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Title
$GABA_A$ Receptor Subtypes Regulate Stress-induced Colon Inflammation in Mice

Short Title
$GABA_A$ receptors and colonic inflammation

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Abbreviations
$\alpha3^{-/-}$, $GABA_AR$ subunit α3 gene-deleted mice
CD163, Cluster of Differentiation 163
CRH, corticotrophin releasing hormone
ELS, early life stress
ENS, enteric nervous system
FITC, fluorescein isothiocyanate
GABA, gamma aminobutyric acid
$GABA_ARs$, $GABA_A$ subtype receptors
GIT, gastrointestinal tract
IBD, inflammatory bowel diseases
MPO, myeloperoxidase
qPCR, quantitative Real-Time Polymerase Chain Reaction
RST, restraint stress
TJP-1, tight junction protein 1
TNFα, tumour necrosis factor α

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MS and JDS designed the study
MS, SR and JDS performed experiments
MS analysed the data
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Abstract

Background & Aims: Psychological stress, in early life or adulthood, is a significant risk factor for inflammatory disorders, including inflammatory bowel diseases. However, little is known about the mechanisms by which emotional factors affect the immune system. Gamma-aminobutyric acid type A receptors ($GABA_ARs$) regulate stress and inflammation, but it is not clear whether specific subtypes of $GABA_ARs$ mediate stress-induced gastrointestinal inflammation. We investigated the roles of different $GABA_AR$ subtypes in mouse colon inflammation induced by 2 different forms of psychological stress.

Methods: C57BL/6J mice were exposed to either early-life stress (ELS) and adult mice were exposed to acute-restraint stress; control mice were not exposed to either form of stress. We collected colon tissues and measured contractility using isometric tension recordings; colon inflammation, based on levels of CD163 and TNF mRNA and protein and myeloperoxidase activity; and permeability, based on levels of tight junction protein 1 and occludin mRNA and protein. Mice were given fluorescently labelled dextran orally and systemic absorption was measured. We also performed studies of mice with disruption of the $GABA_AR$ subunit alpha 3 gene (*Gabra3*$^{-/-}$ mice).

Results: Mice exposed to ELS had significantly altered $GABA_AR$-mediated colonic contractility, impaired barrier function, and their colon tissue had increased levels of *Gabra3* mRNA, compared to control mice. Restraint stress led to colon inflammation in C57/BL6J mice but not *Gabra3*$^{-/-}$ mice. Colonic inflammation was induced *in vitro* by an α3-$GABA_AR$ agonist, demonstrating a pro-inflammatory role for this receptor subtype. In contrast, α1/4/5-$GABA_AR$ ligands decreased the expression of colonic inflammatory markers.

Conclusions: We found stress to increase expression of *Gabra3* and induce inflammation in mouse colon, together with impaired barrier function. The *in vitro* pharmacological activation of α3-$GABA_A$Rs recapitulated colonic inflammation, whilst α1/4/5-$GABA_A$R ligands were anti-inflammatory. These proteins might serve as therapeutic targets for treatment of colon inflammation or inflammatory bowel diseases.

KEY WORDS: IBD; alprazolam; THIP; inflammatory response

Introduction

Psychological stress is a risk factor for gastrointestinal (GI) inflammation [1, 2] as well as psychiatric disorders which are often comorbid with inflammatory bowel diseases (IBD), such as anxiety and depression [3]. As such, identifying the molecular machinery that translates such emotional triggers into GI inflammation is a prerequisite for developing effective treatments for stress-associated GI inflammation. Diverse neural pathways cooperate with the body's organ systems to bring about a coordinated stress response using an array of chemical messengers capable of bridging the respective neuro-immune systems [4]. However, the stress response varies according to the duration of the stimulus and the age of the individual. Indeed, stress experienced during one's childhood appears to have the most profound impact on the immune system later in life [5]. Therefore, given the complexity of stressors encountered through life, and the variability of the ensuing stress response, it is important to identify common biological mechanisms for such diverse processes, if we are to address the associated disorders. One such emerging molecular integrator of the stress, nervous and immune systems is the GABA-$GABA_AR$ system [6].

$GABA_ARs$ are integral membrane ion channel complexes composed of 5 subunits. Up to 19 different subunits have been identified within the mammalian nervous system, and are termed α1-6, β1-3, γ1-3, δ, ε, θ, π and ρ1-3 [7]. Over several decades, this subunit diversity has been shown to result in multiple receptor subtypes, which vary according to their anatomical expression, physiological characteristics and pharmacological profiles within the brain [8]. These $GABA_AR$

expression and functional phenotypes within the brain have been shown to be susceptible to stress-induced plasticity [9, 10]. Emerging evidence now points to various $GABA_AR$ subtypes directly associated with neuroinflammation within the brain [11], and inflammatory disorders in peripheral organs, such as asthma [12]. Furthermore, the anti-epileptic drug topiramate, which possesses $GABA_AR$ agonist properties, has been shown to ameliorate the macroscopic and microscopic GI inflammation score in an animal model of IBD [13]. Despite this convincing evidence for a role of $GABA_ARs$ in contributing to stress and inflammatory responses, their roles in IBD are relatively poorly understood, primarily because of our limited understanding of $GABA_AR$ function in the GI immune system.

We have recently demonstrated the expression of various $GABA_AR$ subunits by neurochemically diverse cell-types of the mouse enteric nervous system (ENS) [14]. Furthermore, we showed that different $GABA_AR$ subtypes had contrasting effects on the spontaneous contractility of the mouse colon [14]. Importantly, acute stress altered not only native colonic contractility, but also $GABA_AR$-mediated contractility[14]. This indicates that stress robustly engages the ENS and alters its influence on GI functions. Since the ENS also plays an important role in regulating the local GI immune system, it is reasonable to speculate that this stress-induced change in $GABA_AR$-mediated ENS function could alter ENS-mediated immune function as well. In the current study, we demonstrate that exposing mice to various forms of stress, robustly induces GI inflammation via α3-$GABA_ARs$ whereas α1/4/5-$GABA_ARs$ have an anti-inflammatory role within mouse colon. Thus, this study positions GI $GABA_ARs$ as dynamic bi directional regulators of intestinal inflammation.

Materials and Methods

All procedures involving animal experiments were approved by the Animal Welfare and Ethical Review Board of the University of Portsmouth and were performed by a personal license holder, under a Home Office-issued project licence, in accordance with the Animals (Scientific Procedures) Act, 1986 (UK) and associated procedures. See Supplementary Information (SI) for a detailed description of Materials and Methods.

Animals

For wild-type (WT) mice, the C57BL/6J strain obtained from the University of Portsmouth Bioresource centre was used. In some experiments, $GABA_AR$ α3 subunit gene deleted (*Gabra3*$^{-/-}$) mice and their WT littermates, raised against the C57BL/6J background, were also used [15]. Animals were bred in-house in a temperature and humidity-controlled environment under a 12-hr light/dark cycle, with free access to standard chow and water. Only male mice were used to preclude any confounds from sex hormones or the oestrous cycle.

Early life stress (ELS)

A validated animal model of ELS, which is based on a fragmented mother-pup interaction during the first week of life [16], was used. Briefly, pregnant dams were housed together with male partners and monitored every 12-hr for the birth of pups. The day of birth was termed postnatal day 0 (PND 0). Both control and ELS dams were left undisturbed until PND 2. On PND 2, litters were adjusted to a maximum of eight pups. Only male offspring were used for analyses. Control dams were housed

in standard sawdust bedding and provided with sufficient nesting material (1 square; Nestlets^{R}, Ancare). In the ELS cages, dams were provided with reduced nesting material (2/3 of a square) placed upon a raised, fine-gauge (5 mm) steel mesh platform. The cage floor was covered with a small amount of sawdust to prevent ammonia build-up. All litters were left undisturbed between PND 2 and PND 9. At PND 9, both control and ELS pups were returned to the dams to cages with standard bedding and nesting material. Offspring remained with the dams until weaning at PND 22-23. Once the animals reached adulthood (PND 90), they were used for further molecular and physiological analyses.

Acute stress in adulthood: 1 hour restraint stress

One week prior to the commencement of the stress protocol, the animals were divided into control and stress experimental groups which allowed adaptation to the new cages. Mice aged postnatal day 60 (PND 60) were inserted tail first into a Broome rodent restrainer (Harvard Apparatus # 52-0407) for 1 hour [9]. Immediately after the period of stress, the control and stress animals were killed by cervical dislocation, and GI tissue harvested for functional and molecular analyses.

Isometric tension recordings of the effects of stress and the $GABA_AR$ ligand alprazolam on the force and frequency of spontaneous contractions in isolated mouse colon segments

Detailed descriptions of the recording and analyses are provided in the SI Methods sections. In brief, adult male mice were killed by cervical dislocation and the distal colon was removed and immediately placed in physiological solution warmed to 37 ℃. Approximately 2 cm-long whole colon segments we re mounted in a Harvard

organ bath (10 ml chamber) filled with the physiological solution (37 ℃) and bubbled with gas containing 95% O_2 and 5% CO_2. The contractile activity for each colon tissue segment was recorded and analysed as described in our previously published protocols [14]. An N value represents one animal and the data are presented as the mean ± SEM.

Immunohistochemistry and confocal microscopy

Mice were anaesthetised with isoflurane and pentobarbitone (1.25 mg/kg of bodyweight; i.p.) and transcardially perfused using a fixative containing 1% w/v paraformaldehyde and 15% v/v saturated picric acid in 0.1 M phosphate buffer (pH 7.4) according to our previously described protocols [17, 18]. Whole-mount preparations of the colon were used for immunohistochemical reactions using cocktails of the following primary antibodies: 1) rabbit anti CD163, 1:250 (Santa Cruz; sc-33560); 2) sheep anti nitric oxide synthase, 1:1000 (Millipore; AB1529), and a mixture of appropriate secondary antibodies conjugated with Alexa Fluor 488 (Invitrogen, Eugene, OR) and indocarbocyanine (Cy3; Jackson ImmunoResearch, West Grove, PA). Sections were examined with a confocal laser-scanning microscope (LSM710; Zeiss, Oberkochen, Germany) according to our previously published protocols [18].

Quantification of CD163-immunopositive cell density

Multiple fields of view were imaged from each piece of tissue and the number of CD163-immunopositive cells were manually counted in each field of view using the Image J software cell count analysis function. The average of all fields of view were calculated for each piece of tissue and was taken as N = 1. One piece of tissue was used per animal.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Adult male mice from control and ELS groups were killed by cervical dislocation and tissue homogenates of the whole colon prepared. RNA was extracted from the samples using an RNeasy mini kit (Qiagen, 74104, Venlo , Netherlands) according to the manufacturer's protocol and then reverse transcribed into cDNA according to our previously published protocols [14]. Quantitative PCR (qPCR) amplification was performed in 96-well plates using FastStart Essential DNA probes master (Roche, Burgess Hill, UK) and run on a LightCycler® 96 System (Roche). See SI for a detailed description of genes investigated and qPCR cycling conditions and analyses.

Fluorescein Isothiocyanate-dextran (FITC-dextran) permeability assay

The effect of ELS on *in vivo* gut permeability was assessed by the oral administration of the permeability marker, FITC-Dextran 3000-5000, and subsequent measurement of its concentration in the systemic circulation, according to previously published protocols [19]. Briefly, mice were fasted for 4 hours prior to oral administration of FITC-dextran (Sigma Aldrich, FD4). Blood was collected 2 hours later and the concentration of FITC-dextran measured using photometric analysis. See SI for a detailed description of the protocol.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed according to manufacturer's protocols to assess the expression of tight junction protein 1 (TJP-1) (Generon, AE14919MO-48), Occludin (Generon, SEC228Mu-48) and TNFα

(Generon, KET7015-48) within colon tissue from control and ELS mice. See SI for a detailed description of the protocol.

Myeloperoxidase activity assay

Myeloperoxidase (MPO) activity assay was performed according to previously published protocols [20]. Briefly, segments of colon tissue were homogenised and centrifuged at 20,000 x g for 15 minutes. The supernatants were removed and analysed for MPO activity in chromogenic reaction with *o*-phenylenediamine (OPD). A 100 µL sample was mixed with an equal amount of substrate buffer (25 mM sodium-citrate, 50 mM sodium-phosphate, 0.45 mg/mL OPD, 0.1% H_2O_2, pH 5.0) and incubated for 30 min at room temperature. Subsequently, the reaction was stopped by the addition of 50 µL of 2 M sulphuric acid and the absorbance was detected at 492 nm. The data were calculated and presented as percentage MPO activity.

$GABA_AR$ pharmacological assays

Adult WT male mice were killed by cervical dislocation, the colons were removed and placed in an organ bath filled with aerated physiological solution as described above. Isolated segments of colon were incubated with various $GABA_AR$ drugs (see SI for details) for 30 minutes. Subsequently, the colons were removed, snap frozen in liquid nitrogen and stored at -80 ^{o} C for further qPCR and MPO activity assay experiments as described above. Control pieces of tissue were treated only with the vehicle. *Gabra3*$^{-/-}$ mice and their WT littermates were used in a subset of experiments.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 7 (GraphPad Inc. La Jolla, CA). Animals were randomly assigned to treatment groups. All results are expressed as mean ± SEM. Statistical comparisons between different animal groups and treatments were assessed using the appropriate statistical tests, indicated in the Results section. A *P* value less than 0.05 was considered statistically significant.

Results

ELS alters colonic contractility and changes the expression and function of the GI $GABA_AR$ system, in adulthood

We recently demonstrated that exposure to acute stress in adulthood alters the spontaneous contractility of the mouse colon [14]. We therefore explored whether prior exposure to stress in early life (ELS) also impacts on colonic contractility, in adulthood. We performed isometric tension recordings of spontaneous longitudinal and circular muscle contractions from isolated segments of whole mouse colon. ELS induced contrasting effects on the spontaneous contractility of longitudinal (Fig. 1 A1) and circular (Fig. 1 B1) muscles within the adult colon. Quantitative analyses confirmed a significantly higher force of longitudinal muscle spontaneous contractions in ELS samples compared to control (P = 0.0018, unpaired Student's t test; N = 10 animals) (Fig. 1 A2). However, there were no significant differences in the frequency of spontaneous contractions (P = 0.1388, unpaired Student's t test; N = 10 animals) (Fig. 1 A3). In contrast, ELS significantly decreased both the force (P = 0.01, unpaired Student's t test; N = 4 animals) (Fig. 1 B2) and frequency (P = 0.0164, unpaired Student's t test; N = 4 animals) (Fig. 1 B3) of circular muscles contractions.

We have also demonstrated that acute stress in adulthood alters the contractile response of the mouse colon to the $GABA_AR$ ligand alprazolam[14]. We therefore explored whether ELS also altered the function and expression of the GI $GABA_AR$ system, in adulthood. While in control tissue, alprazolam significantly decreased the

force of longitudinal muscle contractions, this effect was abolished in tissue from ELS animals ($F_{DFn,\ DFd\ (1.568,\ 14.11)}$ = 18.3; *P* = 0.0002; Repeated measures ANOVA (RMA), with Tukey's post hoc test; N = 10 animals) (Fig. 1 A2). Furthermore, whilst alprazolam significantly increased the frequency of spontaneous colonic contractions in tissue from control animals, this effect was also abolished following ELS ($F_{DFn,\ DFd\ (2.29,\ 20.61)}$ = 40.65; *P* < 0.000, RMA with Tukey's post hoc test; N = 10 animals) (Fig. 1 A3). In a similar manner, alprazolam significantly decreased the force of circular muscle contractions in control but not ELS tissue ($F_{DFn,\ DFd\ (1.217,\ 3.652)}$ = 21.5; *P* = 0.0113; RMA, with Tukey's post hoc test; N = 4 animals) (Fig. 1 B2) as well as their frequency ($F_{DFn,\ DFd\ (1.718,\ 5.154)}$ = 16.09; *P* = 0.0113; RMA, with Tukey's post hoc test; N = 4 animals) (Fig. 1 B3). Apart from its effects on the kinetics of spontaneous contractions, alprazolam has also been shown to reduce the basal tone of the colon. ELS significantly enhanced this effect of alprazolam, compared to control samples (*P* = 0.003, unpaired Student's *t* test; N = 10 animals) (Fig. 1 A4).

Alprazolam is a benzodiazepine with a broad affinity for $GABA_AR$ subtypes. Therefore, its altered response could be due to changes in the expression of α1-3/5-γ2 subunit containing $GABA_ARs$, resulting from ELS. qPCR analyses demonstrated that ELS results in a significant increase in the mRNA expression of the $GABA_AR$ α3 subunit, with no other $GABA_AR$ α or γ subunits altered (*P* = 0.0004; unpaired Student's *t* test; N = 13 animals) (Fig. 1 C). Collectively, the data demonstrate that ELS imparts enduring changes in native colonic contractility and GI α3-$GABA_ARs$.

ELS induces colonic inflammation in adulthood

Changes in GI motility are known to accompany IBD [21]. Considering the altered colonic contractility induced by ELS, we next investigated whether this experience also induces colonic inflammation, in adulthood. Immunohistochemistry revealed a significant increase in the number of CD163-immunoreactive cells within the ENS of tissue from ELS mice (Fig. 2 A). CD163 is a monocyte and M2 type macrophage-specific protein. Its upregulation constitutes one of the principal changes when macrophages switch to an activated phenotype following inflammation [22]. Importantly, elevation of CD163 is a pathological hallmark of IBD [23, 24]. Quantification of the density of CD163-immunopositive cells confirmed a significant increase in tissue from ELS animals (P = 0.0001; unpaired Student's t test; N = 7 animals) (Fig. 2 B). This ELS-induced increase in CD163 was consistent at the mRNA level as well (P = 0.0088; unpaired Student's t test; N = 6 animals) (Fig. 2 B). Further evidence of robust ELS-induced colonic inflammation was the significant increase in the expression of one of the main markers of severe GI inflammation, namely the inflammatory cytokine tumour necrosis factor α (TNFα), at both the mRNA (P = 0.0010; unpaired Student's t test; N = 6 animals) and protein levels (P = 0.0021; unpaired Student's T test; N = 9 animals) (Fig. 2 C). Finally, ELS significantly increased the activity of the enzyme myeloperoxidase which is a marker of sterile inflammation as well as inflammation associated with autoimmune diseases [25] (P = 0.0001; unpaired Student's t test; N = 11 animals) (Fig. 2 D).

Since GI inflammation also impairs GI barrier permeability, we investigated whether ELS alters the expression of key proteins associated with GI mucosal barrier function. ELS significantly reduced the expression of tight junction protein 1 (TJP-1) at both the mRNA (P = 0.0036; unpaired Student's T test; N = 12 animals) and

protein levels (P = 0.020; unpaired Student's T test; N = 8 animals) (Fig. 2 E). TJP-1 is a tight junction-associated protein important in regulating GI permeability [26]. Further evidence for ELS increasing GI permeability was the significant reduction in the tight junction protein, occludin at the mRNA level (P = 0.0064; unpaired Student's t test; N = 11 animals). However, this change did not translate to the protein level (P = 0.1056; unpaired Student's t test; N = 11 animals) (Fig. 2 F). Occludin is an integral plasma-membrane protein located at the tight junctions [27] with changes in its expression evident in various inflammatory GI disorders [28]. Finally, ELS-induced impairment in intestinal barrier permeability was confirmed, *in vivo*, by measuring the systemic concentration of orally-administered FITC-dextran [19]. Indeed, ELS subjects showed an approximately 70% increase in FITC-dextran detected in their blood plasma (P = 0.0042; unpaired Student's t test; N = 8 animals) (Fig. 2 G).

Collectively, these data demonstrate that significant colonic inflammation and increased GI permeability accompanies the ELS-induced changes in colonic contractility and the local $GABA_AR$ system.

Acute (1 hour) restraint stress in adulthood induces colonic inflammation

Prior evidence indicates that ELS results in an enduring chronic hyper-stress phenotype in adulthood, evidenced by elevated basal blood levels of the stress hormone corticosterone [16]. It is therefore unsurprising that such a severe stress phenotype is associated with inflammation. Since our previous data demonstrated that acute stress in adulthood also altered spontaneous colonic contractility and the effect of alprazolam on such contractions, we investigated whether only a single exposure to 1 hour of restraint stress (RST) in adulthood also induces a GI

inflammatory response. Remarkably, this short exposure to stress significantly increased CD163 expression within the ENS of the colon (Fig. 3 A), both at the cellular level (P = 0.0001; unpaired Student's t test; N = 4 animals) (Fig. 3 B1) and the mRNA level (P = 0.0085; unpaired Student's t test; N = 8 animals) (Fig. 3 B2). Further evidence of a stress-induced inflammatory response was the significant increase in myeloperoxidase activity in samples from RST animals (P = 0.0026; unpaired Student's t test; N = 8 animals) (Fig. 3 B3). In contrast to ELS, RST did not significantly alter the expression of TNFα (data not shown).

The genetic deletion of the $GABA_AR$ alpha 3 subunit prevents stress-induced colonic inflammation

Since the various forms of stress not only robustly induce GI inflammation but also engage the local $GABA_AR$ system, we investigated whether $GABA_ARs$ could be involved in stress-induced GI inflammation, focussing on α3-$GABA_ARs$ because of the significant changes in their expression resulting from prior stress (Fig. 1 B). We exposed *Gabra3*$^{-/-}$ mice [15] to acute RST and assessed the degree of inflammation, compared to control *Gabra3*$^{-/-}$ mice. The absence of the α3 subunit gene prevented the stress-induced colonic inflammation (Fig. 4 A). This was confirmed by the absence of any significant differences in: 1) the density of CD163-immunopositive cells within the ENS (P = 0.8664; unpaired Student's t test; N = 5 animals) (Fig. 4 B1); 2) CD163 mRNA expression (P = 0.7720; unpaired Student's t test; N = 10 animals) (Fig. 4 B2); 3) TNFα mRNA expression (P = 0.1733; unpaired Student's t test; N = 10 animals) (Fig. 4 B3) and; 4) myeloperoxidase activity (P = 0.8319; unpaired Student's t test; N = 6 animals) (Fig. 4 B4). Collectively, the data suggest that stress-induced colonic inflammation is mediated by α3-$GABA_ARs$.

The pharmacological activation of α3-GABA$_A$Rs induces colonic inflammation

Since the absence of α3-GABA$_A$Rs prevented stress-induced colonic inflammation, we investigated whether the activation of α3-GABA$_A$Rs *per se* induces colonic inflammation, using the GABA$_A$R ligand TP003. TP003 has been reported to be an α3-GABA$_A$R-selective positive allosteric modulator [29], although this specificity has been called into question [30]. The application of TP003 to isolated segments of WT mouse colon, maintained in an organ bath for 30 minutes, induced significant inflammation, evidenced by the significant increase in CD163 mRNA expression (P = 0.0009; unpaired Student's t test; N = 8 animals) (Fig. 5 A1) and myeloperoxidase activity (P = 0.0001; unpaired Student's t test; N = 8 animals) (Fig. 5 B1). However, TP003 did not significantly alter the mRNA expression of TNFα (P = 0.7143; unpaired Student's t test; N = 8 animals) (Fig. 5 C1). As the α3-selectivity of TP003 has been questioned [30], we confirmed the dependence of the observed effects by testing TP003 in *Gabra3*$^{-/-}$ mouse samples (Figs. 5 A2, B2 and C2).

The pharmacological activation of α1/4/5-GABA$_A$R subtypes has an anti-inflammatory effect on the colon in vitro

We have previously shown that different GABA$_A$R subtypes have vastly contrasting effects on colonic contractility [14]. Given the pro-inflammatory effects of α3-GABA$_A$Rs demonstrated above, we investigated whether other receptor subtypes might also have contrasting neuro-immune effects on the GIT. With a view to enhancing relevance for patient benefit, we selected a clinically available GABA$_A$R ligand alprazolam. Alprazolam is a high potency benzodiazepine clinically prescribed

mainly for anxiety, a condition which is often associated with experience to psychosocial stress as well as GI co-morbidities. Remarkably, the application of alprazolam to isolated segments of WT mouse colon, maintained in an organ bath for 30 minutes, decreased basal inflammation, evidenced by the significant decrease in: 1) CD163 mRNA (P = 0.01; unpaired Student's t test; N = 12 animals); 2) TNFα mRNA (P = 0.001; unpaired Student's t test; N = 12 animals) and; 3) myeloperoxidase activity (P = 0.002; unpaired Student's t test; N = 12 animals) (Fig. 6 A). Given the non-selectivity of alprazolam for individual $GABA_AR$ subtypes, we explored the potential anti-inflammatory effects of α1-$GABA_AR$, α4-$GABA_AR$ and α5-$GABA_AR$ subtypes, using the subunit preferring ligands zolpidem (100 nM) [31], THIP[32] and L-655,708 [33]respectively. Zolpidem, significantly decreased the expression CD163 (P = 0.005; unpaired Student's t test; N = 5 animals), but did not significantly alter TNFα mRNA expression (P = 0.2048; unpaired Student's t test; N = 5 animals) and myeloperoxidase activity (P = 0.4826; unpaired Student's t test; N = 5 animals) (Fig. 6 B). THIP significantly decreased CD163 (P = 0.0082; unpaired Student's t test; N = 5 animals) and TNFα mRNA expression (P = 0.0203; unpaired Student's t test; N = 5 animals) together with myeloperoxidase activity (P = 0.0090; unpaired Student's t test; N = 5 animals) (Fig. 6 C). L-655,708, an inverse agonist selective for the benzodiazepine site at α5-$GABA_ARs$, significantly increased myeloperoxidase activity (P = 0.0074; unpaired Student's t test; N = 5 animals), but not CD163 (P = 0.0761; unpaired Student's t test; N = 5 animals) and TNFα mRNA expression (P = 0.4166; unpaired Student's t test; N = 5 animals) (Fig. 6 D).

This suggests that GI $GABA_AR$ subtypes bi-directionally modulate different inflammatory pathways within the intestinal local neuro-immune system.

Discussion

The data demonstrate that various forms of stress alter colonic contractility and induce robust inflammation of the colon whilst engaging the local $GABA_AR$ system. Genetic and pharmacological evidence indicate that α3-$GABA_ARs$ are central to mediating the pro-inflammatory effects of stress on the colon, whilst the pharmacological activation of α1/4/5-$GABA_ARs$ impart anti-inflammatory effects to varying degrees. Collectively, the data position the GI $GABA_AR$ system as a key molecular link between one's psychological state and the local GI immune system.

The current data suggest that the long-term effects of stress on GI function arise from functional changes within this organ itself. Indeed, both ELS (Fig. 1 A) and acute adulthood stress [14] induced enduring changes in the spontaneous contractility of the isolated colon. However, it is notable that different stress models induced varying levels of GI changes, most likely due to their differences in severity. Indeed, this ELS model induces essentially a chronic stress phenotype throughout life, evidenced by the sustained elevated levels of the stress hormone cortisol [16]. In contrast, effects of acute restraint stress are most likely transient. Such patterns of contractility arise entirely from the interaction of ENS neurons with interstitial cells of Cajal [34] and intestinal smooth muscle, and is devoid of any central nervous system input. Furthermore, ELS induced long-lasting changes in the expression of only one particular $GABA_AR$ subunit, which is the α3 subunit. We have previously demonstrated that within the mouse colon, $GABA_AR$ subunits are enriched within the ENS[14]. This indicates that psychosocial stress imparts long-lasting changes in specific local neural circuits of the mouse colon. However, the underlying

mechanisms of stress-induced GI inflammation and, its relevance to GI disorders such as IBD, still remains unclear.

It is of course debatable how representative stress-induced GI inflammation is with respect to the main forms of IBD. Furthermore, there are variations in the severity of the levels of stress individuals experience and those used in this study. In terms of ELS, the data suggest that the degree of inflammation induced by this form of stress certainly mimics that associated with ulcerative colitis and Crohn's disease because of the significant increase in the expression of TNFα, independent off the other indices of inflammation (Fig. 2). The importance of TNFα in IBD pathology is underscored by the central role that anti-TNFα agents play in treating these conditions [35]. Although the acute adult stress paradigm used in this study induced a milder form of colonic inflammation since TNFα expression was not altered, it is still notable that a single stressful life event imparts such a significant impact on the GI immune system. It would be useful to examine the GIT of animals exposed to chronic adult stress paradigms such as the social defeat model since this has been shown to induce a robust inflammatory response within one of the main stress circuits of the brain, the dorsal raphe, with the degree of inflammation determining the level of depressive-like behaviour [36]. Aligned with this work, we have also shown that repeated, though mild restraint stress significantly increases the expression of α3-$GABA_A$Rs selectively in this very brain region [9]. Given the strong comorbidity of such mental illnesses and IBD, this suggests a degree of commonality of the pathological pathways engaged by stress signals within the brain and GIT, with $GABA_A$Rs appearing to occupy a prominent role in such processes. As such, $GABA_A$R expression and function could prove to be central to the mediation of the

cascade that results in stress-induced GI inflammation. Thus, identifying the potential biological mechanisms through which stress-related signals alter $GABA_AR$ expression are equally important to identifying the individual receptor subtypes associated with such plasticity.

Emerging evidence points to various $GABA_AR$ subtypes having a direct role in various inflammatory disorders. Indeed, multiple studies suggest direct signalling between inflammatory mediators and CNS $GABA_ARs$ [37-39]. Importantly, $GABA_ARs$ have now been revealed to have an important role in peripheral inflammatory disorders such as asthma [40, 41]. It is therefore surprising that the role of $GABA_ARs$ in major peripheral inflammatory disorders such as IBD, are poorly understood, even though preliminary evidence suggests that some $GABA_AR$ ligands could prove therapeutically useful for such conditions [13]. The current study provides proof of concept that α3-$GABA_ARs$, in particular, play a direct role in mediating stress-induced GI inflammation as the constitutive deletion of the $GABA_AR$ α3 subunit gene abolished the stress-induced colonic inflammation (Fig. 4). The obvious caveat is that α3-$GABA_ARs$ are expressed not only in the ENS, but also in various brain regions which are integral to mediating the stress response, such as the locus coeruleus [17] and dorsal raphe [9]. Therefore, to prove the direct association between colonic α3-$GABA_ARs$ and colonic inflammation, we adopted a pharmacological approach using isolated segments of colon tissue which will not be influenced by any centrally-mediated α3-$GABA_ARs$. Remarkably, 30 minutes exposure of the α3-$GABA_AR$ agonist TP003 induced a robust inflammatory response within the colon. Importantly, we confirmed that this effect was mediated via α3-$GABA_ARs$ as TP003 failed to have any such effects in $\alpha3^{-/-}$ mice (Fig. 5). This indicates that the mere

activation of α3-$GABA_A$Rs, whether via stress or any other trigger, engages the GI immune system, and results in a local inflammatory response. In contrast, the benzodiazepine alprazolam which is a widely clinically used $GABA_A$R drug, induced the opposite effects (Fig. 6). Furthermore, other $GABA_A$R subtype preferring ligands also showed significant anti-inflammatory effects, although to varying degrees. THIP, which is strongly associated with α4-$GABA_A$R modulation [32], showed the most robust anti-inflammatory effects. These data are closely aligned with previous reports of an anti-inflammatory role for α4-$GABA_A$Rs in other organs such as lung [40]. This positions various $GABA_A$R subtypes as dynamic regulators of the local GI immune system, being capable of maintaining the balance of inflammation, and therefore as potential targets for treating IBD. The targeting of GI $GABA_A$Rs could provide new opportunities for $GABA_A$R compounds that have been discarded due to their inability to penetrate the CNS as well as repurposing of clinically available ligands for such peripheral disorders.

In summary, the study reveals the significant involvement of the GI $GABA_A$R system as a major contributor to GI disorders, namely stress-induced inflammation. These data provide the scientific platform for future studies in humans, assessing the association of $GABA_A$Rs in GI samples from various populations of IBD patients whilst developing various $GABA_A$R drug moieties for clinical translational studies.

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Figure Legends

Figure 1

ELS alters colonic contractility and changes the expression and function of the GI $GABA_AR$ system, in adulthood

(A1) and (B1) show representative traces of the effects of ELS on the spontaneous contractility of longitudinal and circular muscles, respectively, in the mouse colon, and the effects of the benzodiazepine alprazolam..

Quantification of the comparative effects of alprazolam 10 µM on the (A2) and (B2) force, and (A3) and (B3) frequency of spontaneous longitudinal and circular muscle contractions, respectively, in tissue from control and ELS animals.

(A4) quantification of the effects of alprazolam 10 µM on the colonic baseline.

(C) Quantification of the effect of prior ELS on the mRNA expression of the $GABA_AR$ α1-5 and γ2 subunits in adulthood, relative to the house-keeping gene Gapdh.

The bars represent the means and the error bars represent the SEM. $*P < 0.05$, using repeated measures ANOVA with Tukey's post hoc test for (A2-3; B2-3) and unpaired Student's *t* test for (A4) and (B). Scale bars (A1) vertical 3 mN, horizontal 2.5 minutes; (B1) vertical 10 mN, horizontal 5 minutes.

Figure 2

ELS induces colonic inflammation and impairs barrier permeability in adulthood

(A) immunohistochemical demonstration of ELS-induced inflammation within the ENS of the mouse colon, using immunoreactivity for CD163 (green), a marker of activated monocytes and/or macrophages and nitric oxide synthase (magenta), a

marker of ENS neurons. Note the significant increase in the number CD163-immunopositive profiles in the ELS micrograph.

Quantification of ELS-induced colonic inflammation using (B) the density of CD163-immunoreactive profiles and CD163 mRNA expression, (C) tumour necrosis factor α (TNFα) mRNA and protein expression and (D) myeloperoxidase activity.

Quantification of the effect of ELS on the expression of tight-junction markers associated with GI permeability, namely (E) tight junction protein-1 (TJP-1) mRNA and protein, (F) occludin mRNA and protein. (G) Quantification of the effect of ELS on the systemic absorption of orally administered FITC-dextran.

The bars represent the means and the error bars represent the SEM. * $P < 0.05$, unpaired Student's *t* test. Scale bar 50 µm.

Figure 3

Acute (1 hour) restraint stress (RST) in adulthood induces colonic inflammation

(A) immunohistochemical demonstration of RST-induced inflammation within the ENS of the mouse colon, using immunoreactivity for CD163 (green), and nitric oxide synthase (magenta). Note the significant increase in the number CD163-immunopositive profiles in the RST micrograph.

Quantification of RST-induced colonic inflammation using (B1) the density of CD163-immunoreactive profiles, (B2) CD163 mRNA expression, and (B3) myeloperoxidase activity.

The bars represent the means and the error bars represent the SEM. * $P < 0.05$, unpaired Student's *t* test. Scale bar 50 µm.

Figure 4

The genetic deletion of the $GABA_AR$ α3 subunit prevents stress-induced colonic inflammation

(A) immunohistochemical demonstration of the indistinct levels of inflammation in colon tissue from control $GABA_AR$ α3 subunit gene-deleted mice ($α3^{-/-}$) or $α3^{-/-}$ mice exposed to 1 hour of acute RST, using immunoreactivity for CD163 (green), and nitric oxide synthase (magenta). Note the comparatively similar number of CD163-immunopositive profiles in the representative control and RST micrograph.

Quantification of the expression of various inflammatory markers in control and RST $α3^{-/-}$ mice, namely (B1) the density of CD163-immunoreactive profiles, (B2) CD163 mRNA expression, (B3) TNFα mRNA expression and (B3) myeloperoxidase activity.

The bars represent the means and the error bars represent the SEM. ns = $P > 0.05$, unpaired Student's *t* test. Scale bar 50 µm.

Figure 5

The pharmacological activation of α3-$GABA_A$Rs induces colonic inflammation

Quantification of the effect of the pharmacological activation of $GABA_A$Rs using the α3-$GABA_A$R preferring ligand TP003, on colonic inflammation *in vitro*, using (A1 and A2) CD163 mRNA expression, (B1 and B2) myeloperoxidase activity and (C1 and C2) TNFα mRNA expression in tissue from WT mice and $α3^{-/-}$ mice, respectively.

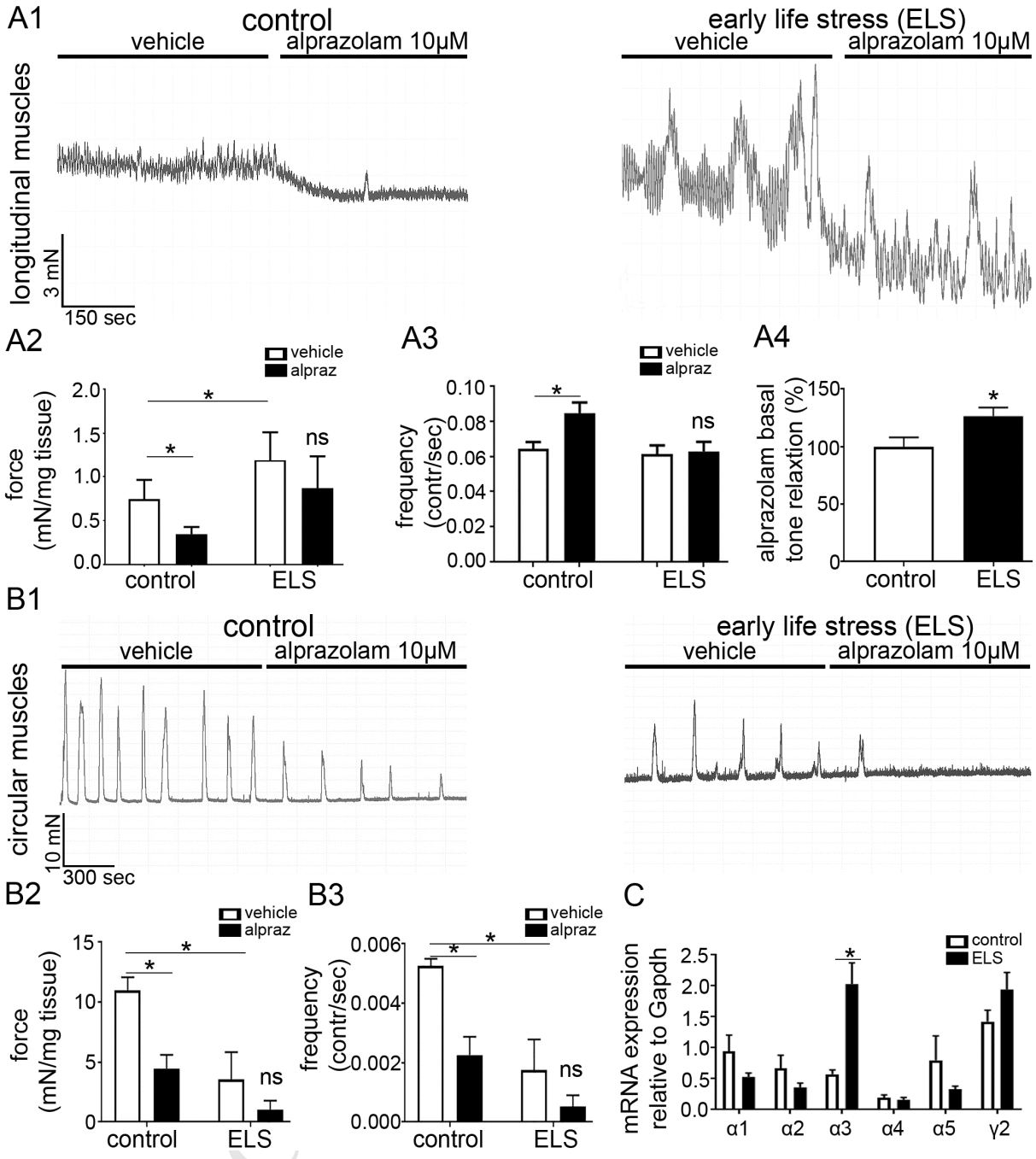
The bars represent the means and the error bars represent the SEM. * $P < 0.05$, unpaired Student's *t* test and ns = $P > 0.05$, unpaired Student's t test.

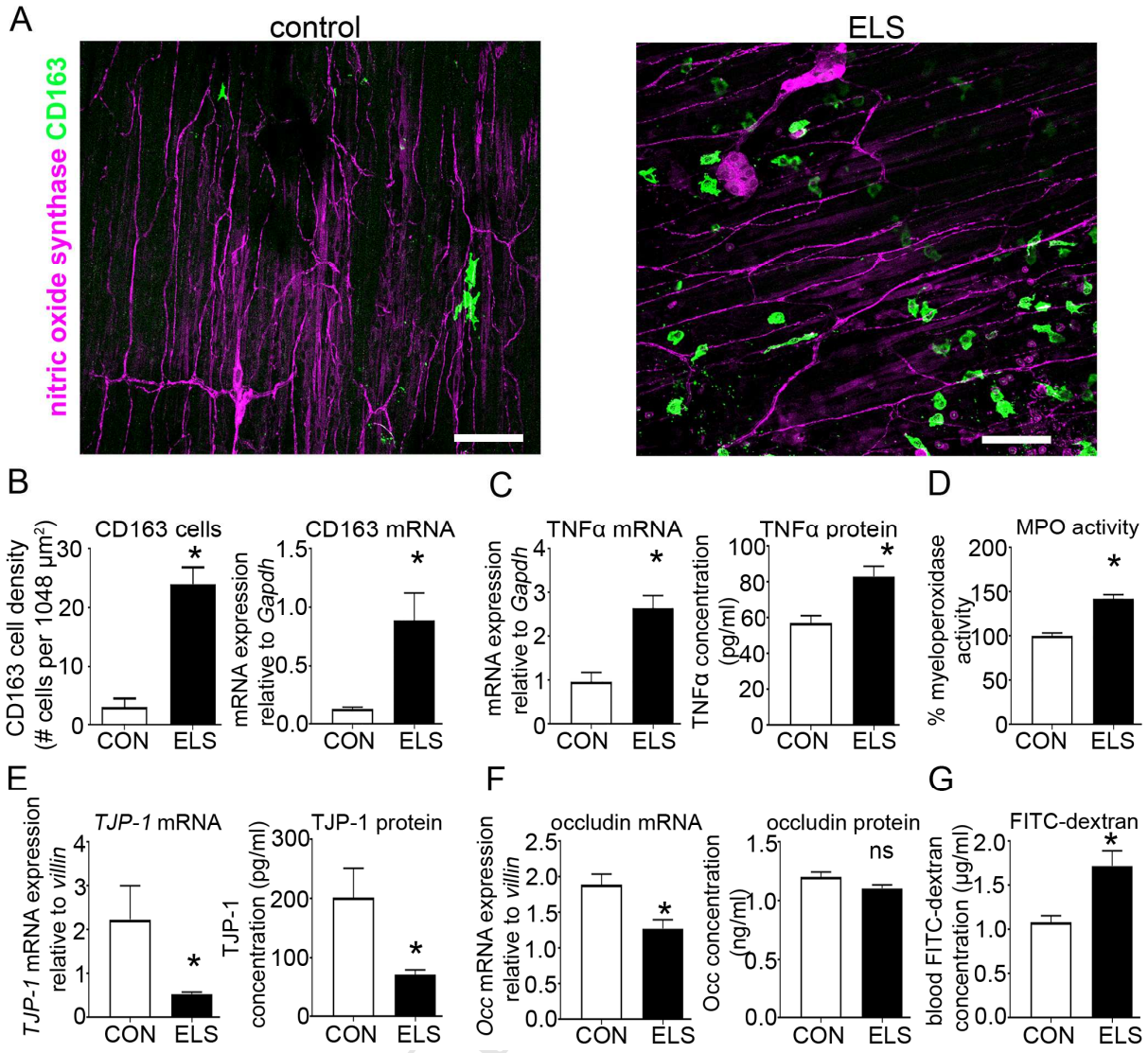
Figure 6

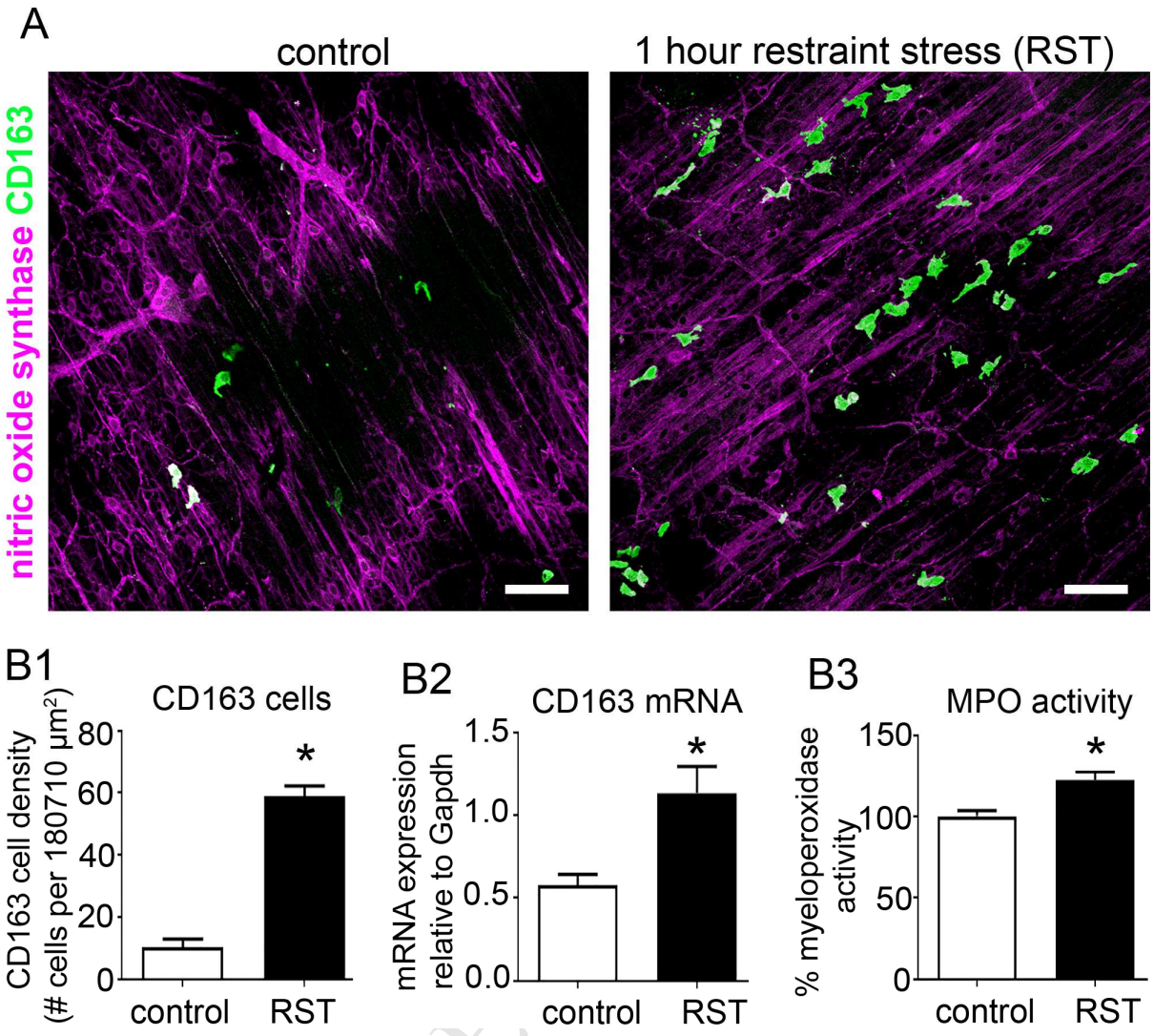
α1/4/5-$GABA_AR$ subtype preferring ligands have varying degrees of anti-inflammatory effects on the colon *in vitro*

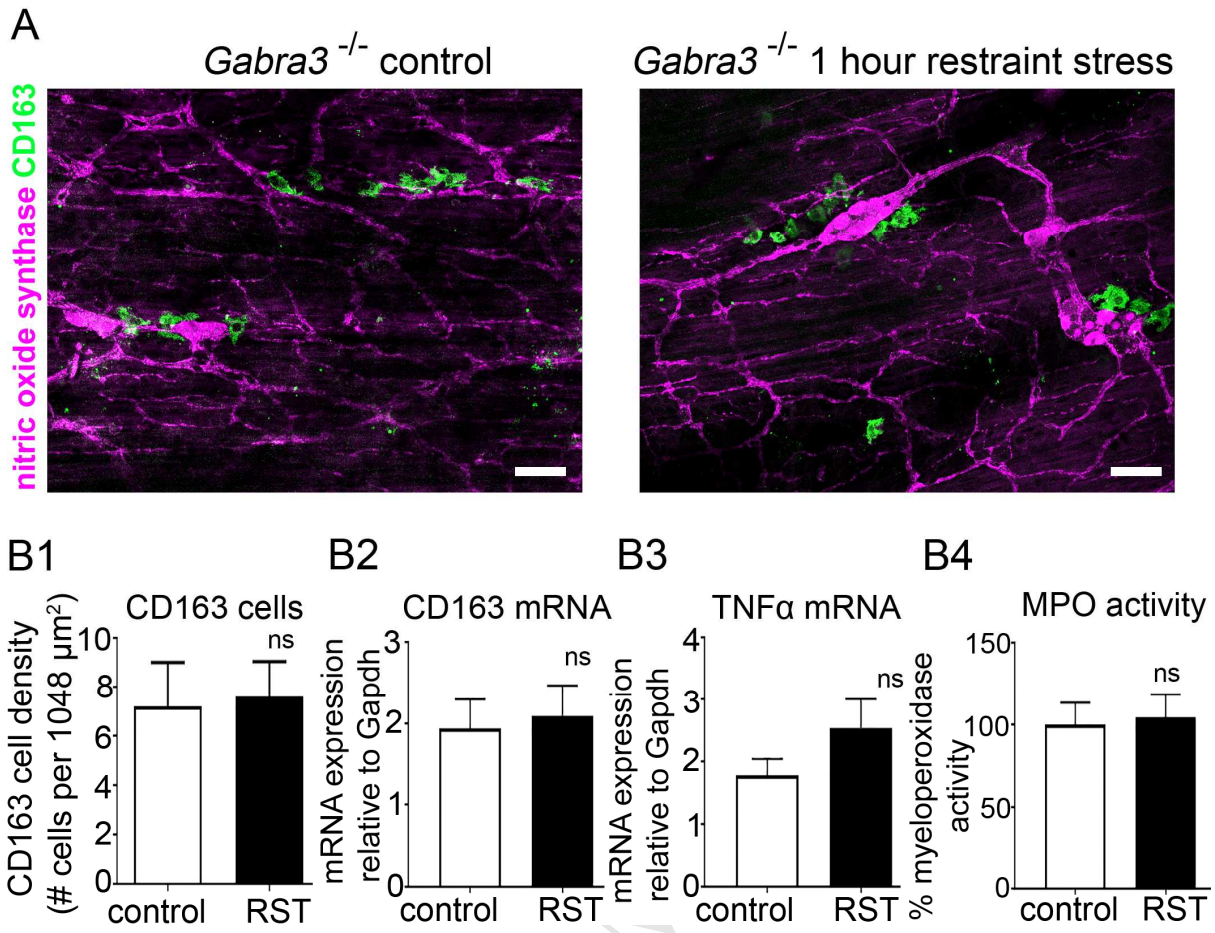
Quantification of the effect of (A) the benzodiazepine alprazolam, (B) zolpidem, which at a concentration of 100 nM is a positive allosteric modulator of α1-$GABA_ARs$, (C) the α4-$GABA_AR$ preferring ligand THIP and (D) the α5-$GABA_AR$ inverse agonist L-655,708 on the expression of CD163 mRNA, TNFα mRNA and myeloperoxidase activity.

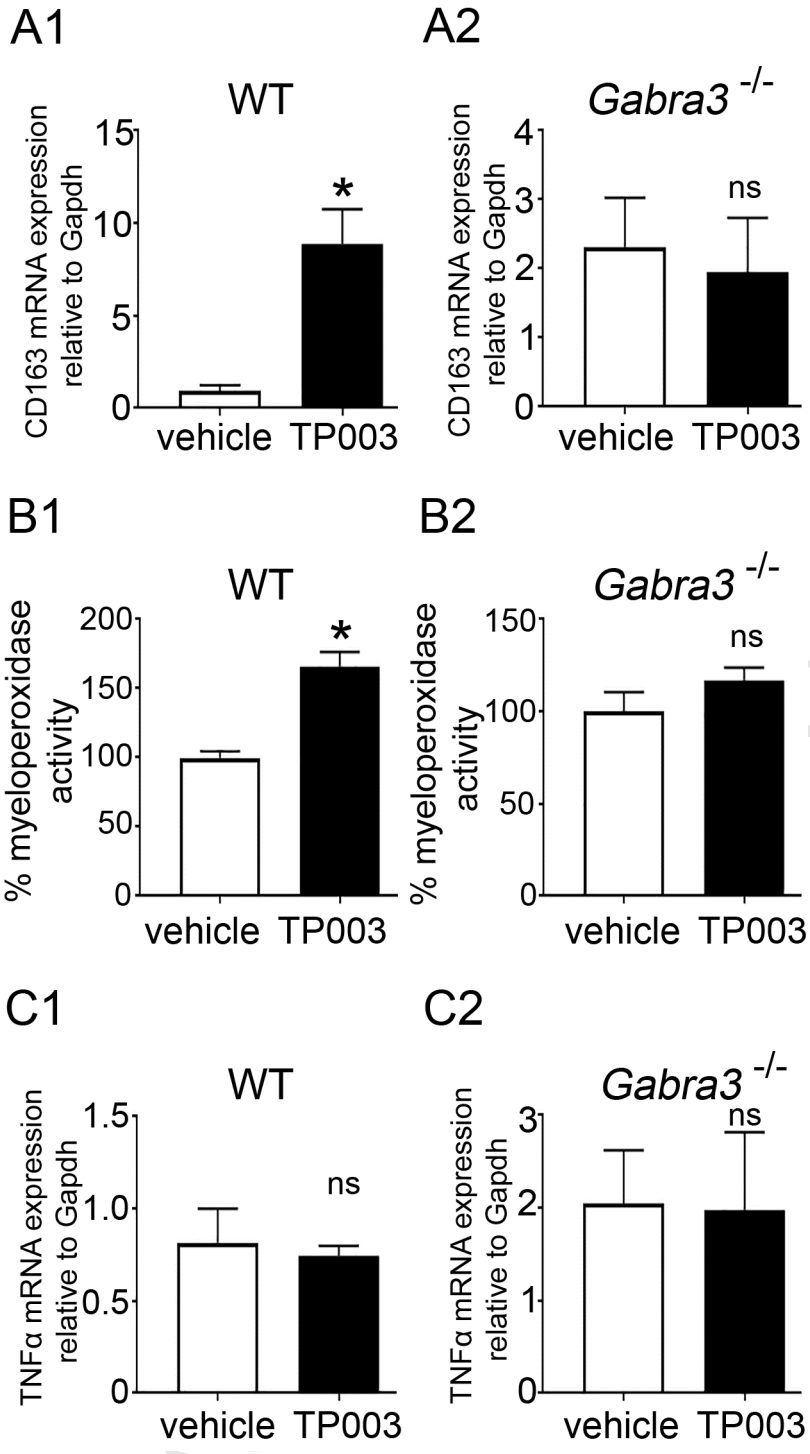
The bars represent the means and the error bars represent the SEM. * $P < 0.05$, unpaired Student's *t* test.


A1
control
vehicle
alprazolam 10μM
early life stress (ELS)
vehicle
alprazolam 10μM
longitudinal muscles
3 mN
150 sec
A2
vehicle
alpraz
force (mN/mg tissue)
2.0
1.5
1.0
0.5
0.0
*
*
ns
control
ELS
A3
vehicle
alpraz
frequency (contr/sec)
0.10
0.08
0.06
0.04
0.02
0.00
*
ns
control
ELS
A4
alprazolam basal tone relaxtion (%)
150
100
50
0
*
control
ELS
B1
control
vehicle
alprazolam 10μM
early life stress (ELS)
vehicle
alprazolam 10μM
circular muscles
10 mN
300 sec
B2
vehicle
alpraz
force (mN/mg tissue)
15
10
5
0
*
*
ns
control
ELS
B3
vehicle
alpraz
frequency (contr/sec)
0.006
0.004
0.002
0.000
*
*
ns
control
ELS
C
control
ELS
mRNA expression relative to Gapdh
2.5
2.0
1.5
1.0
0.5
0.0
*
α1
α2
α3
α4
α5
γ2


A
control
ELS
nitric oxide synthase CD163
B
CD163 cells
CD163 cell density (# cells per 1048 µm²)
CD163 mRNA
mRNA expression relative to Gapdh
C
TNFα mRNA
mRNA expression relative to Gapdh
TNFα protein
TNFα concentration (pg/ml)
D
MPO activity
% myeloperoxidase activity
E
TJP-1 mRNA
TJP-1 mRNA expression relative to villin
TJP-1 protein
TJP-1 concentration (pg/ml)
F
occludin mRNA
Occ mRNA expression relative to villin
occludin protein
Occ concentration (ng/ml)
ns
G
FITC-dextran
blood FITC-dextran concentration (µg/ml)
CON
ELS


A
control
1 hour restraint stress (RST)
nitric oxide synthase CD163
B1
CD163 cells
CD163 cell density
(# cells per 180710 µm²)
80
60
40
20
0
*
control
RST
B2
CD163 mRNA
mRNA expression
relative to Gapdh
1.5
1.0
0.5
0.0
*
control
RST
B3
MPO activity
% myeloperoxidase
activity
150
100
50
0
*
control
RST




A1
WT
CD163 mRNA expression relative to Gapdh
15
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5
0
*
vehicle
TP003
A2
Gabra3 -/-
CD163 mRNA expression relative to Gapdh
4
3
2
1
0
ns
vehicle
TP003
B1
WT
% myeloperoxidase activity
200
150
100
50
0
*
vehicle
TP003
B2
Gabra3 -/-
% myeloperoxidase activity
150
100
50
0
ns
vehicle
TP003
C1
WT
TNFα mRNA expression relative to Gapdh
1.5
1.0
0.5
0
ns
vehicle
TP003
C2
Gabra3 -/-
TNFα mRNA expression relative to Gapdh
3
2
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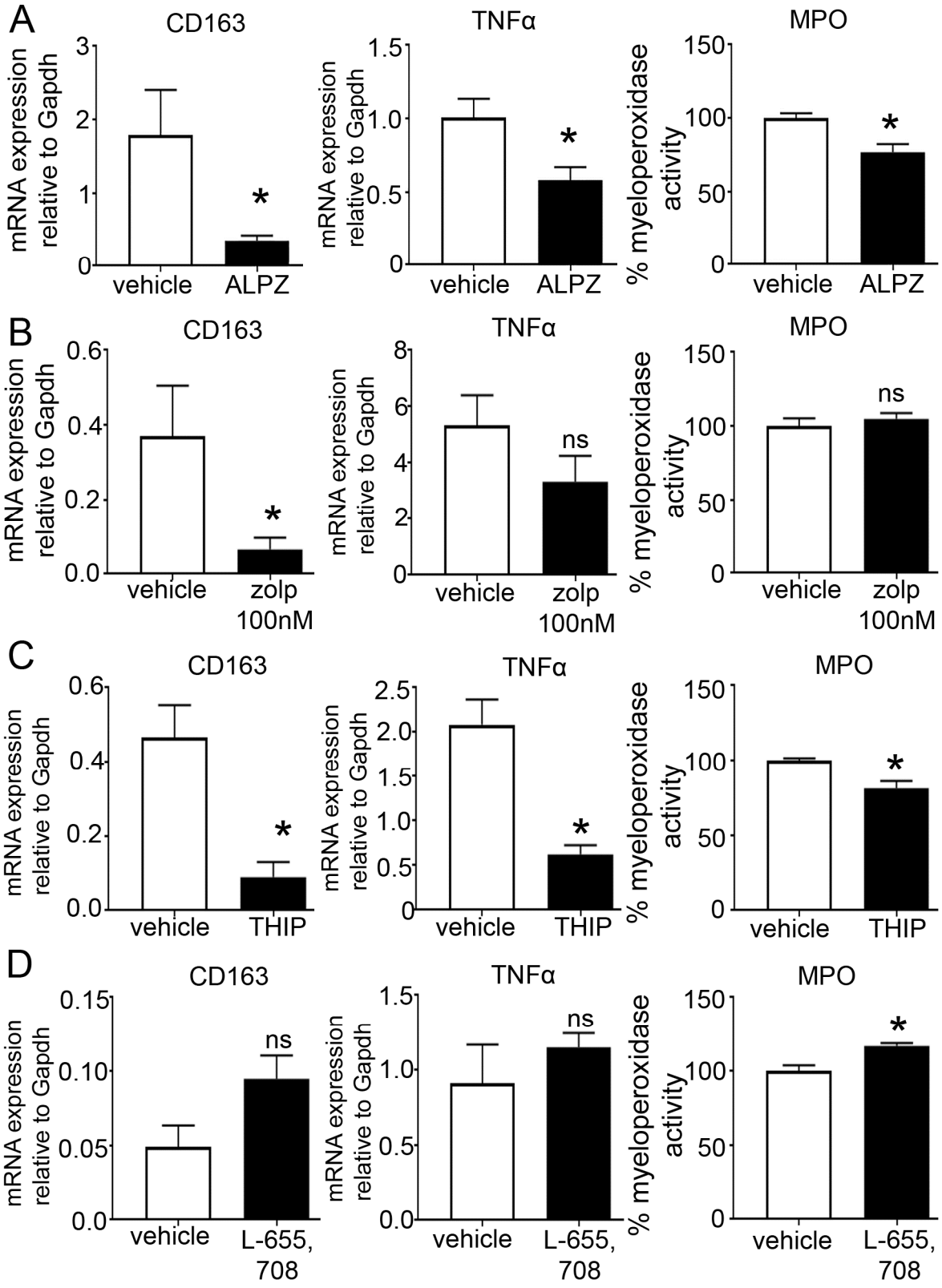
vehicle
TP003


A
CD163
mRNA expression relative to Gapdh
3
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1
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*
vehicle
ALPZ
TNFα
mRNA expression relative to Gapdh
1.5
1.0
0.5
0
*
vehicle
ALPZ
MPO
% myeloperoxidase activity
150
100
50
0
*
vehicle
ALPZ
B
CD163
mRNA expression relative to Gapdh
0.6
0.4
0.2
0.0
*
vehicle
zolp 100nM
TNFα
mRNA expression relative to Gapdh
8
6
4
2
0
ns
vehicle
zolp 100nM
MPO
% myeloperoxidase activity
150
100
50
0
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vehicle
zolp 100nM
C
CD163
mRNA expression relative to Gapdh
0.6
0.4
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0.0
*
vehicle
THIP
TNFα
mRNA expression relative to Gapdh
2.5
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1.0
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vehicle
THIP
MPO
% myeloperoxidase activity
150
100
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vehicle
THIP
D
CD163
mRNA expression relative to Gapdh
0.15
0.10
0.05
0.0
ns
vehicle
L-655, 708
TNFα
mRNA expression relative to Gapdh
1.5
1.0
0.5
0.0
ns
vehicle
L-655, 708
MPO
% myeloperoxidase activity
150
100
50
0
*
vehicle
L-655, 708

Supplementary Information

$GABA_A$ receptor subtypes dynamically regulate stress-induced mouse gastrointestinal inflammation

Mohsen Seifi, Scott Rodaway, Uwe Rudolph, Jerome D Swinny

Materials and Methods

All procedures involving animal experiments were approved by the Animal Welfare and Ethical review body of the University of Portsmouth and were performed by a personal license holder, under a Home Office-issued project licence, in accordance with the Animals (Scientific Procedures) Act, 1986 (UK) and associated procedures.

Acute stress in adulthood: 1 hour restraint stress

One week prior to the commencement of the stress protocol, the animals were divided into control and stress experimental groups which allowed adaptation to the new cages. Mice aged postnatal day 60 (PND 60) were inserted tail first into a Broome rodent restrainer (Harvard Apparatus # 52-0407) for 1 hour. The mice were restrained within the tube but not completely immobilised and thus were able to move slightly backwards and forwards in the tube. During restraint stress, mice were kept in individual cages containing a thin layer of corn cob. Immediately after the period of stress, the control and stress animals were killed by cervical dislocation, and GI tissue harvested for physiological and molecular analyses.

Isometric tension recordings of the effects of stress and the $GABA_AR$ ligand alprazolam on the force and frequency of spontaneous contractions in isolated mouse colon segments

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 37℃. In a subset of experiment $\alpha3^{-/-}$ mice and their WT littermates were used. The intraluminal contents were removed by gently flushing the colon with the physiological solution. In order to measure longitudinal and circular muscle contractions, approximately 2 cm-long whole segments of colon were mounted vertically and horizontally respectively, in a Harvard organ bath (10 ml chamber) filled with the physiological solution (37^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. At this stage, in order to assess the noise produced by the electrical equipment and as an experimental control, a long piece of cotton was tied to the tissue hook placed in an aerated organ bath at one end and the other end was passed through the transducer which picked up any movement in the piece of cotton due to noise. This was represented on the computer as a trace with peaks up to maximum of 0.02 grams of tension. Therefore, in any subsequent analysis of contractile activities produced by pieces of colon, any peak less than 0.02 grams of

force was disregarded in order to produce accurate account for the force and frequency of spontaneous contractions. 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 calculated on LabChart Reader software by measuring the difference between the baseline and the peak of every individual contraction. This was done for the all the contractions before and after the drug additions and the average for that animal was determined. The mean value for each animal was then normalised against the weight of the tissue used in the experiment. A mean value for the individual averages was then obtained for each drug. Thus, an N value represents one animal and the data are presented as the mean ± SEM.

Immunohistochemistry and confocal microscopy

Mice were anaesthetised with isoflurane and pentobarbitone (1.25 mg/kg of bodyweight; i.p.) and transcardially perfused using a fixative containing 1% w/v paraformaldehyde and 15% v/v saturated picric acid in 0.1 M phosphate buffer (pH 7.4) according to previously described protocols [2]. After perfusion, the colons were removed and post-fixed in the same fixative over night at 4^{o} C. The next day, tissue was washed in 0.1 M 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, and stored in 0.1 M phosphate buffer containing 0.05% w/v sodium azide. Non-specific binding of secondary antibodies was blocked by incubating the tissue with 20% v/v normal horse serum for 2 hours at room temperature. The tissue was incubated with cocktails of the following primary antibodies: 1) rabbit anti CD163, 1:250 (Santa Cruz; sc-33560); 2) sheep anti nitric oxide synthase, 1:1000 (Millipore; AB1529), diluted in Tris buffer saline containing 0.3% w/v Triton X-100 (TBS-Tx) and 20% v/v normal horse serum, overnight at 4℃. 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) and indocarbocyanine (Cy3; 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. 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 (Adobe Systems, San José , CA). Only brightness and contrast were adjusted for the whole frame, and no part of a frame was enhanced or modified in any way.

Quantification of CD163-immunopositive cell density

Multiple fields of view were imaged from each piece of tissue and the number of CD163-immunopositive cells were manually counted in each field of view using the Image J software cell count analysis function. The average of all fields of view were calculated for each piece of tissue and it was taken as N = 1. One piece of tissue was used per animal.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Adult male mice from control and ELS groups were killed by cervical dislocation and tissue homogenates of the whole colon prepared. RNA was extracted from the samples using an RNeasy mini kit (Qiagen, 74104) according to the manufacturer's protocol. Equal amount of RNA from each tissue was reverse-transcribed into first-stand cDNA in the following reaction: 2 µl of reverse transcription buffer (BioLabs), 1 µl of oligo(dT)s (ThermoFisher Scientific), 1µ DNTPs (ThermoFisher Scientific), 0.5 µl of M-Mulv reverse transcriptase (Applied Biosystems) and 0.5 µl of RiboLock RNAse Inhibitor (ThermoFisher Scientific). Quantitative PCR (qPCR) amplification was performed in 96-well plates in a mastermix for probes (Roche, Burgess Hill, UK) and run on a LightCycler® 96 System (Roche). The qPCR amplifications for the mouse *Gabra1* (assay ID: Mm00439046_m1), *Gabra2* (assay ID: Mm00433435_m1), *Gabra3* (assay ID: Mm01294271_m1), *Gabra4* (assay ID: Mm00802631_m1), *Gabra5* (assay ID: Mm00621092_m1), *Gabrg2* (assay ID: Mm00433489_m1), *CD163* (assay ID: Mm00474091_m1), *TNFα* (assay ID: Mm00443258_m1), *ZO-1* (assay ID: Mm00493699_m1) and *Occludin* (Mm00500912_m1) genes were performed using pre-designed Taqman primers/probes purchased from Life Technologies (ThermoFisher scientific). *Gapdh*

(assay ID: Mm99999915_g1) and Villin (assay ID: Mm00494146_m1) gene expression was used as the housekeeping gene in various reactions. The qPCR cycling conditions entailed 95℃ for 10 mins and 40 cycles of 95℃ for 15 sec and 60℃ for 60 seconds (LightCycler® 96 System, Roche). Standard curves were generated for each gene using serial dilutions of a known amount of mRNA extracted from each organ which were then reverse transcribed into cDNA. Each measurement was performed in duplicate and each Ct value was then converted into ng mRNA using linear regression analysis of the standard curve (Microsoft Excel). Each ng mRNA value was then normalised against the ng housekeeping gene level within the same sample and the mean mRNA levels for every sample was finally calculated and compared across all experimental groups.

Fluorescein Isothiocyanate-Dextran (FITC-Dextran) permeability assay

FITC-Dextran permeability assay is widely used in rodents to assess the integrity of the intestinal barrier function [3]. FITC-dextran test was performed according to published protocols [4]. Briefly, mice were fasted for 4 hours prior to the start of the experiments with free access to drinking water. Subsequently, FITC-dextran (Sigma Aldrich, FD4) was administered to mice orally at a concentration of 600 mg/kg of body weight and a volume of 250 µl. Mice were then returned to their cages without access to food. Two hours after oral administration of FITC-dextran, the mice were rendered unconscious with increasing concentration of carbon dioxide and blood was collected from each mouse by cardiac puncture and placed in heparinized blood collection tubes. Blood samples were immediately centrifuged at (10 min, 12,000g, 4 ^{0}C), and the plasma was light protected and stored at -80 C for photometric analysis. Subsequently, each plasma sample was diluted in an equal volume of phosphate-

buffered saline (PBS, pH 7.4). Standards (range 50–0.01 µg/ml) were obtained by diluting appropriate amount of FITC-dextran in PBS. An amount of 100 µl of both diluted animal samples and standards, as well as blanks (PBS and diluted plasma from untreated animals), were transferred to black 96-well microplates (Greiner Bio-one, Frickenhausen, Germany). Analysis for the FITC-dextran concentration was carried out with a fluorescence spectrophotometer, at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Finally, a standard curve was blotted using the range of standards used and the concentration of FITC-dextran in each plasma sample was extrapolated from this standard curve.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed to assess the expression of tight junction protein 1 (Generon, AE14919MO-48), Occludin (Generon, SEC228Mu-48) and TNFα (Generon, KET7015-48) within colon tissue from control and ELS mice. Briefly, total protein extracts were obtained from fresh colon tissue from control and ELS mice, using lysis buffer composed of 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, 1 mM EDTA, 10 mM Na4P2O7 and a cocktail of protease inhibitors, pH 8.0. Subsequently, a 100 µl volume of each sample containing equal amount of total protein were used in each ELISA according to the manufacturer's protocol.

Drugs

The following drugs were used in this study: zolpidem (Tocris Biosciences, 0655), alprazolam (Sigma Aldrich, A8800), TP003 (Tocris Biosciences, 4414), THIP hydrochloride (Tocris Biosciences, 0807), L-655, 708 (Tocris Biosciences, 1327).

Apart from THIP hydrochloride which was dissolved in distilled water, all other drugs were dissolved in DMSO.

References

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