GABA<sub>A</sub> Receptor Subtypes Regulate Stress-Induced Colon Inflammation in Mice

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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. γ-Aminobutyric acid type A receptors (GABA<sub>A</sub>Rs) regulate stress and inflammation, but it is not clear whether specific subtypes of GABA<sub>A</sub>Rs mediate stress-induced gastrointestinal inflammation. We investigated the roles of different GABA<sub>A</sub>R subtypes in mouse colon inflammation induced by 2 different forms of psychological stress.

METHODS: C57BL/6J mice were exposed to early-life stress, 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 cluster of differentiation 163 and tumor necrosis factor messenger RNA (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 labeled dextran orally and systemic absorption was measured. We also performed studies of mice with disruption of the GABA<sub>A</sub>R subunit α3 gene (Gabra3<sup>−/−</sup> mice).

RESULTS: Mice exposed to early-life stress had significantly altered GABA<sub>A</sub>R-mediated colonic contractility and impaired barrier function, and their colonic tissue had increased levels of Gabra3 mRNA compared with control mice. Restraint stress led to colon inflammation in C57/BL6J mice but not Gabra3<sup>−/−</sup> mice. Colonic inflammation was induced in vitro by an α3-GABA<sub>A</sub>R agonist, showing a proinflammatory role for this receptor subtype. In contrast, α1/4/5-GABA<sub>A</sub>R 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 pharmacologic activation of α3-GABA<sub>A</sub>Rs recapitulated colonic inflammation, whereas α1/4/5-GABA<sub>A</sub>R ligands were anti-inflammatory. These proteins might serve as therapeutic targets for treatment of colon inflammation or inflammatory bowel diseases.

Keywords: Alprazolam; IBD; Inflammatory Response; THIP.

Abbreviations used in this paper: α3<sup>−/−</sup>, γ-aminobutyric acid subtype A receptor α3 gene–deleted mice; CD163, cluster of differentiation 163; ELS, early-life stress; ENS, enteric nervous system; FITC, fluorescein isothiocyanate; GABA, γ-aminobutyric acid; GABA<sub>A</sub>R, γ-aminobutyric acid subtype A receptor; GI, gastrointestinal; IBD, inflammatory bowel disease; MPO, myeloperoxidase; mRNA, messenger RNA; PND, postnatal day; qPCR, quantitative real-time polymerase chain reaction; RMA, repeated measures analysis of variance; RST, restraint stress; TNF-α, tumor necrosis factor α; WT, wild type.

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Psychological stress is a risk factor for gastrointestinal (GI) inflammation and psychiatric disorders that are often comorbid with inflammatory bowel diseases (IBDs), such as anxiety and depression. Therefore, 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 neuroimmune systems. 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. Therefore, given the complexity of stressors encountered through life and the variability of the ensuing stress response, we must 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 4 receptor (GABAAR) system.

GABAARs are integral membrane ion channel complexes composed of 5 subunits. Up to 19 different subunits have been identified within the mammalian nervous system, and these are termed GABAARs have an anti-inflammatory role within the colon. Thus, this study positions GABAARs as dynamic bidirectional 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 license, in accordance with the Animals (Scientific Procedures) Act, 1986 (UK) and associated procedures.

See Supplementary Material 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 center was used. In some experiments, GABAAR a3 subunit gene-deleted (Gabra3 −/−) mice and their WT littermates, raised against the C57BL/6J background, were also used. Animals were bred in house in a temperature- and humidity-controlled environment under a 12-hour light/dark cycle, with free access to standard chow and water. Only male mice were used to preclude any confounding from sex hormones or the estrous cycle.

Early-Life Stress
A validated animal model of ELS, which is based on a fragmented mother-pup interaction during the first week of life, was used. Briefly, pregnant dams were housed together with male partners and monitored every 12 hours 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 8 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, Ancare, Bellmore, NY). In the ELS cages, dams were provided with reduced nesting material (two thirds of a square) placed on a raised, fine-gauge (5-mm) steel mesh platform. The cage floor was covered with a small amount of sawdust to prevent ammonia buildup. 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 before 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 PND 60 were inserted tail first into a Broome rodent restrainer (no. 52-0407; Harvard Apparatus, Holliston, MA) for 1 hour. Immediately after the period of stress, the control and stress animals were killed by cervical dislocation, and GI tissue was harvested for functional and molecular analyses.

Isometric Tension Recordings of the Effects of Stress and the GABA<sub>R</sub> 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 Supplementary Material. 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°C. Approximately 2-cm–long colon segments were mounted in an organ bath (Harvard Apparatus) (10-ml chamber) filled with the physiological solution (37°C) and bubbled with gas containing 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The contractile activity for each colon tissue segment was recorded and analyzed as described in our previously published protocols. An n value represents 1 animal, and the data are presented as the mean ± standard error of the mean.

Immunohistochemistry and Confocal Microscopy

Mice were anesthetized with isoflurane and pentobarbital (1.25 mg/kg of body weight, intraperitoneal) and transcardially perfused using a fixative containing 1% weight/volume paraformaldehyde and 15% volume/volume saturated picric acid in 0.1 mol/L phosphate buffer (pH 7.4) according to our previously described protocols. Whole-mount preparations of the colon were used for immunohistochemical reactions using cocktails of the following primary antibodies: (1) rabbit anti CD163, 1:250 (sc-33560; Santa Cruz Biotechnology, Dallas, TX) and (2) sheep anti–nitric oxide synthase, 1:1000 (AB1529; Millipore, Burlington, MA), along with a mixture of appropriate secondary antibodies conjugated with Alexa Fluor 488 (Invitrogen, Eugene, OR) and indocarboxyamine (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.

Quantification of Cluster of Differentiation 163–Immunopositive Cell Density

Multiple fields of view were imaged from each piece of tissue and the number of cluster of differentiation 163 (CD163)–immunopositive cells were manually counted in each field of view using the Image J software (National Institutes of Health, Bethesda, MD) cell count analysis function. The average of all fields of view was 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

Adult male mice from control and ELS groups were killed by cervical dislocation, and tissue homogenates of the whole colon were prepared. RNA was extracted from the samples using an RNeasy mini kit (74104; Qiagen, Venlo, The Netherlands) according to the manufacturer’s protocol and then reverse transcribed into cDNA according to our previously published protocols. Quantitative real-time 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 the Supplementary Material for a detailed description of the protocol.

Fluorescein Isothiocyanate–Dextran Permeability Assay

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

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay was performed according to the manufacturer’s protocols to assess the expression of tight junction protein 1 (AE14919MO-48; Generon, Slough, UK), occludin (SEC228Mu-48; Generon,) and tumor necrosis factor α (TNF-α) (Generon, KET7015-48) in colon tissue from control and ELS mice. See Supplementary Material for a detailed description of the protocol.

Myeloperoxidase Activity Assay

Myeloperoxidase activity assay was performed according to previously published protocols. Briefly, segments of colon tissue were homogenized and centrifuged at 20,000g for 15 minutes. The supernatants were removed and analyzed for myeloperoxidase activity in chromogenic reaction with o-phenylenediamine. A 100-μL sample was mixed with an equal
amount of substrate buffer (25 mmol/L sodium-citrate, 50 mmol/L sodium-phosphate, 0.45 mg/mL o-phenyленediamine, 0.1% H2O2; pH 5.0) and incubated for 30 minutes at room temperature. Subsequently, the reaction was stopped by the addition of 50 μL of 2 mol/L sulfuric acid, and the absorbance was detected at 492 nm. The data were calculated and presented as percentage myeloperoxidase activity.

**GABAAR Pharmacologic Assays**

Adult WT male mice were killed by cervical dislocation, and the colons were removed and placed in an organ bath filled with aerated physiological solution as described earlier. Isolated segments of colon were incubated with various GABAAR drugs (see Supplementary Material for details) for 30 minutes. Subsequently, the colons were removed, snap frozen in liquid nitrogen, and stored at −80°C for further qPCR and myeloperoxidase activity assay experiments, as described. 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, La Jolla, CA). Animals were randomly assigned to treatment groups. All results are expressed as mean ± standard error of the mean. Statistical comparisons between different animal groups and treatments were assessed using the appropriate statistical tests, indicated in the Results section. A P value <.05 was considered statistically significant.

**Results**

**ELS Alters Colonic Contractility and Changes the Expression and Function of the GI GABAAR System in Adulthood**

We recently showed that exposure to acute stress in adulthood alters the spontaneous contractility of the mouse colon. We therefore explored whether prior exposure to ELS also affects 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 (Figure 1A) and circular (Figure 1B) muscles within the adult colon. Quantitative analyses confirmed a significantly higher force of longitudinal muscle spontaneous contractions in ELS samples compared with control (P = .0018, unpaired Student t test; n = 10 animals) (Figure 1Aii). However, there were no significant differences in the frequency of spontaneous contractions (P = .1388, unpaired Student t test; n = 10 animals) (Figure 1Aiii). In contrast, ELS significantly decreased both the force (P = .01, unpaired Student t test; n = 4 animals) (Figure 1Bii) and frequency (P = .0164, unpaired Student t test; n = 4 animals) (Figure 1Biii) of circular muscle contractions.

We have also shown that acute stress in adulthood alters the contractile response of the mouse colon to the GABAAR ligand alprazolam. We therefore explored whether ELS also altered the function and expression of the GI GABAAR system in adulthood. Although in control tissue alprazolam significantly decreased the force of longitudinal muscle contractions, this effect was abolished in tissue from ELS animals (FDFn, DFd (1,568, 14,11) = 18.3, P = .0002, repeated measures analysis of variance (RMA) with Tukey post hoc test; n = 10 animals) (Figure 1Aii). Furthermore, although alprazolam significantly increased the frequency of spontaneous colonic contractions in tissue from control animals, this effect was also abolished after ELS (FDFn, DFd (2,29, 20.63) = 40.65, P < .001, RMA with Tukey post hoc test; n = 10 animals) (Figure 1Aiii). In a similar manner, alprazolam significantly decreased the force of circular muscle contractions in control but not ELS tissue (FDFn, DFd (1,217, 3,652) = 21.5, P = .0113, RMA with Tukey post hoc test; n = 4 animals) (Figure 1Bii) as well as their frequency (FDFn, DFd (1,718, 5,154) = 16.09, P = .0113, RMA with Tukey post hoc test; n = 4 animals) (Figure 1Biii). 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 with control samples (P = .003, unpaired Student t test; n = 10 animals) (Figure 1Aiv).

Alprazolam is a benzodiazepine with a broad affinity for GABAAR subtypes. Therefore, its altered response could be due to changes in the expression of α1-3/5-γ2 subunit-containing GABAARs, resulting from ELS. qPCR analyses showed that ELS results in a significant increase in the messenger RNA (mRNA) expression of the GABAAR α3 subunit, with no other GABAAR α or γ subunits altered (P = .0004, unpaired Student t test; n = 13 animals) (Figure 1C). Collectively, the data show that ELS impacts enduring changes in native colonic contractility and GI α3-GABAARs.

**ELS Induces Colonic Inflammation in Adulthood**

Changes in GI motility are known to accompany IBD. Considering the altered colonic contractility induced by ELS, we next investigated whether this experience also induces colonic inflammation in adulthood. Immunohistochemistry showed a significant increase in the number of CD163-immunoreactive cells within the ENS of tissue from ELS mice (Figure 2A). CD163 is a monocyte and M2 type macrophage-specific protein. Its up-regulation constitutes one of the principal changes when macrophages switch to an activated phenotype after inflammation. Elevation of CD163 is a pathologic hallmark of IBD. Quantification of the density of CD163-immunopositive cells confirmed a significant increase in tissue from ELS animals (P = .0001, unpaired Student t test; n = 7 animals) (Figure 2B). This ELS-induced increase in CD163 was consistent at the mRNA level as well (P = .0088, unpaired Student t test; n = 6 animals) (Figure 2B). 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 TNF-α, at both the mRNA (P = .0010, unpaired Student t test; n = 6 animals) and protein levels (P = .0021; unpaired Student t test; n = 9 animals) (Figure 2C). Finally, ELS significantly increased the activity of the enzyme myeloperoxidase,
Figure 1. ELS alters colonic contractility and changes the expression and function of the GI GABA<sub>A</sub>R system, in adulthood. (Ai and Bi) 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 μmol/L on the (Aii and Bii) force and (Aiii and Biii) frequency of spontaneous longitudinal and circular muscle contractions, respectively, in tissue from control and ELS animals. (Aiv) Quantification of the effects of alprazolam 10 μmol/L on the colonic baseline. (C) Quantification of the effect of prior ELS on the mRNA expression of the GABA<sub>A</sub>R α1–5 and γ2 subunits in adulthood, relative to the housekeeping gene Gapdh. The bars represent the means, and the error bars represent the standard error of the mean. *P < .05, using RMA with (Aii, Aiii, Bii, Biii) Tukey post hoc test and (Aiv, C) unpaired Student t test. Scale bars in Ai: vertical, 3 mN; horizontal, 2.5 minutes. Scale bars in Bi: vertical, 10 mN; horizontal, 5 minutes. M, mol/L; ns, not significant; sec, second.
which is a marker of sterile inflammation and inflammation associated with autoimmune diseases\(^{25}\) \((P = .0001,\) unpaired Student \(t\) test; \(n = 11\) animals) (Figure 2D).

Because 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 at both the mRNA \((P = .0036,\) unpaired Student \(t\) test; \(n = 12\) animals) and protein levels \((P = .020;\) unpaired Student \(t\) test; \(n = 8\) animals) (Figure 2E). Tight junction protein 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 \((P = .0064,\) unpaired Student \(t\) test; \(n = 11\) animals). However, this change did not

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. (B–D) Quantification of ELS-induced colonic inflammation using (B) the density of CD163-immunoreactive profiles and CD163 mRNA expression, and (D) myeloperoxidase activity. (E, F) Quantification of the effect of ELS on the expression of tight junction markers associated with GI permeability, namely (E) tight junction protein 1 mRNA and protein and (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 standard error of the mean. *\(P < .05,\) unpaired Student \(t\) test. Scale bar, 50 \(\mu m.\) CON, control; MPO, myeloperoxidase.
translate to the protein level \( (P = .1056; \text{unpaired Student } t\text{ test}; n = 11 \text{ animals}) \) (Figure 2F). 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 \( (P = .0042; \text{unpaired Student } t\text{ test}; n = 8 \text{ animals}) \) (Figure 2G).

Collectively, these data show that significant colonic inflammation and increased GI permeability accompanies the ELS-induced changes in colonic contractility and the local GABA\(_{A}\)R system.

**Acute (1-Hour) Restraint Stress in Adulthood Induces Colonic Inflammation**

Prior evidence indicates that ELS results in an enduring chronic hyperstress 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. Because our previous data showed 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 \( (P = .0001, \text{unpaired Student } t\text{ test}; n = 4 \text{ animals}) \) (Figure 3A), both at the cellular level \( (P = .0026, \text{unpaired Student } t\text{ test}; n = 8 \text{ animals}) \) (Figure 3Bi). Further evidence of a stress-induced inflammatory response was the significant increase in myeloperoxidase activity in samples from RST animals \( (P = .0026, \text{unpaired Student } t\text{ test}; n = 8 \text{ animals}) \) (Figure 3Biii). In contrast to ELS, RST did not significantly alter the expression of TNF-\(\alpha\) (data not shown).

**The Genetic Deletion of the GABA\(_{A}\)R \(\alpha3\) Subunit Prevents Stress-Induced Colonic Inflammation**

Because the various forms of stress not only robustly induce GI inflammation but also engage the local GABA\(_{A}\)R system, we investigated whether GABA\(_{A}\)Rs could be involved in stress-induced GI inflammation, focusing on \(\alpha3\)-GABA\(_{A}\)Rs because of the significant changes in their expression resulting from prior stress \( (P = .0085, \text{unpaired Student } t\text{ test}; n = 8 \text{ animals}) \) (Figure 3Bii). We exposed \(Gabra3^{-/-}\) mice\(^{15}\) to acute RST and assessed the degree of inflammation compared with control \(Gabra3^{-/-}\) mice. The absence of the \(\alpha3\) subunit gene prevented stress-induced colonic inflammation \( (P = .0001, \text{unpaired Student } t\text{ test}; n = 8 \text{ animals}) \).
confirmed by the absence of any significant differences in (1) the density of CD163-immunopositive cells within the ENS \( (P = .8664, \text{unpaired Student } t \text{ test; } n = 5 \text{ animals}) \) (Figure 4Bi), (2) CD163 mRNA expression \( (P = .7720, \text{unpaired Student } t \text{ test; } n = 10 \text{ animals}) \) (Figure 4Bii), (3) TNF-\( \alpha \) mRNA expression \( (P = .1733, \text{unpaired Student } t \text{ test; } n = 10 \text{ animals}) \) (Figure 4Biii), and (4) myeloperoxidase activity \( (P = .8319, \text{unpaired Student } t \text{ test; } n = 6 \text{ animals}) \) (Figure 4Biv). Collectively, the data suggest that stress-induced colonic inflammation is mediated by \( \alpha 3 \)-GABA\(_A\)Rs.

**The Pharmacological Activation of \( \alpha 3 \)-GABA\(_A\)Rs Induces Colonic Inflammation**

Because the absence of \( \alpha 3 \)-GABA\(_A\)Rs prevented stress-induced colonic inflammation, we investigated whether the activation of \( \alpha 3 \)-GABA\(_A\)Rs per se induces colonic inflammation using the GABA\(_A\)R ligand TP003. TP003 has been reported to be an \( \alpha 3 \)-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 \( (P = .0009, \text{unpaired Student } t \text{ test; } n = 8 \text{ animals}) \) (Figure 5Ai) and myeloperoxidase activity \( (P = .0001, \text{unpaired Student } t \text{ test; } n = 8 \text{ animals}) \) (Figure 5Bi). However, TP003 did not significantly alter the mRNA expression of TNF-\( \alpha \) \( (P = .7143, \text{unpaired Student } t \text{ test; } n = 8 \text{ animals}) \) (Figure 5Ci). Because the \( \alpha 3 \)-selectivity of TP003 has been questioned,\(^ {30}\) we confirmed the dependence of the observed effects by testing TP003 in Gabra3\(^{-/-}\) mouse samples (Figure 5Ai, Bi, and Ci).
The Pharmacologic Activation of α1/4/5-GABA<sub>A</sub>R Subtypes Has an Anti-inflammatory Effect on the Colon in Vitro

We have previously shown that different GABA<sub>A</sub>R subtypes have vastly contrasting effects on colonic contractility. Given the proinflammatory effects of α3-GABA<sub>A</sub>Rs described earlier, we investigated whether other receptor subtypes might also have contrasting neuroimmune effects on the gastrointestinal tract. With a view to enhance relevance for patient benefit, we selected a clinically available GABA<sub>A</sub>R ligand alprazolam. Alprazolam is a high-potency benzodiazepine clinically prescribed mainly for anxiety, a condition that is often associated with experience of 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 = .01, unpaired Student t test; n = 12 animals), (2) TNF-α mRNA (P = .001, unpaired Student t test; n = 12 animals), and (3) myeloperoxidase activity (P = .002, unpaired Student t test; n = 12 animals) (Figure 6A). Given the nonselectivity of alprazolam for individual GABA<sub>A</sub>R subtypes, we explored the potential anti-inflammatory effects of α1-GABA<sub>A</sub>R, α4-GABA<sub>A</sub>R, and α5-GABA<sub>A</sub>R subtypes, using the subunit-prefering ligands zolpidem (100 nmol/L), THIP<sup>31</sup> and L-655,708<sup>32</sup> respectively. Zolpidem significantly decreased the expression CD163 (P = .005, unpaired Student t test; n = 5 animals) but did not significantly alter TNF-α mRNA expression (P = .2048, unpaired Student t test; n = 5 animals) and myeloperoxidase activity (P = .4826, unpaired Student t test; n = 5 animals) (Figure 6B). THIP significantly decreased CD163 (P = .0082, unpaired Student t test; n = 5 animals) and TNF-α mRNA expression (P = .0203, unpaired Student t test; n = 5 animals), together with myeloperoxidase activity (P = .0090, unpaired Student t test; n = 5 animals) (Figure 6C). L-655,708, an inverse agonist selective for the benzodiazepine site at α5-GABA<sub>A</sub>Rs, significantly increased myeloperoxidase activity (P = .0074, unpaired Student t test; n = 5 animals) but not CD163 (P = .0761, unpaired Student t test; n = 5 animals) and TNF-α mRNA expression (P = .4166, unpaired Student t test; n = 5 animals) (Figure 6D). This suggests that GI GABA<sub>A</sub>R subtypes bi-directionally modulate different inflammatory pathways within the intestinal local immune system.

Discussion

The data show that various forms of stress alter colonic contractility and induce robust inflammation of the colon while engaging the local GABA<sub>A</sub>R system. Genetic and pharmacologic evidence indicate that α3-GABA<sub>A</sub>Rs are central to mediating the proinflammatory effects of stress on the colon, whereas the pharmacologic activation of α1/4/5-GABA<sub>A</sub>Rs impart anti-inflammatory effects to varying degrees. Collectively, the data position the GI GABA<sub>A</sub>R 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 (Figure 1A) and acute adulthood stress<sup>14</sup> 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 because of 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.<sup>16</sup> In contrast, effects of acute RST are most likely transient. Such patterns of contractility arise entirely from the interaction of ENS neurons with interstitial cells of Cajal<sup>34</sup> and intestinal smooth muscle and are devoid of any central nervous
Furthermore, ELS induced long-lasting changes in the expression of only 1 particular GABAAR subunit, which is the $\alpha_3$ subunit. We have previously shown that within the mouse colon, GABAAR subunits are enriched within the ENS. 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 remain unclear.

It is 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 TNF-$\alpha$, independent of the other indices of inflammation (Figure 2). The importance of TNF-$\alpha$ in IBD pathology is underscored by the central role that anti–TNF-$\alpha$ agents play in treating these conditions. Although the acute adult stress paradigm used in this study induced a milder form of colonic inflammation, because TNF-$\alpha$ 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 gastrointestinal tract of animals exposed to chronic adult stress paradigms such as the social defeat model, because this has been shown to

![Figure 6. $\alpha_1/4/5$-GABAAR subtype-prefering ligands have varying degrees of anti-inflammatory effects on the colon in vitro.](image-url)
induce a robust inflammatory response within 1 of the main stress circuits of the brain, the dorsal raphe, with degree of inflammation determining the level of depressive-like behavior.\textsuperscript{36} Aligned with this work, we have also shown that repeated, though mild, RST significantly increases the expression of \(\alpha_3\)-GABA\(_A\)Rs selectively in this very brain region.\textsuperscript{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 gastrointestinal tract, 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\(_A\)R expression is equally important to identifying the individual receptor subtypes associated with such plasticity.

Emerging evidence points to various GABA\(_A\)R subtypes having a direct role in various inflammatory disorders. Indeed, multiple studies suggest direct signaling between inflammatory mediators and central nervous system GABA\(_A\)Rs.\textsuperscript{37–39} GABA\(_A\)Rs have now been shown to have an important role in peripheral inflammatory disorders such as asthma.\textsuperscript{40,41} It is therefore surprising that the role of GABA\(_A\)Rs in major peripheral inflammatory disorders such as IBD is poorly understood, even though preliminary evidence suggests that some GABA\(_A\)R ligands could prove therapeutically useful for such conditions.\textsuperscript{13} The current study provides proof of concept that \(\alpha_3\)-GABA\(_A\)Rs, in particular, play a direct role in mediating stress-induced GI inflammation, because the constitutive deletion of the GABA\(_A\)R \(\alpha_3\) subunit gene abolished stress-induced colonic inflammation (Figure 4). The obvious caveat is that \(\alpha_3\)-GABA\(_A\)Rs are expressed not only in the ENS but also in various brain regions that are integral to mediating the stress response, such as the locus coeruleus\textsuperscript{17} and dorsal raphe.\textsuperscript{7} Therefore, to prove the direct association between colonic \(\alpha_3\)-GABA\(_A\)Rs and colonic inflammation, we adopted a pharmacologic approach using isolated segments of colon tissue, which will not be influenced by any centrally mediated \(\alpha_3\)-GABA\(_A\)Rs. Remarkably, 30 minutes of exposure of the \(\alpha_3\)-GABA\(_A\)R agonist TP003 induced a robust inflammatory response within the colon. We confirmed that this effect was mediated via \(\alpha_3\)-GABA\(_A\)Rs, because TP003 failed to have any such effects in GABA\(_A\)R subunit \(\alpha_3\) gene–deleted (\(\alpha_3^{-/-}\)) mice (Figure 5). This indicates that the mere activation of \(\alpha_3\)-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 (Figure 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 \(\alpha_4\)-GABA\(_A\)R modulation,\textsuperscript{32} showed the most robust anti-inflammatory effects. These data are closely aligned with previous reports of an anti-inflammatory role for \(\alpha_4\)-GABA\(_A\)Rs in other organs such as lung.\textsuperscript{42} This positions various GABA\(_A\)R subtypes as dynamic regulators of the local GI immune system, 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 because of their inability to penetrate the central nervous system and repurposing of clinically available ligands for such peripheral disorders.

In summary, the study shows 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 and developing various GABA\(_A\)R drug moieties for clinical translational studies.

**Supplementary Material**

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2018.05.033.

**References**


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Conflicts of interest
The authors disclose no conﬂicts.

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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 license, in accordance with the Animals (Scientific Procedures) Act, 1986 (UK) and associated procedures.

Acute Stress in Adulthood: 1-Hour RST

One week before commencement of the stress protocol, the animals were divided into control and stress experimental groups, which allowed adaptation to the new cages. Mice aged PND 60 were inserted tail first into a Broome rodent restrainer (no. 52-0407; Harvard Apparatus) for 1 hour. The mice were restrained within the tube but not completely immobilized and thus were able to move slightly backward and forward in the tube. During RST, 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 was harvested for physiological and molecular analyses.

Isometric Tension Recordings of the Effects of Stress and the GABA<sub>A</sub>R 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 NaCl 140 mmol/L, NaHCO<sub>3</sub> 11.9 mmol/L, D+ glucose 5.6 mmol/L, KCl 2.7 mmol/L, MgCl<sub>2</sub> 6H<sub>2</sub>O 1.05 mmol/L, NaH<sub>2</sub>PO<sub>4</sub> 2H<sub>2</sub>O 0.5 mmol/L, and CaCl<sub>2</sub> 1.8 mmol/L, warmed to 37°C. In a subset of the experiment, α3<sup>-/−</sup> mice and their WT littermates were used. The intraluminal contents were removed by gently flushing the colon with the physiological solution. To measure longitudinal and circular muscle contractions, approximately 2-cm-long whole segments of colon were mounted vertically and horizontally, respectively, in a Harvard Apparatus organ bath (10-mL chamber) filled with the physiological solution (37°C) and bubbled with gas containing 95% O<sub>2</sub> and 5% CO<sub>2</sub>. 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, Sydney, Australia). 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 1-g weight to express the changes in the amplitude detected by the transducer into grams of force. At this stage, 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 g of tension. Therefore, in any subsequent analysis of contractile activities produced by pieces of colon, any peak less than 0.02 g of force was disregarded to produce an accurate account for the force and frequency of spontaneous contractions. The tissue was then placed under 1 g of resting tension and allowed to equilibrate for 30 minutes. The AD Instruments LabChart 7 program installed on a personal computer was used to monitor, record, and analyze 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 (AD Instruments) 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 normalized 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 1 animal, and the data are presented as the mean ± standard error of the mean.

Immunohistochemistry and Confocal Microscopy

Mice were anaesthetised with isoflurane and pentobarbitone (1.25 mg/kg of body weight, intraperitoneal) and transcardially perfused using a fixative containing 1% weight/volume paraformaldehyde and 15% volume/volume saturated picric acid in 0.1 mol/L phosphate buffer (pH 7.4) according to previously described protocols. After perfusion, the colons were removed and postfixed in the same fixative overnight at 4°C. The next day, tissue was washed in 0.1 mol/L 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 mol/L phosphate buffer containing 0.05% weight/volume sodium azide. Nonspecific binding of secondary antibodies was blocked by incubating the tissue with 20% volume/volume 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 (sc-33560; Santa Cruz Biotechnology, Dallas, TX); (2) sheep anti nitric oxide synthase, 1:1000 (AB1529; Millipore, Burlington, MA), diluted in Tris buffer saline containing 0.3% weight/volume Triton X-100 (TBS-Tx) and 20% volume/volume 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) and indocarbocyanine (Cy3; Jackson ImmunoResearch,
West Grove, PA) for 2 hours at room temperature. The tissue was washed in TBS-Tx and mounted on glass slides in Mowiol mounting medium (Polysciences, Warrington, PA) and then cover-slipped. Sections were examined with a confocal laser-scanning microscope (LSM710; Zeiss, Oberkochen, Germany) using either a Plan Apochromatic 40× DIC oil objective (NA1.3) (pixel size, 0.29 μm), a Plan Apochromatic 63× DIC oil objective (NA1.4) (pixel size, 0.13 μm) or a Plan Apochromatic 100× DIC oil objective (NA1.46) (pixel size, 0.08 μm). Z-stacks were used for routine evaluation of the labeling. 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 1 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 Image J software (National Institutes of Health, Bethesda, MD) cell count analysis function. The average of all fields of view was 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**

Adult male mice from control and ELS groups were killed by cervical dislocation, and tissue homogenates of the whole colon were prepared. RNA was extracted from the samples using an RNaseasy mini kit (74104; Qiagen, Venlo, The Netherlands) according to the manufacturer’s protocol. An 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 (Thermo Fisher Scientific, Waltham, MA), 1 μL dNTPs (Thermo Fisher Scientific), 0.5 μL M-Mulv reverse transcriptase (Applied Biosystems, Foster City, CA), and 0.5 μL of Riboblock RNase Inhibitor (Thermo Fisher Scientific). qPCR amplification was performed in 96-well plates in a master mix for probes (Roche, Burgess Hill, UK) and run on a LightCycler 96 System (Roche). The qPCR amplifications for the mouse *Gabra1* (assay identification [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 (Thermo Fisher Scientific). *Gapdh* (assay ID: Mm99999915_g1) and *Villin* (assay ID: Mm00494146_m1) gene expressions were used as the housekeeping genes in various reactions. The qPCR cycling conditions entailed 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C 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 cycle threshold value was then converted into nanograms of mRNA using linear regression analysis of the standard curve (Microsoft Excel, Microsoft, Redmond, WA). Each nanogram of mRNA value was then normalized against the nanograms of housekeeping gene level within the same sample, and the mean mRNA levels for every sample were finally calculated and compared across all experimental groups.

**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 before the start of the experiments with free access to drinking water. Subsequently, FITC-dextran (FD4; Sigma-Aldrich, St Louis, MO) 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 for 10 minutes at 12,000g and 4°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 the 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 plotted with 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 was performed to assess the expression of tight junction protein 1 (AE14919MO-48, Generon), occludin (SEC228Mu-48; Generon) and TNF-α (KET7015-48; Generon) 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 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Triton X-100, 0.5% NP-40, 1 mmol/L EDTA, 10 mmol/L Na4P2O7, and a cocktail of protease inhibitors, pH 8.0. Subsequently, a 100-μL volume of each sample containing an equal amount of total protein was used in each ELISA according to the manufacturer’s protocol.

**Drugs**

The following drugs were used in this study: zolpidem (0655; Tocris Biosciences, Minneapolis, MN), alprazolam (A8800, Sigma Aldrich), TP003 (4414, Tocris Biosciences), THIP hydrochloride (0807, Tocris Biosciences), and L-655,708 (1327, Tocris Biosciences). Apart from THIP hydrochloride, which was dissolved in distilled water, all other drugs were dissolved in dimethyl sulfoxide.

**References**