodent restrainer (Harvard Apparatus # 52-0470). During the period of restraint, the mice were kept individually in standard housing cages containing a thin layer of corn cob. Control mice remained in their original cages and were left undisturbed in their home environment. Immediately after the period of restraint, the animals were killed by cervical dislocation and used for isometric tension recordings.

Drugs

The following drugs were used in this study: zolpidem (Tocris Biosciences), alprazolam (Sigma Aldrich), TP003 (Tocris Biosciences), THIP hydrochloride (Tocris Biosciences), L-655, 708 (Tocris Biosciences). Apart from THIP hydrochloride which was dissolved in distilled water, all other drugs were dissolved in DMSO. DMSO at the bath concentrations used had no effect on the amplitude or frequency of colonic spontaneous contractions in agreement with previous evidence (Bayer et al., 2002).

Statistical analysis

All data are presented as the arithmetic mean ± SEM unless stated otherwise. Statistical comparisons were made using either Student's *t* test (paired or unpaired where appropriate) or repeated-measures ANOVA (one-way), followed by the Tukey's post hoc test.

Results

$GABA_AR$ subunit mRNA expression in the mouse colon

Currently, up to 19 different $GABA_AR$ subunits are known to be expressed within the CNS (Sieghart, 2006). Previous studies on $GABA_AR$ subunit expression in the PNS did not specifically report expression patterns within the mouse colon (Akinci and Schofield, 1999; Poulter et al., 1999). RT-PCR performed on homogenates of mouse colon revealed mRNA expression for 14 out of the 16 $GABA_AR$ subunits investigated (Fig. 1) (N= 3 animals). No corresponding signal of the same size was detected for the $GABA_AR$ alpha6 and epsilon subunits in the colon with mouse whole brain homogenates serving as a positive control (Fig. 1).

Immunolocalisation of GABAergic synaptic marker proteins in the ENS of the mouse colon

Immunoreactivity for putative pre- and postsynaptic GABAergic marker proteins was used to determine the distribution of GABAergic innervation across neuronal and non-neuronal cell-types in whole mount preparations of the mouse colon. Microtubule associated protein 2 (MAP2), a pan-neuronal marker protein was used to visualise the somato-dendritic domains of neurons located within the myenteric and submucosal plexuses. Immunoreactivity for the vesicular GABA transporter (VGAT), a protein which within the CNS is selectively expressed in GABAergic axon terminals was used to locate presumptive GABAergic input to different sub-cellular domains while immunoreactivity for neuroligin2 (NL2), a protein which in the CNS is selectively localised to GABAergic and glycinergic inhibitory synapses (Varoqueaux et al., 2004) was used to locate putative inhibitory postsynaptic domains with the

caveat that ultrastructural studies are required to unequivocally demonstrate that, as is the case in the CNS, VGAT and NL2 are located at inhibitory pre- and postsynaptic junctions. Immunoreactivity for the tyrosine-protein kinase Kit, c-Kit, was used to detect the non-neuronal interstitial cells of Cajal (ICC) (Maeda et al., 1992) which provide pace-maker activity in terms of colonic contractility (Garcia-Lopez et al., 2009). Immunoreactivity for VGAT was widely distributed amongst MAP2 immunoreactive somata and dendrites as well as c-Kit immunoreactive profiles located within myenteric and submucosal plexuses (Fig. 2A). Immunoreactivity for NL2 presented as individual clusters which were concentrated on somatic and dendritic compartments of myenteric and submucosal plexus neurons and were closely apposed to VGAT immunoreactive puncta (Fig. 2B). VGAT immunoreactive clusters were also evident within colonic muscle layers and were distinctly associated with nitric oxide synthase (NOS) immunoreactive axon terminals which appeared to innervate c-Kit immunoreactive ICC (Fig. 2C). Thus, the putative sites of GABA release and predictive location of GABAergic receptors within the ENS of the mouse colon includes the neurons of the myenteric and submucosal plexuses as well as the non-neuronal ICCs.

Guided by the patterns of GABAergic innervation and the $GABA_AR$ subunit mRNA expression patterns, immunohistochemistry and confocal microscopy was used to localise the expression of the $GABA_AR$ gamma2 (γ2) and alphas1-5 (α 1-5) subunits within neurochemically defined cell-types of the ENS of the mouse colon. $GABA_AR$-subunit preferring ligands were then used to investigate the consequences of $GABA_AR$ activation on spontaneous colonic longitudinal muscle contractions *in vitro*.

Expression of the γ2 subunit in the mouse colon and its role in the regulation of longitudinal smooth muscle spontaneous contractions

Immunoreactivity for the γ2 subunit was widely distributed across both neuronal and non-neuronal cell-types of myenteric (Fig. 3A), and submucosal (Fig. 3B) plexuses as well as the intramuscular layer (Fig. 3C). Within the myenteric plexus, immunoreactivity for the γ2 subunit presented as distinct clusters almost exclusively located on the somatic and dendritic plasma membranes of nitric oxide synthase (NOS)-, serotonin (5HT)-, corticotrophin releasing hormone (CRH)-, somatostatin (SOM) and choline acetyl transferase (Chat)-immunoreactive neurons (Fig. 3A). In contrast to the membrane-bound location of γ2 subunit immunoreactivity in myenteric neurons, the location of the signal in NOS-immunopositive submucosal plexus neurons was predominantly cytoplasmic which might be suggestive of a presynaptic locus of expression (Fig. 3B). Thus, the targeting of γ2 subunit–containing $GABA_A$Rs (γ2-$GABA_A$Rs) to specific sub-cellular domains of ENS neurons is cell-type specific. Apart from expression in neurons, γ2 subunit immunoreactivity was also evident on putative ICC immunopositive for c-Kit located in proximity to the submucosal plexus (Fig. 3B) and muscle layers (Fig. 3C).

ICC are hypothesised to be the cellular links between ENS neurons and intestinal smooth muscle (Sanders and Ward, 2006; Huizinga et al., 2009) and are thus predominantly involved in GI contractility. Intestinal smooth muscle cells possess spontaneous rhythmic oscillations in their membrane potential, or slow waves which are the source of spontaneous contractions (Iino and Horiguchi, 2006). Myenteric and submucosal ICC are reportedly involved in the generation and propagation of these slow waves (Hirst and Ward, 2003; Sanders et al., 2004). In addition, the

intramuscular ICC which are distributed amongst smooth muscle cells act as mediators of neurotransmission from the ENS to intestinal muscle cells (Ward et al., 2004). Importantly, it has been demonstrated that GABA and the $GABA_AR$ agonist muscimol can modulate the amplitude of these spontaneous contractions in the rat colon (Bayer et al., 2002). The location of γ2 subunit immunoreactivity at the interface between cell-types which are implicated in regulating colon contractility suggests a possible involvement of γ2 subunit containing $GABA_ARs$ (γ2-$GABA_ARs$) in such functions. Since the effects of $GABA_AR$ ligands on the activity of GI circular smooth muscles have been reported on extensively (Tonini et al., 1989b; Tonini et al., 1989a; Bayer et al., 2002; Bayer et al., 2003), we focussed exclusively on their effects on longitudinal smooth muscle contractility. We therefore applied the benzodiazepine alprazolam to whole segments of mouse colon in a conformation that detects predominantly longitudinal smooth muscle activity and determined the changes in the force and frequency of spontaneous contractions; thus our future reference in the manuscript to colonic contractility refers to longitudinal smooth muscle activity. Benzodiazepines as a class act as positive allosteric modulators at α1/2/3/5-β-γ2-$GABA_ARs$ and therefore enhance the endogenous effects of GABA (Rudolph and Knoflach, 2011) with alprazolam in particular being a high potency benzodiazepine widely prescribed for the treatment of generalized anxiety, panic attacks and depression. Alprazolam at a bath concentration of 10 μM induced a significant decrease in the basal tone of the tissue (from - 0.42 ± 0.09 grams to, -0.52 ± 0.1 grams, N = 4 animals*; P* = 0.003, paired Student's *t* test). Alprazolam also significantly decreased the force of spontaneous contractions (from 0.19 ± 0.06 grams to 0.08 ± 0.04 grams, N = 4 animals; *P* = 0.007, paired Student's *t* test) and increased their frequency (from 0.054 ± 0.003 Hz to 0.071 ± 0.009 Hz, *P* = 0.0244,

paired Student's *t* test). Thus, the activation of γ2-$GABA_A$Rs (i.e., $GABA_A$Rs containing - amongst others - the γ2 subunit) has a direct effect on the amplitude and frequency of spontaneous colonic longitudinal muscle contractions as well as the basal tone of the colon.

The intricate expression patterns of the γ2 subunit within the neurochemically diverse cell networks of the ENS raises the question whether the effects of alprazolam on colonic contractility occur directly or via secondary mediators. Two key neurochemical mediators of colonic contractility are acetylcholine which, within the intestine, signals primarily via cholinergic muscarinic receptors to cause intestinal contraction (Furness, 2006) and nitric oxide (NO) which acts via various intra- and inter-cellular pathways to cause intestinal relaxation (Shah et al., 2004). To explore this further, we investigated the effects of alprazolam on the basal tone of the colon as well as the force and frequency of spontaneous colonic contractions in the presence of either atropine a cholinergic muscarinic receptor antagonist or L-NAME, an inhibitor of the NO synthesising enzyme nitric oxide synthase. Alprazolam significantly [$F_{(4, 12)}$ 16.93, $P < 0.0001$; Repeated measures ANOVA, RMA] reduced the basal tone of the colon both alone ($P < 0.05$, RMA) as well as in the presence of atropine ($P < 0.05$, RMA; N = 5 animals) (Fig. 3 D1). Furthermore, alprazolam significantly [$F_{(4, 12)}$ 52, $P < 0.0001$; RMA] decreased the force of spontaneous colonic contractions on its own ($P < 0.05$, RMA) as well as in the presence of atropine ($P < 0.05$, RMA; N = 5 animals) (Fig. 3 D1, 2). In contrast, while alprazolam significantly [$F_{(4, 12)}$ 4.22, $P = 0.02$; RMA] increased the frequency of colonic contractions on its own ($P < 0.05$, RMA), this effect was blocked in the presence of atropine ($P > 0.05$, RMA; N = 5 animals) (Fig. 3 D3). Thus, the muscarinic

cholinergic system is required for the effect of alprazolam on the frequency but not the force of colonic contractions.

Whilst alprazolam significantly [$F_{(6, 18)}$ 11, P = 0.0064; RMA] reduced the basal tone of the colon on its own (P < 0.05, RMA; N = 7 animals), this effect was abolished in the presence of L-NAME (P > 0.05, RMA). In contrast to atropine, the alprazolam-induced [$F_{(6, 18)}$ 5.78, P = 0.0017; RMA] decrease in the force of colonic contraction (P < 0.05, RMA) was blocked in the presence of L-NAME (P > 0.05, RMA; N = 7 animals) (Fig. 3 E1, 2). However, the significant [$F_{(6, 18)}$ 6.45, P = 0.0001; RMA] alprazolam-induced increase in the frequency of colonic contraction (P < 0.05, RMA) still persisted in the presence of L-NAME (P < 0.05, RMA; N = 7 animals) (Fig. 3 E3). Thus, the nitric oxide system is engaged in mediating the effects of alprazolam on the basal tone as well as the force of colonic contractions.

Whilst the direct readout of the preparation used is smooth muscle contraction, it would be informative to confirm the involvement of the ENS in such effects. We therefore directly engaged neural elements by transmurally stimulating the colon segments using electrical field stimulation and measured the evoked contractile response (Fig. 4A). The application of tetrodotoxin (TTX), a blocker of voltage-gated sodium channels, which in this preparation, are expressed by neuronal elements, significantly reduced the amplitude of the evoked response (P = 0.003, paired Student's t test) (Fig. 4B) confirming that neural activity underlies the evoked response. The application of alprazolam mimicked the effect of TTX by significantly reducing the amplitude of the evoked response (P = 0.01, paired Student's t test) (Fig. 4B). There was no significant difference between the evoked responses

produced by TTX and alprazolam (P = 0.07, unpaired Student's t test). This suggests that alprazolam directly engages the ENS and dampens overall neuronal excitability. Dedicated microelectrode studies are required to dissect the effects of $GABA_AR$ subtype function at the single cell or cellular network which manifest in regulating ENS out as a whole.

Confirmation of the specificity of $GABA_AR$ subunit immunoreactivity in brain and colon tissue from $GABA_AR$ α subunit specific gene-deleted ($\alpha^{-/-}$) and WT mice

The specificity of the immunoreactivity patterns obtained by the antibodies against the α1-5 subunits was confirmed in tissue from the brain (Fig. 5) and colon (Fig. 6) of WT and $\alpha1\text{-}5^{-/-}$ mice.

Expression of the α1 subunit in the mouse colon and its role in the regulation of longitudinal smooth muscle spontaneous contractions

Immunoreactivity for the α1 subunit was located on neurons of both the myenteric and submucosal plexuses (Fig. 7A, B). Clustered immunoreactivity for the α1 subunit was evident on MAP2 immunopositive myenteric plexus neurons closely mirroring the expression pattern of the γ2 subunit signal (Fig. 7A1). Immunoreactivity for the α1 subunit presented as distinct clusters associated with VGAT immunoreactive clusters in close proximity to somato-dendritic plasma membranes, which were delineated by the voltage-gated potassium channel 2.1 (Kv2.1), thus implying expression at inhibitory synaptic junctions (Fig. 7A2). In addition, this clustered somato-dendritic pattern of α1 subunit immunoreactivity was also evident on NOS, Chat-, 5HT-, and CRH-immunopositive myenteric neurons (Fig. 7A3-6). Furthermore,

α1 subunit immunoreactivity was clustered on Chat-immunopositive varicosities in the muscle layer (Fig. 7A7). Immunoreactivity for the α1 subunit within submucosal plexus neurons also closely mirrored the pattern of the γ2 subunit, appearing wholly cytoplasmic in NOS-immunoreactive neurons, with distinct α1 subunit immunoreactive clusters evident on NOS-immunoreactive axonal varicosities (Fig. 7B). This immunolocalisation pattern suggests that α1-$GABA_A$Rs are located postsynaptically on myenteric plexus neurons and presynaptically on submucosal plexus neurons.

In order to investigate whether the activation of α1-$GABA_A$Rs influences colonic contraction, we applied the $GABA_A$R subunit-selective imidazopyridine zolpidem to isolated mouse colon segments and measured the changes in the force and frequency of spontaneous contractions. Within the CNS, zolpidem at a concentration of 100nM is a selective positive allosteric modulator (PAM) of α1-γ2-$GABA_A$Rs, whereas a concentration of 1μM zolpidem has affinity not only for α1-γ2, but additionally α2/3-γ2-$GABA_A$Rs (Langer et al., 1990; Crestani et al., 2000; Peden et al., 2008). Zolpidem at a bath concentration of 100nM significantly increased the force of spontaneous contractions (P = 0.0246, paired Student's t test, N = 4 animals) (Fig. 7C1, 2). However, zolpidem at this concentration had no significant effect on the frequency of spontaneous contractions (P = 0.4228, paired Student's t test; N = 4 animals) (Fig. 7C1, 3).

Expression of the α2, 3 subunits in the mouse colon and their role in the regulation of longitudinal smooth muscle spontaneous contractions

Immunoreactivity for the α2 subunit was more restricted compared to other subunits investigated and was localised preferentially on MAP2-immunopositive neurons of

the myenteric plexus (Fig. 8A). In addition, within this region, α2 subunit immunoreactive clusters also decorated c-Kit immunopositive profiles, the putative ICC (Fig. 8A). There was a noticeable gradient in the comparative levels of α2 subunit immunoreactivity in NOS-immunopositive neurons of the myenteric and submucosal plexuses with the latter exhibiting strikingly higher levels of signal, which, in a similar manner to other $GABA_AR$ subunits, was located cytoplasmically (Fig. 8B, C). Finally, somatostatin immunoreactive varicosities were closely apposed to α2 subunit immunoreactive clusters within the myenteric plexus (Fig. 8D) suggesting that GABA released from somatostatin-expressing neurons may signal via α2-$GABA_ARs$. Indeed, somatostatin is a neurochemical signature of GABAergic interneurons within the ENS (Furness, 2006). Immunoreactivity for the α3 subunit was restricted to the somatic and dendritic domains of somatostatin-immunopositive neurons (Fig. 8E) as well as neurons contacted by Chat-immunopositive varicosities (Fig. 8F) within the myenteric plexus. Furthermore α3 subunit immunoreactivity clusters were evident within the muscle layer and distinctly associated with NOS-immunopositive varicosities and c-Kit Immunopositive ICCs (Fig. 8G).

In order to investigate the potential functional roles of α2/3-$GABA_ARs$ in colonic contractility we applied zolpidem 1 μM to isolated mouse colon and measured the changes in the force and frequency of spontaneous contractions. At this concentration, zolpidem is expected to enhance the function of α2/3-γ2 in addition to α1-γ2-$GABA_ARs$ (Peden et al., 2008). Zolpidem at a bath concentration of 1 μM significantly decreased the force of spontaneous contractions (P = 0.0133, paired Student's t test; N = 4 animals) (Fig. 8H1) and increased their frequency (P = 0.0237, paired Student's t test; N= 4 animals) (Fig. 8H2). To dissect the potential contrasting

roles of α2- and α3-$GABA_A$Rs on the force and frequency of spontaneous colonic contractions, we utilised the $GABA_A$R ligand TP003 which in recombinant systems is a selective PAM of α3-γ2-$GABA_A$Rs (Dias et al., 2005). A caveat is that TP003 may lack this α3 subunit selectively in native $GABA_A$R expression systems (Peden et al., 2008). TP003 at a bath concentration of 100 μM significantly decreased the force of spontaneous contractions (P = 0.024, paired Student's t test; N = 4 animals) (Fig. 8I1) but had no significant effect on their frequency (P = 0.294, paired Student's t test; N = 4 animals) (Fig. 8I2). Collectively, the effects of zolpidem 1 μM and TP003 suggest that the activation of α2-$GABA_A$Rs influences the frequency of spontaneous colonic contractions whereas the activation of α3-$GABA_A$Rs influences the force of spontaneous colonic contractions. We were unable to fully reverse the effects of both zolpidem and TP003 by washout and thus not able to use atropine or L-NAME to evaluate the potential roles of muscarinic cholinergic receptors and nitric oxide pathways in mediating the effects of these drugs.

Expression of the α4 subunit in the mouse colon and its role in the regulation of longitudinal smooth muscle spontaneous contractions

In contrast to that of the γ2 subunit, α4 subunit immunoreactivity was restricted to the neurons and ICC of the myenteric plexus and was not detectable within the submucosal plexus (Fig. 9A). Clusters immunoreactive for the α4 subunit were located on somato-dendritic domains of NOS, Chat, 5HT and CRH immunopositive neurons (Fig. 9A, B). Thus, within the ENS of the mouse colon, $GABA_A$R subunit expression varies not only according to cell-type and sub-cellular domain but also according to distinct regions of the ENS delineated by the myenteric and submucosal plexuses.

The lack of availability of a selective α4-$GABA_AR$ ligand precluded the unequivocal determination of the contribution of α4-$GABA_AR$ activation to colonic contractility. We therefore utilised the $GABA_AR$ agonist THIP which will be selective for those α4-$GABA_ARs$ which are co-assembled with δ subunits (Brown et al., 2002; Storustovu and Ebert, 2006) with the caveat that $GABA_ARs$ not composed of γ or δ subunits (i.e. α-β pentamers) might also be engaged. THIP, at a bath concentration of 10 μM significantly increased the force of spontaneous contractions (from 0.11 ± 0.04 grams to 0.19 ± 0.09 grams, N = 5 animals; P = 0.0299, paired Student's *t* test) but did not significantly alter their frequency (from 0.052 ± 0.005 Hz to 0.051 ± 0.009 Hz, N = 5 animals; *P* = 0.5583, paired Student's *t* test).

We then evaluated the effects of THIP in the presence of atropine and L-NAME. Whilst THIP significantly increased the force of colonic contractions on its own (*P* < 0.05, RMA), this effect was abolished in the presence of atropine (*P* > 0.05, RMA; N = 5 animals) (Fig. 9 C1, 2). In accordance with above, THIP had no significant effect on the frequency of spontaneous contractions either alone (*P* > 0.05, RMA) or in the presence of atropine (*P* > 0.05, RMA) (Fig. 9 C3).

In contrast to atropine, the significant [$F_{(2, 6)}$ 13.6; *P* = 0.0059. RMA] THIP-induced increase in the force of colonic contractions (*P* < 0.05, RMA) persisted in the presence of L-NAME (*P* < 0.05, RMA; N = 3 animals) (Fig. 9 D1, 2). Once again, THIP had no significant effect on the frequency of spontaneous contractions either alone (*P* > 0.05, RMA) or in the presence of L-NAME (*P* > 0.05, RMA) (Fig. 9 D3). Thus, the muscarinic cholinergic system but not the nitric oxide system appears to be involved in mediating the effects of THIP on the force of colonic contractions.

Expression of the α5 subunit in the mouse colon and its role in the regulation of longitudinal smooth muscle spontaneous contractions

In a similar pattern to α4 subunit immunoreactivity, signal for the α5 subunit was restricted to neurons and putative ICC of the myenteric plexus with no α5 subunit immunoreactivity detectable in the submucosal plexus (Fig. 10A). Within the myenteric plexus, immunoreactivity for the α5 subunit was located on the somato-dendritic domains of NOS, CRH and 5HT immunopositive neurons as well as apposed to Chat immunoreactive varicosities (Fig. 10Ai, B).

L-655,708, an inverse agonist selective for the benzodiazepine site at α5-γ2-$GABA_A$Rs (Quirk et al., 1996), was used to investigate the functional implications of α5-$GABA_A$Rs activity on the force and frequency of spontaneous contractions of the mouse colon. L-655,708 at a bath concentration of 10 μM induced a profound reduction in the basal tone of the tissue (Fig. 10C1, double arrow) (from - 0.28 ± 0.16 grams to -0.58 ± 0.18 grams, N = 8 animals*; P* < 0.0001, paired Student's *t* test). Furthermore, L-655,708 10 μM significantly decreased the force of spontaneous contractions (from 0.13 ± 0.05 grams to 0.10 ± 0.02 grams, N= 8 animals; *P* = 0.0316, paired Student's *t* test). However, L-655,708 did not significantly alter the frequency of contractions (from 0.058 ± 0.010 Hz to 0.058 ± 0.011 Hz, N = 8 animals; *P* = 0.8398, paired Student's *t* test). Notably, out of all the $GABA_A$R ligands tested L-655,708 produced the most robust reduction in the basal tone of the tissue with only alprazolam mimicking such an effect, although to a much lesser degree. This suggests a central role for α5-$GABA_A$Rs in setting the muscle tone of the mouse colon.

In a separate experiment, we then evaluated the effects of L-655,708 in the presence of atropine and L-NAME. L-655,708 significantly [$F_{(5, 15)}$ 3.23; P = 0.03, RMA] reduced the basal tone of the colon, both on its own (P < 0.05, RMA) and in the presence of atropine (P < 0.05, RMA; N = 6 animals). The effect of L-655,708 in significantly [$F_{(5, 15)}$ 4.79; P = 0.0081, RMA] reducing the force of colonic contractions (P < 0.05, RMA) persisted in the presence of atropine (P < 0.05, RMA) (Fig. 10 C1, 2). In accordance with above, L-655,708 had no significant effect on the frequency of spontaneous contractions either alone (P > 0.05, RMA) or in the presence of atropine (P > 0.05, RMA) (Fig. 10 C3). The data suggest that the muscarinic cholinergic system is not associated with the effect of L-655,708 on the basal tone or force of colonic contractions.

Whilst L-655,708 significantly [$F_{(5, 15)}$ 5.8; P = 0.003, RMA] reduced the basal tone of the colon on its own (P < 0.05, RMA), this effect was abolished in the presence of L-NAME (P > 0.05, RMA; N = 6 animals). In contrast to atropine, the significant L-655,708-induced [$F_{(5, 15)}$ 4.9; P = 0.007, RMA] decrease in the force of colonic contractions (P < 0.05, RMA) was abolished in the presence of L-NAME (N = 6 animals; P > 0.05, RMA) (Fig. 10 D1, 2). Once again, L-655,708 had no significant effect on the frequency of spontaneous contractions either alone (P > 0.05, RMA) or in the presence of L-NAME (P > 0.05, RMA; N = 6 animals) (Fig. 10 D3). Thus, the nitric oxide system is involved in mediating the effects of L-655,708 on both basal tone and the force of colonic contractions.

The effect of $GABA_AR$ activation on the stressed induced alterations in colonic longitudinal smooth muscle spontaneous contractions

599 Psychosocial stress is a key contributor to the underlying pathology of a number of
600 GI disorders (Konturek et al., 2011) such as inflammatory bowel disease (IBD) and
601 irritable bowel syndrome (IBS) (Tache et al., 2004; Mawdsley and Rampton, 2005;
602 Fichna and Storr, 2012). With a view to elucidating a potential role for $GABA_AR$
603 ligands in influencing stress-induced alterations in colonic contractions, we
604 compared the effects of alprazolam in tissue from control animals and animals
605 exposed to 1 hour of restraint stress. While alprazolam at a bath concentration of 10
606 µM predictably (see Fig. 3) reduced the basal tone of tissue from control animals
607 (Fig. 11 A1, double arrow), this effect was negligible in tissue from stress animals
608 (Fig. 11 A2) (control, - 0.17 ± 0.07 grams *versus* stress, -0.06 ± 0.01 grams, N = 8
609 animals*; P* = 0.0021, unpaired Student's *t* test). The force of baseline spontaneous
610 contractions were significantly larger in tissue from stress animals compared with
611 control (control, 0.11 ± 0.01 *versus* stress, 0.19 ± 0.01, N =7, *P* < 0.001, RMA) with
612 large rhythmic contractions superimposed on smaller contractions evident in tissue
613 from stress animals (Fig. 11A2, arrows). Alprazolam significantly decreased [$F_{(2.401,}$
614 $_{14.40)}$ = 44.48, P < 0.0001, RMA] the force of spontaneous colonic contractions in
615 both control (N = 7 animals; *P* < 0.001, RMA) and stress tissue (*P* < 0.001, RMA; N =
616 7 animals). Although the same concentration of alprazolam induced a greater
617 percentage reduction in the force of spontaneous colonic contractions in tissue from
618 stress animals compared to control tissue (mean ± SD % reduction; control, 39.76 ±
619 11.4 % versus stress, 53.26 ± 14.5 %), the effect did not reach statistical significance
620 (*P* = 0.07, unpaired Student's *t* test). However, it is notable that alprazolam reduced
621 the force of spontaneous contractions in tissue from stress animals to the levels
622 exhibited at baseline for control tissue (Fig. 11 B1). Stress did not significantly alter
623 the frequency of spontaneous contractions (*P* > 0.05, RMA; N =7). While alprazolam

predictably significantly increased the frequency of spontaneous colonic contractions in tissue from control mice ($P < 0.001$, RMA), this effect was not evident in tissue from stress animals ($P > 0.05$, RMA). Collectively, these data suggest that drugs targeting γ2-$GABA_AR$ have the potential to reverse changes in the force of colonic contractions arising from exposure to stressors.

Discussion

The study shows that immunoreactivity for the α1 and γ2 subunits was the most widespread compared to the other subunits investigated, being located on chemically diverse neurons of both myenteric and submucosal plexuses. This preponderance of α1-γ2 subunit immunoreactivity within the ENS mirrors $GABA_AR$ expression patterns within the CNS where α1-γ2-$GABA_A$Rs are thought to be the major subtypes (Wisden et al., 1992). While immunoreactivity for the α2 and 3 subunits was also evident in myenteric and submucosal plexuses, their signals were restricted to smaller sub-sets of neurochemically defined enteric neurons. In stark contrast, immunoreactivity for the α4-5 subunits was only detectable in myenteric plexus neurons. $GABA_AR$ subunit immunoreactivity was also located on non-neuronal cells which are likely to be the ICC that act as pace-makers of the GIT and are involved in the creation of slow wave potentials which leads to the contraction of smooth muscle (Garcia-Lopez et al., 2009). The application of $GABA_AR$ subunit preferring ligands induced contrasting effects on the force and frequency of spontaneous contraction of longitudinal smooth muscles of the colon *in vitro*. Finally, α-γ2-$GABA_AR$ activation reversed the stress-induced increase in the force of spontaneous contractions. The study reveals the rich molecular and functional diversity of the $GABA_AR$ system within the ENS of the mouse colon and provides a platform for the design of $GABA_AR$-based formulations targeted specifically for GI disorders

Implications of $GABA_AR$ subunit expression patterns for colon contractility

The ENS is capable of providing complete neural control of GI functions independent of input from the CNS (Furness, 2006). Within the mammalian ENS, over thirty functionally distinct types of neurons have been discovered which communicate using more than 25 different neurotransmitters (McConalogue and Furness, 1994; Furness, 2000), including GABA (Jessen et al., 1986). At the organ level, GABA, released predominantly from interneurons and endocrine cells (Krantis, 2000; Furness, 2006), influences various GI functions including motility (Cherubini and North, 1984), secretion (Luzzi et al., 1987) and mucosal function (Hardcastle et al., 1991; MacNaughton et al., 1996). At the single cell level, applied GABA depolarises myenteric neurons and thus exerts excitatory postsynaptic effects in the ENS (Cherubini and North, 1984) via $GABA_ARs$ (Cherubini and North, 1985). However, the precise effects of various $GABA_AR$ subtypes on the excitability of the functionally and neurochemically diverse ENS neurons remain to be revealed, necessitating a detailed description of their expression patterns in the first instance.

A striking feature of the $GABA_AR$ subunit immunoreactivity patterns revealed by this study was the plexus-dependent location of the signal. $GABA_AR$ subunit immunoreactivity in myenteric plexus neurons was always located on somato-dendritic cell surfaces, irrespective of the neurochemical content of the cell. This clustering on postsynaptic domains is the conventional $GABA_AR$ subunit expression pattern of the CNS (Fritschy and Mohler, 1995; Nusser et al., 1996; Somogyi et al., 1996) and serves to regulate the neuron which is postsynaptic to the GABA release site (Farrant and Nusser, 2005). In contrast, $GABA_AR$ subunit immunoreactivity in submucosal neurons was invariably located cytoplasmically and on axonal

varicosities. This suggests a presynaptic locus of expression for $GABA_A$Rs in submucosal neurons which is likely to result in an auto-regulatory function that could influence the further release of co-expressed neurotransmitters (Kullmann et al., 2005). It is difficult to speculate what eventual net effect $GABA_A$R activation will have on, for example, myenteric plexus output such as colonic contractility given the fact that non-overlapping populations of NOS-, somatostatin- or encephalin-immunopositive GABAergic interneurons innervate both excitatory as well as inhibitory neurons (Krantis, 2000). An added layer of complexity was the association of $GABA_A$R subunit immunoreactivity with non-neuronal cells, which, based on their immunoreactivity, are likely to be the ICC. ICC are thought to provide pacemaker activity in terms of intestinal contractions (Garcia-Lopez et al., 2009) suggesting a clear role for $GABA_A$Rs in intestinal motility. Collectively, this cell-type-specific targeting of $GABA_A$Rs to either pre- or postsynaptic compartments of submucosal and myenteric plexuses respectively is likely to result in contrasting effects on the excitability of the neurons, the ensuing overall output of the plexuses as a whole, and thus GI function, following the application of GABA or the ingestion of $GABA_A$R ligands.

To gain a perspective on the potential contributions of various $GABA_A$R subtypes to GI function, we concentrated on the myenteric plexus in light of its readily measurable physiological output, namely colonic spontaneous contractility. Despite the widespread expression of various $GABA_A$R subunits throughout the ENS, it is notable that the $GABA_A$R subunit-preferring ligands had such distinctly opposing effects on longitudinal smooth muscle contractility. Indeed, the pharmacological activation of α1-γ2-$GABA_A$Rs and α4-$GABA_A$Rs increased the force of spontaneous

contractions, α2-γ2-$GABA_A$Rs increased their frequency, α3-γ2-$GABA_A$Rs decreased their force and an inverse agonist at α5-γ2-$GABA_A$Rs decreased their force. This suggests that the engagement of various $GABA_A$R subtypes within the cellular networks of the ENS cooperate to modulate the distinct physiological processes which underlie coordinated contractility. It would be beneficial to understand which particular $GABA_A$R-cellular pathway modulates distinct facets of the contractile process such as amplitude or frequency. While the current study suggests the overlap of multiple $GABA_A$R subtypes on neurochemically diverse cell-types, such as those expressing NOS and Chat, these combinatorial pharmacological analyses allow us to draw cautious conclusions on the neurochemical and cellular pathways mediating the $GABA_A$R-subtype dependent effects on the force and frequency of longitudinal muscle colonic contractions. For example, alprazolam, which is likely to preferentially engage α2-3-γ2-$GABA_A$Rs, appeared to induce a decrease in the force of contractions via NO pathways. In accordance, Furthermore, α2/3-$GABA_A$R immunoreactivity was associated with somatostatin-immunopositive neurons, the activation of which via $GABA_A$Rs is linked to the release of nitric oxide and vasoactive intestinal peptide (VIP) from inhibitory motor neurons (Krantis, 2000) and a consequent decrease in intestinal motility, an effect manifested by the pharmacological activation of α2/3-γ2-$GABA_A$Rs. The obvious caveat is that the pharmacology of the $GABA_A$R subunit-preferring ligands has been demonstrated predominantly in either recombinant systems or CNS preparations. Thus, the future characterisation of these ligands in GI tissue from $GABA_A$R subunit-specific mutant mouse models will be instrumental in confirming their pharmacological profiles in colon tissue.

$GABA_AR$s and stress-induced GI disorders

Dysregulation of the ENS contributes to the pathophysiology of a number of GI disorders including IBS and IBD (Margolis and Gershon, 2009; Ohman and Simren, 2010). A key component of such disorders as well as other GI disorders is psychosocial stress (Mawdsley and Rampton, 2005; Santos et al., 2008). CRH, released primarily from the hypothalamus, is the key mediator of the body's response to stress (Bale and Vale, 2004). However, there are a number of extra-hypothalamic sources of CRH throughout the body, including the ENS (Liu et al., 2006), presumably functioning to mediate the stress response at a local level (Stengel and Tache, 2010). Importantly, changes in GI CRH and CRH receptor expression within certain disorders of the GIT have been reported (Tache et al., 2004; Tache and Perdue, 2004; Yuan et al., 2012). The excitability of CRH-containing ENS neurons is likely to determine CRH release within the GIT and is thus integral to GI homeostasis following exposure to stressors. It is notable that robust $GABA_AR$ subunit expression was evident on enteric CRH neurons. Since the $GABA_AR$s provide such a central role in regulating neuronal activity, and thus the release of neuronal contents, the modulation of $GABA_AR$ activity specifically on enteric CRH-expressing neurons might provide a highly specific strategy for targeting stress-induced GI disorders. Based on the immunoreactivity patterns within this study, drugs targeting α1/4/5-$GABA_AR$ are likely to influence the activity of at least the CRH-expressing neurons of the ENS. Thus, determining the precise effects of various $GABA_AR$ ligands on the excitability of defined sets of ENS neurons is essential for the further judicious design and use of such agents in GI disorders.

Importantly, stressors have been shown to cause a decrease in gastric emptying, an increase in distal colonic motility and acceleration of intestinal transit (Mayer, 2000). It is thus promising that alprazolam in this study was able to reverse the stress-induced increase in the force of colonic contractions. However, it is currently unclear what the contribution of such stress-induced increase in contractile responses is to stress-related GI pathology, if any. Surprisingly, out of the number of therapeutic agents considered for conditions such as IBS or IBD, $GABA_AR$ ligands are largely overlooked (Hammerle and Surawicz, 2008; Saad and Chey, 2008), although recent evidence is promising (Salari and Abdollahi, 2011). The rich field of $GABA_AR$ pharmacology (Rudolph and Mohler, 2006; Rudolph and Knoflach, 2011) is littered with agents that showed promising pharmacological profiles but translated poorly to the clinic due to either unacceptable central side effects or poor CNS penetration. The current study provides the scientific rationale for the re-evaluation of such agents with a view to reformulating them specifically for delivery to the GIT. In conclusion, the study provides a detailed description of the location of diverse $GABA_AR$ subunits expressed within the complex network of neurons composing the ENS of the mouse colon. The fledgling functional analyses provides a firm mandate for further exploring the individual roles of specific $GABA_AR$ subtypes in GI functional and associated disorders.

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Figure Legends

Figure 1

$GABA_AR$ subunit mRNA expression in the mouse colon

Representative gel electrophoresis images of mRNA transcripts for various $GABA_AR$ subunits using RT-PCR and homogenates from whole mouse brain and colon. Corresponding amplicons of the same size to those obtained from brain samples were consistently detected for the $GABA_AR$ α1-5, β1-3, γ1-3 and δ subunits but not the α6 and ε subunits in colon samples (N = 3 animals). A negative control, no RT reaction was performed with every experiment.

Figure 2

Immunolocalisation of putative inhibitory synaptic marker proteins in the ENS of the mouse colon

(Ai) immunoreactivity for the somato-dendritic marker protein microtubule associated protein 2 (MAP2) (blue) demonstrates the location of neurons within ganglia of the myenteric plexus. Immunoreactivity for the GABAergic presynaptic marker protein vesicular GABA transporter (VGAT) (red) shows the widespread GABAergic innervation of neurons throughout the ENS. (Aii) shows immunoreactivity for the interstitial cells of Cajal (ICC) marker protein c-Kit (green) within the same field of view as (Ai). (Aiii) is an overlay of (Ai and Aii) and demonstrates the association of VGAT immunoreactivity with neuronal and non-neuronal cells of the ENS within the mouse colon. (Bi) shows immunoreactivity for the voltage-gated potassium channel 2.1 (Kv2.1) (blue) which delineates somato-dendritic plasma membranes as well as immunoreactivity for neuroligin2 (NL2), a protein which in the CNS is located exclusively in inhibitory synapses (green). (Bii) shows immunoreactivity for VGAT

within the same field of view as (Bi). (Biii) is an overlay of (Bi and Bii) demonstrating the close association between putative presynaptic VGAT and postsynaptic NL2 immunoreactive clusters (arrowheads) and thus the likely locations of GABAergic synapses. (Ci) shows nitric oxide synthase (NOS) immunoreactive axon terminals (blue) which are also immunopositive for VGAT (red). (Cii) VGAT immunoreactive puncta are apposed to cellular profiles immunoreactive for c-Kit which are likely to be ICC. (Ciii) is an overlay of (Ci and Cii) showing the close association between GABAergic axon terminals and the profiles of ICC. The insert is a magnified view of the boxed area. Scale bars: (A) 30 µm; (B) 5 µm; (C) 20 µm; insert, 2 µm.

Figure 3

Immunolocalisation of the $GABA_AR$ γ2 subunit in the ENS of the mouse colon and the pharmacological effect of activating γ2-$GABA_A$Rs on spontaneous colonic longitudinal smooth muscle contractions

(A1) shows immunoreactivity for the γ2 subunit is widely distributed on neurons of the myenteric plexus visualised by NOS immunoreactivity (insert). Immunoreactivity for the γ2 subunit was located on somato-dendritic surfaces of (A2) serotonergic-immunopositive (5HT)-, (A3) corticotrophin releasing hormone (CRH)-immunopositive, (A4) somatostatin-immunopositive (SOM)- and (A5) choline acetyl transferase (Chat)-immunopositive myenteric plexus neurons. (B) in contrast to the surface location of γ2 subunit immunoreactivity on myenteric plexus neurons (A), the signal in submucosal plexus neurons (asterisks) identified by NOS immunoreactivity, was located predominantly within the cytoplasm as well as in c-Kit immunopositive profiles (arrowheads). The inserts are magnified views of the cell identified by the arrow. (C) within the muscle layer, γ2 subunit immunoreactivity was closely

associated with (Ci) NOS-immunopositive axon terminals and (Cii) c-Kit immunopositive profiles (asterisks). (D1) representative trace demonstrating the effect of the benzodiazepine alprazolam 10 µM on spontaneous contractions in a piece of isolated mouse colon in the absence and presence of the muscarinic cholinergic receptor antagonist atropine 1 µM. Quantification of the effects of alprazolam 10 µM, before and after the co-application of atropine on (D2) the force and (D3) the frequency of spontaneous colonic contractions. (E1) representative trace demonstrating the effect of alprazolam 10 µM on spontaneous contractions in a piece of isolated mouse colon in the absence and presence of the nitric oxide synthase inhibitor L-NAME 10 µM. Quantification of the effects of alprazolam 10 µM, before and after the co-application of L-NAME on (E2) the force and (E3) the frequency of spontaneous colonic contractions. Bars represent means and the lines represent the SD. N = 7 animals, * $P < 0.05$, repeated measures ANOVA with posthoc Tukey's test. Scale bars: (A1) 20 µm; (A2-5) 10 µm; (B, C) 20 µm; (D1, E1) vertical 0.25 grams, horizontal 2 minutes.

Figure 4

Effect of alprazolam on electrically evoked contractile responses of colon longitudinal smooth muscles

(A) shows representative records of the contractile responses of a colon segment following electrical field transmural stimulation (EFS) either alone or in the presence of tetrodotoxin (TTX), which blocks neural activity, or alprazolam. Note that both TTX and alprazolam attenuate the evoked response largely to the same degree. (B) quantification of the effects of TTX and alprazolam on the evoked contractile responses. Bars represent the mean percentage of the maximal response and the

lines represent the SD. N = 4 animals, * $P < 0.05$, paired Student's *t* test. Scale bars (A1) vertical 0.1 grams, horizontal 30 seconds.

Figure 5

Confirmation of the specificity of the $GABA_AR$ α subunit immunoreactivity using tissue from the brains of wild type (WT) ($\alpha^{+/+}$) and $GABA_AR$ α1-5 subunit-specific gene-deleted mice ($\alpha^{-/-}$)

(A1, B1, D1, E1) shows characteristic immunoreactivity patterns for the α1-2, 4-5 subunits in the hippocampus and neocortex of WT mouse brain respectively. (C1) shows the characteristic enrichment of α3 subunit immunoreactivity within the reticular nucleus of the thalamus (nRT). (A2, B2, C2, D2, and E2) no specific signal was detectable in brain tissue from the appropriate $\alpha^{-/-}$ mice. Scale bars 200 µm.

Figure 6

Confirmation of the specificity of the $GABA_AR$ α subunit immunoreactivity using tissue from the colons of wild type (WT) ($\alpha^{+/+}$) and $GABA_AR$ α1-5 subunit-specific gene-deleted mice ($\alpha^{-/-}$)

(A1-E1) are images of whole-mount preparations of the ENS of WT mouse colon demonstrating myenteric plexus neurons identified by (A1i-E1i) NOS immunoreactivity. (A1ii-E1ii) in the corresponding fields of view, α1-5 subunit immunoreactivity respectively is strongly associated with myenteric plexus neurons. (A2i-E2i) are images of whole-mount preparations of the ENS of $\alpha 1\text{-}5^{-/-}$ colon respectively demonstrating myenteric plexus neurons identified by NOS immunoreactivity. (A2ii-E2iii) in the corresponding fields of view, no specific α1-5 subunit signal respectively was detectable. Scale bars: (A, B) 40 µm.

Figure 7

Immunolocalisation of the $GABA_AR$ α1 subunit in the ENS of the mouse colon and the pharmacological effect of activating α1-$GABA_ARs$ on spontaneous colonic longitudinal smooth muscle contractions

(A1) shows clustered α1 subunit immunoreactivity (red) widely distributed on the somato-dendritic surfaces of MAP2-immunopositive myenteric plexus neurons (blue). (A2i) shows that α1 subunit immunoreactivity (red) on plasma-membrane surfaces, identified by Kv2.1 immunoreactivity (blue) is closely apposed to (A2ii, iii) VGAT immunoreactive puncta (green) and thus likely GABAergic synaptic junctions. Immunoreactivity for the α1 subunit was located on somato-dendritic surfaces of (A3) NOS-immunopositive, (A4) Chat-immunopositive, (A5) 5HT-immunopositive and (A6) CRH-immunopositive myenteric plexus neurons as well as (A7) Chat-immunopositive axon terminals in the muscle layer. (B) shows that α1 subunit immunoreactivity within neurons of the submucosal plexus was located on cytoplasmic and axonal compartments. (Bii) is a magnified view of the boxed area in (Bi). (C1) representative trace demonstrating the effects of the application of zolpidem at a concentration of 100nM (α1-$GABA_AR$ selective agonist) on the spontaneous contractions in a piece of isolated colon. Quantification of the effects of zolpidem 100nM on (C2) the force and (C3) the frequency of spontaneous colonic contractions. Boxes represent means, the lines represent the SD and the small squares represent the individual data points. N= 4 animals. * $P < 0.05$ paired Student's *t* test. Scale bars: (A) 10 µm; (A7 insert) 2 µm; (B) 10 µm; (C1) vertical 0.5 grams, horizontal 5 minutes.

Figure 8

Immunolocalisation of the $GABA_AR$ α2 and 3 subunits in the ENS of the mouse colon and the pharmacological effect of activating α2/3-$GABA_ARs$ on spontaneous colonic longitudinal smooth muscle contractions

(A) shows the association of α2 subunit immunoreactivity with neuronal and non-neuronal cellular profiles in ENS of the mouse colon. (Ai) is an overlay of immunoreactivity patterns for MAP2 (blue) a marker of neurons, c-Kit (green) a marker of ICC and the α2 subunit (red). (Aii) is a magnified view of the boxed area in (Ai) showing the significant association between α2 subunit immunoreactive clusters with MAP2-immunopositive somata and dendrites. (Aiii) in the corresponding field of view numerous α2 subunit immunoreactive clusters are located on c-Kit immunopositive profiles (arrowheads). (B) shows α2 subunit immunoreactivity on the somato-dendritic surfaces of NOS-immunopositive myenteric plexus neurons. (C) shows the comparative cytoplasmic immunoreactivity pattern for the α2 subunit in NOS-immunopositive submucosal plexus neurons. (D) shows α2 subunit immunoreactivity clusters closely apposed to SOM-immunopositive puncta within the myenteric plexus. The insert is a magnified view of the area highlighted by the asterisk. (E) shows α3 subunit immunoreactive clusters on the cell body of a SOM-immunopositive myenteric plexus neuron. (F) shows α3 subunit immunoreactive clusters closely associated with Chat-immunopositive varicosities. (Gi) shows α3 subunit immunoreactive clusters decorating NOS-immunopositive axon terminals in the muscle layer. (Gii) in the corresponding field of view, α3 subunit immunoreactive clusters are located in close proximity to c-Kit-immunopositive profiles. The inserts on the left of (Gi and Gii) are magnified views of the boxed area. The insert on the right of (Gii) is a magnified merged image of all three channels demonstrating the

juxtaposition of α3 subunit immunoreactive clusters between NOS-immunopositive axon terminals and c-Kit immunopositive profiles which are likely to be ICC. (H) quantification of the effects of zolpidem 1 µM on (H1) the force and (H2) the frequency of spontaneous colonic contractions (N = 4 animals). (I) quantification of the effects of TP003 100 µM on (I1) the force and (I2) the frequency of spontaneous colonic contractions (N = 4 animals). Boxes represent means, the lines represent the SD and the small squares represent the individual data points. * $P < 0.05$ paired Student's *t* test. Scale bars: (Ai) 20 µm; (Aii, iii) 10 µm; (B-D) 10 µm; (E, F) 5 µm; (G) 50 µm.

Figure 9

Immunolocalisation of the $GABA_AR$ α4 subunit in the ENS of the mouse colon and the pharmacological effect of activating α4-$GABA_A$Rs on spontaneous colonic longitudinal smooth muscle contractions

(Ai) shows the association of α4 subunit immunoreactive clusters (red) with NOS-immunopositive neurons (blue) of the myenteric plexus. (Aii) shows the association of α4 subunit immunoreactive clusters (red) with c-Kit immunopositive profiles (green) in the same field of view. (Aiii) is a magnified view of the boxed areas in (Ai, ii) demonstrating that α4 subunit immunoreactive clusters decorate the surfaces of NOS-immunopositive somata and dendrites as well as c-Kit immunopositive processes. (B1) shows that α4 subunit immunoreactive clusters are located in the close vicinity of Chat-immunopositive varicosities in the myenteric plexus. Immunoreactivity for the α4 subunit was also detectable on the somato-dendritic domains of (B2) 5HT- and (B3) CRH-immunopositive myenteric plexus neurons. (C1) representative trace demonstrating the effect of THIP 10 µM on spontaneous

contractions in a piece of isolated mouse colon in the absence and presence of the muscarinic cholinergic receptor antagonist atropine 1 µM. Quantification of the effects of THIP, before and after the co-application of atropine on (C2) the force and (C3) the frequency of spontaneous colonic contractions. (D1) representative trace demonstrating the effect of THIP 10 µM on spontaneous contractions in a piece of isolated mouse colon in the absence and presence of the nitric oxide synthase inhibitor L-NAME 10 µM. Quantification of the effects of THIP, before and after the co-application of L-NAME on (D2) the force and (D3) the frequency of spontaneous colonic contractions. Bars represent means and the lines represent the SD. N = 5 animals, * $P < 0.05$, repeated measures ANOVA with post-hoc Tukey's test. Scale bars: Scale bars: (Ai, ii) 50 µm; (Aiii3) 10 µm; (B) 10 µm; (C1, D1) vertical 0.25 grams, horizontal 2 minutes.

Figure 10

Immunolocalisation of the $GABA_AR$ α5 subunit in the ENS of the mouse colon and the pharmacological effect of activating α5-$GABA_A$Rs on spontaneous colonic longitudinal smooth muscle contractions

(Ai) shows the association of α5 subunit immunoreactive clusters (red) with NOS-immunopositive neurons (blue) of the myenteric plexus. Note the significant number of α5 subunit immunoreactive clusters located towards the centre of the field of view which are not associated with neuronal profiles. (Aii) shows the strong association of α5 subunit immunoreactive clusters (red) with c-Kit immunopositive profiles in the same field of view. (Aiii) is an overlay of (Ai and ii). (B1) shows that α5 subunit immunoreactive clusters are located in the close vicinity of Chat-immunopositive varicosities in the myenteric plexus. Immunoreactivity for the α5 subunit was also

detectable on the somato-dendritic domains of (B2) CRH- and (B3) 5HT-immunopositive myenteric plexus neurons. (C1) representative trace demonstrating the effect of L-655,708 10 µM on spontaneous contractions in a piece of isolated mouse colon in the absence and presence of the muscarinic cholinergic receptor antagonist atropine 1 µM. Quantification of the effects of L-655,708, before and after the co-application of atropine on (C2) the force and (C3) the frequency of spontaneous colonic contractions. (D1) representative trace demonstrating the effect of L-655,708 on spontaneous contractions in a piece of isolated mouse colon in the absence and presence of the nitric oxide synthase inhibitor L-NAME 10 µM. Quantification of the effects of L-655,708, before and after the co-application of L-NAME on (D2) the force and (D3) the frequency of spontaneous colonic contractions. Bars represent means and the lines represent the SD. N = 5 animals, * $P < 0.05$, repeated measures ANOVA with post-hoc Tukey's test. Scale bars (A) 20 µm; (B) 10 µm; (C1, D1) vertical 0.25 grams, horizontal 2 minutes

Figure 11

The effects of $GABA_AR$ activation on the stress induced alterations in the force and frequency of colonic spontaneous contractions

(A) representative traces of the effects of alprazolam 10 µM on the contractile responses of colon tissue obtained from (A1) control and (A2) stress animals. Note in (A2) the stress-induced large amplitude rhythmic baseline contractions (arrows) and the absence of the alprazolam-induced reduction in basal tone of the tissue which is evident in the trace from control tissue (double arrow in A1). (B) quantification of the comparative effects of alprazolam 10 µM on the (B1) force and (B2) frequency of spontaneous contractions in tissue from control and stress

animals. Bars represent means and the lines represent the SD. N = 7 animals. $P < 0.05$, repeated measures ANOVA with post-hoc Tukey's post-hoc test. Scale bars (A) vertical 0.3 grams, horizontal 2.5 minutes

1273 Table 1

1274 Table of RT-PCR primer sequences

Gene	Primer sequence	RT-PCR Product length (bp)	Reference
$GABA_AR$ α1	CCA AGT CTC CTT CTG GCT CAA CA GGG AGG GAA TTT CTG GCA CTG AT	111	(Tan S et al., 2011).
$GABA_AR$ α2	TTA CAG TCC AAG CCG AAT GTC CC ACT TCT GAG GTT GTG TAA GCG TAG C	103	(Tan S et al., 2011).
$GABA_AR$ α3	CAA GAA CCT GGG GAC TTT GTG AA AGC CGA TCC AAG ATT CTA GTG AA	119	(Tan S et al., 2011).
$GABA_AR$ α4	GAG ACT GGT GGA TTT TCC TAT GG GGT CCA GGT GTA GAT CAT CTC ACT	94	(Tan S et al., 2011).
$GABA_AR$ α5	CCC TCC TTG TCT TCT GTA TTT CC TGA TGT TGT CAT TGG TCT CGT CT	99	(Tan S et al., 2011).
$GABA_AR$ α6	TAC AAA GGA AGA TGG GCT ATT ACG ATG GGC AAA GTC AGA GAG	439	(Glassmeier G et al., 1998).
$GABA_AR$ β1	GGG GCT TCT CTC TTT TCC CGT GA GGT GTC TGG TAC CCA GAG TTG GT	334	(Gustincich S et al., 1999).
$GABA_AR$ β2	CAA CTC TGG GTG CCT GAC ACC TA TCC TAA TGC AAC CCG TGC AGC AG	495	(Gustincich S et al., 1999).
$GABA_AR$ β3	GGT TTG CTG CGC TCA GAG CGT AA TAC AGC ACT GTC CCA TCA GGG T	390	(Gustincich S et al., 1999).
$GABA_AR$ γ1	CAG TTT GCA TTT GTA GGG TTA CG AGA CAC CCA GGA AAG AAC CAC TG	165	(Gustincich S et al., 1999).
$GABA_AR$ γ2	GGT GGA GTA TGG CAC CCT GCA TT AGG CGG TAG GGA AGA AGA TCC GA	322	(Gustincich S et al., 1999).
$GABA_AR$ γ3	TGC TCG GTC CAG GAG GGT AGA CTG ATC AGC TGC CTC AAC TGA ATT TTT	592	(Gustincich S et al., 1999).
$GABA_AR$ δ	GAC TAC GTG GGC TCC AAC CTG GA ACT GTG GAG GTG ATG CGG ATG CT	398	(Gustincich S et al., 1999).
$GABA_AR$ ε	CAA TGC GAA GAA CAC TTG GAA GC CTG GCA GCA GCA GCT TCT ATC TT	225	(Gustincich S et al., 1999).
β-actin	AGG CCA ACC GTG AAA AGA TG ACC AGA GGC ATA CAG GGA CAA	101	(Gustincich S et al., 1999).

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1277 Table 2

1278 Details of primary antibodies used in the study

Antibody	Host	Dilution	Source	Specificity/Reference
ChAT	Goat	1:100	Millipore (AB144P)	(Heinze et al., 2007). (Härtig et al., 2007).
c-Kit	Rat	1:250	eBioscience (14-1172)	(Sato et al., 1996). (Torihashi et al., 1995).
CRF	Guinea-pig	1:1000	Peninsula Labs (T-5007)	(Stanic et al., 2010). (Armstrong et al., 2009).
$GABA_AR$ α1	Rabbit	1:5000	Synaptic systems (224203)	(Wisłowska-Stanek et al., 2013). No signal in knockout mouse, this study.
$GABA_AR$ α2	Rabbit	1:1000	Werner Sieghart, antigen sequence α2L amino acids 322-357. R # 28/16 Bleed # 01/10/2002	(Pirker et al., 2000). No signal in knockout mouse, this study.
$GABA_AR$ α3	Guinea-pig	1:3000	Jean-Marc Fritschy, Antigen sequence α3N amino acids 1–15.	(Fritschy and Mohler, 1995). No signal in knockout mouse, this study.
$GABA_AR$ α4	Rabbit	1:500	Werner Sieghart, antigen sequence α4 amino acids 379-421. R # 25/1 Bleed # 19/03/2001	(Pirker et al., 2000). No signal in knockout mouse, this study.
$GABA_AR$ α5	Rabbit	1:1000	Werner Sieghart, antigen sequence α5 amino acids 337-388. R # 34/30 Bleed # 17/12/2007	(Pirker et al., 2000). No signal in knockout mouse, this study.
$GABA_AR$ γ2	Rabbit	1:3000	Synaptic system (224003)	(Fish et al., 2013). No signal in knockout mice, This study.
Kv2.1	Mouse	1:1000	Neuromab (75-014)	Western blot; band at 105-125 kDa. No signal in knockout mice. (Hermanstyne et al., 2010).
Map-2	Chicken	1:500	Avēs Labs (MAP0607)	Expression patterns as shown in previous studies
Neuroligin2	Rabbit	1:1000	Synaptic Systems (129203)	(Chih et al., 2005). (De Jaco et al., 2006).
NOS	Sheep	1:1000	Millipore (AB1529)	(Liu et al., 2008). (Cauli et al., 2004).
Serotonin transporter	Guinea-pig	1:250	Chemicon (AB1772)	(Häring et al., 2007). (Collin et al., 2000).
Somatostatin	Rat	1:500	Millipore (MAB354)	(Tanaka et al., 2011). (Dimitrov and Usdin, 2010).
VGAT	Guinea-pig	1:1000	Synaptic Systems (131004)	(Schock et al., 2012). (Geis et al., 2010).

1279