Title:

Developmental and age-dependent plasticity of $GABA_A$ receptors in the mouse colon: implications in colonic motility and inflammation

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Abbreviations:

GIT, gastrointestinal tract; GI, gastrointestinal; ENS, enteric nervous system; GABA, gamma-aminobutyric acid; $GABA_AR$, gamma-aminobutyric acid A receptor; P, postnatal day; KO, knockout; WT, wild-type; TNFa, tumour necrosis factor alpha; CNS, central nervous system.

Abstract

Lifelong functional plasticity of the gastrointestinal (GI) tract is essential for health, yet the underlying molecular mechanisms are poorly understood. The enteric nervous system (ENS) regulates all aspects of the gut function, via a range of neurotransmitter pathways, one of which is the GABA-$GABA_A$ receptor ($GABA_AR$) system. We have previously shown that $GABA_A$ receptor subunits are differentially expressed within the ENS and are involved in regulating various GI functions. We have also shown that these receptors are involved in mediating stress-induced colonic inflammation. However, the expression and function of intestinal $GABA_ARs$, at different ages, is largely unexplored and was the focus of this study. Here we show that the impact of $GABA_AR$ activation on colonic contractility changes from early postnatal period through to late adulthood, in an age-dependant manner. We also show that the highest levels of expression for all $GABA_AR$ subunits is evident at postnatal day (P) 10 apart from the α3 subunit which increased with age. This increase in the α3 subunit expression in late adulthood (18 months old) is accompanied by an increase in the expression of inflammatory markers within the mouse colon. Finally, we demonstrate that the deletion of the α3 subunit prevents the increase in the expression of colonic inflammatory markers associated with healthy ageing. Collectively, the data provide the first demonstration of the molecular and functional plasticity of the GI $GABA_AR$ system over the course of a lifetime, and its possible role in mediating the age-induced colonic inflammation associated with healthy ageing.

Key words:

postnatal; alprazolam; alpha 3 subunit; enteric nervous system; inflammatory bowel diseases

1 Introduction

The integrity of the mammalian gastrointestinal tract (GIT) is fundamental for nutrition and barrier function over the course of a lifetime. The GIT exhibits remarkable plasticity during development and the ageing process, despite the changing nutritional needs of the individual, or the local environment in the form of microbiota and immune function (1). Nevertheless, ageing is associated with a general decline in intestinal function, manifesting in motility disorders such as constipation, or altered immune function, such as inflammation (2). However, the molecular mechanisms underlying this age-related GI plasticity and the ensuing associated disorders have yet to be fully understood (3). Age-specific changes in the intrinsic nervous system of the GIT, namely the enteric nervous system (ENS) and its associated neurotransmitters systems are most likely central to lifelong GI health (4).

The ENS is integral to all aspects of coordinated GI function (5, 6). It is a large collection of neurochemically diverse neurons (7, 8) located within the submucosal layer and muscle wall of the GIT. These neurons form intricate cellular networks with neuronal and non-neuronal cell-types and provide the intrinsic neural control of virtually all GI functions such as peristalsis (9), secretion (10), barrier function (11, 12) and local immune function (13-15). Despite our considerable insight into ENS-mediated GI function in adulthood, relatively less is known about how this differs throughout the process of ageing, from early postnatal days to late adulthood. The most consistent finding in the aged ENS is altered local immune function (16) and degeneration of neurochemically-distinct cell-types (4, 17, 18). However, there is considerably less known about the age-related changes in the neurotransmitter receptor systems through which different ENS neurons mediate their effects on the GIT. One of these neurotransmitter-receptor systems, the GABA-$GABA_AR$ system has been shown to be involved in virtually all ENS-mediated GI functions (19).

$GABA_ARs$ are chloride permeable integral membrane ion channels composed of five interacting subunit proteins which mediate the rapid effects of the neurotransmitter GABA (20). While only five subunits are required to form a functional receptor, up to nineteen molecularly distinct $GABA_AR$ subunits (α1-6; β1-3; γ1-3; δ; ε; ρ) have been identified, which underpin the expression of ~ 20-30 main distinct $GABA_AR$ isoforms

(21). Within the central nervous system, these receptor subtypes display a differential regional expression or cellular location (22) and exhibit specific physiological (20) and pharmacological properties (23). Previous studies in adult animals indicate that the GABA-$GABA_AR$ system directly alters the excitability of ENS neurons (24), spontaneous colonic contractility (25-27) and GI motility (28, 29). However, the expression and functional plasticity of this neurotransmitter system at different maturational stages of the colon, is largely unexplored. Furthermore, we have recently reported that different $GABA_AR$ subtypes have contrasting effects on stress-induced colonic inflammation (30). Given the consistent finding of altered immune function in the elderly, that may give rise to colonic inflammation (31), the question arises whether $GABA_ARs$ may be associated. In the current study, we show that the force and frequency of native spontaneous colonic contractions change significantly during early postnatal development stages. Furthermore, colonic $GABA_AR$ expression changes dramatically in a subunit and age-dependent manner. Finally, the deletion of the $GABA_AR$ α3 prevents age-related colonic inflammation.

2 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.

2.1 Animals

For wild-type (WT) mice, the C57BL/6J strain obtained from the University of Portsmouth Bioresources centre was used. In some experiments, $GABA_AR$ α3 subunit gene deleted (α3 KO) mice, on a C57/BL6J background, were also used. For such experiments, WT littermates of the α3 KO mice were used as controls. The generation of these mice has been previously described (32). 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.

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

Isometric tension recordings of isolated mouse colon were performed according to our previously published protocols (27, 33). Mice aged postnatal day 10 (P10), P15, P60 and 18 months old were killed by cervical dislocation. The distal colon removed and immediately placed in physiological solution containing (mM): NaCl 140, $NaHCO_3$ 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°C. The intraluminal contents were removed by gently flushing the colon with physiological solution. Approximately 2 cm long segments were mounted in a Harvard organ bath (10 ml chamber) filled with physiological solution (37ºC) and bubbled with gas containing 95% O_2 and 5% CO_2. Contractile activity for each colon tissue segment 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 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 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 colonic contractility, any peak less than 0.02 grams of force was disregarded, thereby revealing only intrinsic 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 was installed on a PC in order to monitor, record and analyse the activity. After a stable baseline was established, 1 µM flumazenil (Tocris Bioscience; 1328) or 10 µM alprazolam (Sigma-Aldrich; A8800) was added to the bath and the tissue was allowed to reach maximum response. We measured the time it takes to achieve a full response on contractile activity using alprazolam. We observed full response by 10 minutes after adding alprazolam. Therefore, 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 value was then subtracted from the noise level 0.02 g in order to account for the electrical noise produced by equipment. This was done for the all the contraction before and after the drug additions and the average for that animal was determined. Due to differences in the patterns of colonic spontaneous contractions across ages we were unable to separate large amplitude contractions from smaller oscillations. Therefore, there data presented in the paper are an average of both large and smaller spontaneous contractions together. 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 a particular drug. In addition, the effect of 10 µM alprazolam on the basal tone of the tissue was also determined. An N value thus represents one animal and the data are presented as the mean ± SEM.

2.3 Quantitative reverse transcription Polymerase Chain Reaction (qPCR)

qPCR performed on colon tissue was carried out according to our previously published protocols (34). Mice aged P10, P15, P60 and 18 months were killed by cervical dislocation and tissue homogenates of the whole colon prepared. RNA was extracted from the samples using a RNeasy mini kit (Qiagen, 74104) according to the manufacturer's protocol. Equal amounts of RNA from each tissue was reverse-transcribed into first-strand cDNA in the following reaction: 2 µl of reverse transcription buffer (BioLabs), 1 µl of oligo(dT) (ThermoFisher Scientific), 1µ dNTP (ThermoFisher Scientific), 0.5 µl of M-MuLV reverse transcriptase (Applied Biosystems) and 0.5 µl of RiboLock (ThermoFisher Scientific). The reactions were then made up to 20 µl with nuclease free-PCR grade water. 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) and *TNFα* (assay ID: Mm00443258_m1) genes were performed using pre-designed Taqman primers/probes purchased from Life Technologies (ThermoFisher scientific). *Gapdh* (assay ID: Mm99999915_g1) gene expression was used as the housekeeping gene in every reaction. The qPCR cycling conditions entailed 95°C for 10 mins and 40 cycles of 95°C for 15 sec and 60°C for 60 sec (LightCycler® 96 System, Roche). Standard curves were generated for each gene using serial dilutions of a known amount of mRNA which was 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.

2.4 Immunohistochemistry and confocal microscopy

Mice were anaesthetised first with isoflurane and then pentobarbitone (1.25 mg/kg of bodyweight; i.p.), transcardially perfused first with 0.9% saline and then 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 (35). After perfusion, the colons were removed and post-fixed in the same fixative over night at 4^{o} C. The next day, the 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, which were then stored in 0.1 M phosphate buffer containing 0.05% w/v sodium azide. Staining for the inflammatory marker CD163 on whole-mount preparations of the colon was performed as described in our previously published protocols (36). Briefly, 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°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) 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). All images presented represent a single optical section. These images were acquired using sequential acquisition of the different channels to avoid cross-talk between fluorophores, with the pinholes adjusted to one airy unit. Images were processed with the software Zen2008 Light Edition (Zeiss, Oberkochen, Germany) and exported into Adobe Photoshop. Only brightness and contrast were adjusted for the whole frame, and no part of a frame was enhanced or modified in any way.

2.5 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 was calculated for each piece of tissue and considered as N = 1. One piece of tissue was used per animal.

2.6 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.

3 Results

3.1 Spontaneous colonic contractions and their degree of modulation by $GABA_AR$s, changes dynamically with age

We first characterised the changes in the force and frequency of spontaneous colonic contractions, at P 10, 15, 60 and 18 months. We then investigated the impact of $GABA_AR$ activation on spontaneous colonic contraction across these ages. There were striking differences in the patterns of spontaneous colonic contractility across all ages investigated (Fig. 1 A). Quantification of the force of spontaneous contractions revealed significant differences at different ages ($p < 0.0001$, one way ANOVA, N = 8 animals per age group). Post-hoc analysis revealed a significant decrease in the force of spontaneous contractions between P 10 and P 15 ($p < 0.0001$, Tukey's), and P 60 ($p < 0.0001$, Tukey's) and 18 months old ($p < 0.0001$, Tukey's) (Fig. 1 B1). The frequency of spontaneous colonic contractions also changed significantly with age ($p < 0.0001$, one way ANOVA, N = 8 animals per age group). Post-hoc analysis revealed a significant increase in the frequency of spontaneous contractions between P 10 and P 15 ($p < 0.0001$, Tukey's), P 10 and P 60 ($p < 0.0001$, Tukey's) and P 10 and 18 months ($p < 0.0001$, Tukey's). There was also a significant increase between P 15 and P 60 ($p < 0.0001$, Tukey's). However, there was no significant difference between P 60 and 18 months ($p = 0.1760$, Tukey's). Therefore, the changes observed in the longitudinal spontaneous colonic contractility were mostly associated with developmental stages rather than ageing during adulthood.

We have previously demonstrated that in adult mice (P 60), the benzodiazepine alprazolam, which is likely to positively allosterically modulate α1-3/5-γ2-β subunit-containing $GABA_AR$s, significantly decreases the force of spontaneous colonic contractions (27). We therefore assessed whether this effect of colonic $GABA_AR$ activation persists at all ages. There was a significant interaction between the effect of alprazolam and age on the force of contractions ($p = 0.003$, two way ANOVA with repeated measures, N = 8 animals per age group). Post-hoc analysis revealed that alprazolam significantly decreased the force of contractions at P 10 ($p <0.0001$, Tukey's) and at P 60 ($p <0.0001$, Tukey's). However, this effect of alprazolam was not evident at P 15 (, $p = 0.333$, Tukey's) and 18 months ($p = 0.482$, Tukey's) (Fig. 1 B1).

There was also a significant interaction between the effect of alprazolam and age on the frequency of contractions ($p < 0.0001$, two way ANOVA, N = 8 animals per age group). Post-hoc analysis revealed that alprazolam significantly increased the frequency of contractions at P 60 ($p < 0.0001$, Tukey's). However, alprazolam had no significant effect at, P 10 ($p > 0.9999$), P 15 ($p = 0.8786$) and 18 months ($p = 0.9735$) (Fig. 1 B2). Alprazolam has also been shown to decrease the basal tone of the adult mouse colon (27), and demonstrated in Fig. 1 A. The current study revealed that this effect of $GABA_AR$ activation on colonic tone is significant at all ages compared to the baseline which was taken as zero ($p < 0.0001$, one way ANOVA, N = 8 animals per age group) (Fig. 1 B3). However, this relaxant effect of alprazolam on the colonic tone was significantly increased between P 15 to P 60 (P < 0.0001, Tukey's) and 18 months old ($p < 0.0001$, Tukey's). The pre-application of the benzodiazepine antagonist flumazenil (1 µM) abolished the contractile effects of alprazolam (Fig. 1 A, boxed trace), thus confirming that the effect of alprazolam is mediated via benzodiazepine sites on colonic $GABA_ARs$. This suggests that the modulation of spontaneous colonic contractility by the local $GABA_AR$ system, changes dynamically during early development extending into later adulthood.

3.2 $GABA_AR$ subunit expression changes with age

Given the contrasting *functional* effects of alprazolam on the mouse colon at different ages, we next explored whether the *expression* of these receptors might also vary with age, using qPCR to measure the mRNA expression of the α 1 – 5 and γ2 subunits, in homogenates containing all tissue layers of the colon. The α1 ($p = 0.0003$, ANOVA) (Fig. 2 A), α2 ($p = 0.0025$, ANOVA) (Fig. 2 B), α5 ($p < 0.0001$, ANOVA) (Fig. 2 E) and γ2 ($p = 0.0001$, ANOVA) (Fig. 2 F) subunits all exhibited a significant decrease in expression with age. In stark contrast, the α3 subunit showed a significant increase in expression with age ($p = 0.0143$, ANOVA) (Fig. 2 C). There were no significant differences between ages for the α4 subunit ($p > 0.05$, ANOVA) (Fig. 2 D). This indicates that $GABA_AR$ expression within the mouse colon is age and subunit specific.

3.3 The $GABA_AR$ α3 subunit as a mediator of colonic inflammation in late adulthood

Altered local immune function is a consequence of healthy ageing of the intestine (3, 16). We have recently demonstrated that the $GABA_AR$ α3 subunit promotes stress-

induced inflammation in the mouse colon (30). In light of the striking increase in the expression of the $GABA_AR$ α3 subunit at 18 months, we explored whether this subunit could be involved in age-related colonic inflammation. Firstly, we investigated the expression levels of inflammatory mediators in the colon of young adult (2 months) and older adult (18 months) WT mice. We then investigated this in age-matched older WT and α3 KO adult mice. Immunohistochemical analysis for CD163, within the ENS of the colon revealed a significant increase in expression between 2 and 18 months of age (Fig. 3). 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 (37). Quantification of the density of CD163-immunopositive profiles revealed a significant increase between 2 and 18 month old subjects, reacted and imaged under identical conditions ($p < 0.0001$, unpaired Student's *t* test, N = 5) (Fig. 4 A). There was also a significant increase in the mRNA expression of CD163 with age (ANOVA, N = 6) (Fig. 4 B). Finally, there was a significant increase in the mRNA expression of another key marker of intestinal inflammation, tumour necrosis factor alpha (TNFα), at 18 months, compared to younger ages (ANOVA, N = 6) (Fig. 4 C).

In order to investigate the possible link between the observed inflammation in older adult mice and $GABA_AR$ α3 subunit, we repeated these experiments in α3 KO mice. In comparative analyses using aged-matched (12 months) WT and α3 KO mice, whilst widespread CD163 immunoreactivity was evident in WT tissue, significantly lower levels were evident in tissue from α3 KO mice (Fig. 5). Quantification of the density of CD163-immunoreactive profiles revealed a significant difference between WT and α3 KO mice ($p = 0.0002$, unpaired Student's *t* test, N = 6) (Fig. 6 A). There was also a significant decrease in CD163 mRNA between WT and α3 KO mice ($p = 0.0001$, unpaired Student's *t* test, N = 6) (Fig. 6 B). Whilst the TNF α mRNA showed a trend towards decreased expression, this difference was not statistically significant ($p = 0.5459$, unpaired Student's *t* test, N = 6) (Fig. 6 C). This suggests a possible role for the $GABA_AR$ α3 subunit in mediating the colonic inflammation associated with the process of ageing.

4 Discussion

The data demonstrate that mouse longitudinal spontaneous colonic contractility patterns change significantly during early postnatal developmental stages but not from young to old adulthood. Furthermore, the effect of $GABA_AR$ activation on these contractions was age-dependent and the expression of $GABA_AR$ subunits changed dynamically in a subunit-specific and age-dependant manner. Finally, the deletion of the $GABA_AR$ α3 subunit prevented an increase in the expression of colonic inflammatory markers associated with healthy ageing. Collectively, the data provide the first demonstration of the molecular and functional plasticity of the GI $GABA_AR$ system over the course of a lifetime, and its possible role in mediating the age-induced colonic inflammation associated with healthy ageing.

Central to ensuring optimal nutritional requirements, at different ages, is an intestinal motility pattern appropriate for the changes in diet that occur from the neonate through to the elderly. This study focussed on only one aspect of motility, namely, contractility of longitudinal smooth muscles. We have previously shown two different patterns of longitudinal contractions within the mouse colon. These include large spontaneous contractions superimposed on smaller, more frequent contractions (27). The data presented in this study are an average of both large and smaller spontaneous contractions together. This study shows that the pattern of these contractions changes significantly from P 10 to P 15 and onwards. This coincides with the age at which young mice open their eyes and start intake of solid food in addition to milk. Interestingly, previous studies have shown that myenteric neurons of the intestine undergo significant morphological and electrophysiological changes from P 10 to adulthood (38). Indeed, neurotransmitter systems such as dopaminergic and purinergic system undergo developmental changes shifting from contraction to relaxation just before and during weaning (39, 40). In addition, during these early postnatal days, the microbiota and immune cell community of the GIT are constantly changing (41, 42). Therefore, the altered pattern of colonic contractility at P15 may be the result of this changing landscape of GIT function during development. Furthermore, the process of ageing has been associated with drastic changes in GI motility and development of gastrointestinal disorder (17). Indeed, colonic motility has been shown to be impaired in aged (24 month old) mice (43). Since colonic motility

arises from the coordinated contractions and relaxations of the colonic smooth muscles, we therefore expected age-induced changes in the force of colonic spontaneous contractions. However, in the current study, we did not detect any significant changes in the force of spontaneous colonic contractions of 18 months old mice in comparison to young adults. The most likely explanation is the differences in the age of old mice used in the present study. In addition, methodological differences could also be a factor as we did not measure overall colonic motility *per se*, merely one contributor to motility, namely longitudinal muscle contractility. Nonetheless, numerous studies (4, 17, 43) have suggested that changes in the neurotransmitter systems of the ENS are a contributing factor to the developmental and the age-induced decline in GI motility. However, it is important to note that mucosal signalling through the 5-HT system has also been shown to play an important role in age-induced changes in GI motility (44).

In the present study, we focussed on investigating the expression and function of the enteric GABA-$GABA_AR$ system during development and at different ages. Here, we show that the relaxant effect of alprazolam on the spontaneous colonic contractions is significant at P 60 and P 10, but not P 15 or 18 months old. Furthermore, alprazolam was only able to significantly alter the frequency of colonic longitudinal spontaneous contraction at P 60. An explanation for this could be differences in the amount of $GABA_ARs$ expressed at various ages. However, we only observed significantly higher levels of $GABA_ARs$ expression at P10, with no significant differences between P 15, P 60 and 18 months old. Another likely explanation could be differences in the amount of ambient tonic GABA within the ENS and GIT. Since alprazolam is a benzodiazepine, it will induce an effect only if local GABA is already bound to the receptor. Therefore, differences in the endogenous extracellular GABA within the gut at different ages will likely impact on the overall effect of alprazolam on colonic contractility. Importantly, ageing induced decline in GABA concentration have been previously shown within the brain (45). This could in turn impact on the regulation of ENS $GABA_AR$ numbers, and thus $GABA_AR$-mediated colonic function. Therefore, future studies focussed on characterising the changes in enteric GABAergic neurons, extracellular GABA concentrations and $GABA_AR$ subtype expression, across ages, could reveal novel insights into gut homeostasis and how this is impaired in age-specific gut dysfunction, such as GI inflammation in the elderly.

The most striking finding was that the majority of the $GABA_AR$ subunits examined showed the highest levels of expression at early postnatal ages. This suggests a potential role for this system in development of the ENS and the GIT. This is not surprising as the GABA-$GABA_AR$ system is implicated in the development of the CNS. In early postnatal stages of the brain, GABA, signalling via $GABA_ARs$, is thought to have a depolarising effect on postsynaptic membranes due to the relatively high concentration of intracellular chloride ions (46). It is postulated that this initial excitatory effect of $GABA_ARs$ makes major a contribution to the development of brain circuitry prior to the development of glutamate inputs (47). In contrast to the CNS, GABA is generally considered to be predominantly excitatory in the ENS (19), thereby indicating a potential role as a modulator of neural circuitry development. Furthermore, the highest levels of intestinal GABA expression, in rat at least, are detected at early developmental stages (48). The earliest age we examined was P 10, by which stage, a significant degree of development of the ENS would have already ensued (49). It would therefore be useful to examine $GABA_AR$ subunit expression possibly at embryonic or earlier postnatal stages to determine whether a changing landscape of the $GABA_AR$ system coincides with specific developmental time points of the ENS. Coupled with functional analysis using $GABA_AR$ subunit-specific knockout mice, such studies might allow for the possible exploitation of this neurotransmitters system in medical conditions associated with impaired development of the ENS, such as Hirschsprung's disease (50).

Numerous studies have shown developmental and age-induced alteration in the expression and function of $GABA_ARs$ within the CNS, in a subunit, brain region and disease specific manner (51-54). In this study, we provide the first demonstration of such changes in the expression of $GABA_ARs$ within the GIT. Interestingly, in contrast to all other subunits examined which showed a decreasing trajectory of expression with age, the $GABA_AR$ α3 subunit exhibited an expression profile that increased, exclusively in late adulthood (18 months). In conjunction with this, we also observed a significant increase in the expression of inflammatory markers in late adulthood (18 months). This is important, as we have recently shown that the activation of colonic $GABA_AR$ α3 subunit induces inflammation and plays a direct role in stress-induced GI inflammation (30). Furthermore, other studies have also shown that activation of colonic $GABA_ARs$ exacerbates acute colitis (55). Therefore, our data suggests that

GABA$_A$R α3 subunit may play a role in mediating age-related increase in colonic inflammation. Remarkably, the deletion of this subunit prevented the increase in the expression of colonic inflammatory markers in 12 months old mice. Although 12 months old mice may not be classified as aged mice, this data suggests an important role for the GABA$_A$R α3 subunit as a potential contributor to age-induced GI disorders associated with immune dysfunction. There is a disproportionate prevalence of GI disorders in the elderly, compared to younger individuals (3, 56). Whilst the underlying changes are complex, a consistent finding is alterations in the local immune system, manifesting in increased infections and inflammation (31). Therefore, further investigations of the role of GABA$_A$R α3 subunit as potential target for the treatment of GI inflammatory disorders in the elderly, could provide novel therapeutics for alleviation of associated symptoms. The other associated GABA$_A$R subtypes should not be overlooked, potentially as therapeutic targets for impaired GI immune function. Indeed, we have also demonstrated that the activation of α1/2 subunit-containing GABA$_A$Rs induces an anti-inflammatory effect in the colon (30). Furthermore, the expression profiles across age of these subunits were diametrically opposite to that of the α3 subunit. Collectively, that data reveals a dynamic GI GABA-GABA$_A$R system that adapts, over the course of a lifetime, to mediate various GI functions at different ages. This study also provides a platform for further investigation of the GABA$_A$R system as potential therapeutic targets for the treatment of inflammatory disorders associated with ageing.

Acknowledgments

The expert technical assistance of Torquil Jackson, Stewart Gallacher, Daniel Arthur, Scott Rodaway, Angela Scutt and Andy Milner is gratefully acknowledged. This study was funded by a Research Development Fund award to JDS from the University of Portsmouth.

Author contributions

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

Disclosures

The authors declare no conflict of interests.

References

1. Salles N. Basic mechanisms of the aging gastrointestinal tract. Dig Dis. 2007;25(2):112-7. PubMed PMID: 17468545.
2. Rao SS, Go JT. Update on the management of constipation in the elderly: new treatment options. Clinical interventions in aging. 2010;5:163-71. PubMed PMID: 20711435. Pubmed Central PMCID: 2920196.
3. Saffrey MJ. Aging of the mammalian gastrointestinal tract: a complex organ system. Age (Dordr). 2014 Jun;36(3):9603. PubMed PMID: 24352567. Pubmed Central PMCID: 4082571.
4. Saffrey MJ. Cellular changes in the enteric nervous system during ageing. Developmental biology. 2013 Oct 1;382(1):344-55. PubMed PMID: 23537898. Epub 2013/03/30. eng.
5. Furness JB. The enteric nervous system: normal functions and enteric neuropathies. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society. 2008 May;20 Suppl 1:32-8. PubMed PMID: 18402640. Epub 2008/04/18. eng.
6. Di Nardo G, Blandizzi C, Volta U, Colucci R, Stanghellini V, Barbara G, et al. Review article: molecular, pathological and therapeutic features of human enteric neuropathies. Alimentary pharmacology & therapeutics. 2008 Jul;28(1):25-42. PubMed PMID: 18410560. Epub 2008/04/16. eng.
7. Furness JB. Types of neurons in the enteric nervous system. Journal of the autonomic nervous system. 2000 Jul 3;81(1-3):87-96. PubMed PMID: 10869706. Epub 2000/06/28. eng.
8. Noorian AR, Taylor GM, Annerino DM, Greene JG. Neurochemical phenotypes of myenteric neurons in the rhesus monkey. The Journal of comparative neurology. 2011 Dec 1;519(17):3387-401. PubMed PMID: 21618236. Pubmed Central PMCID: 4033411. Epub 2011/05/28. eng.
9. Grider JR. Identification of neurotransmitters regulating intestinal peristaltic reflex in humans. Gastroenterology. 1989 Dec;97(6):1414-9. PubMed PMID: 2479588. Epub 1989/12/01. eng.
10. Riegler M, Castagliuolo I, Wang C, Wlk M, Sogukoglu T, Wenzl E, et al. Neurotensin stimulates Cl(-) secretion in human colonic mucosa In vitro: role of adenosine. Gastroenterology. 2000 Aug;119(2):348-57. PubMed PMID: 10930370. Epub 2000/08/10. eng.
11. Neunlist M, Toumi F, Oreschkova T, Denis M, Leborgne J, Laboisse CL, et al. Human ENS regulates the intestinal epithelial barrier permeability and a tight junction-associated protein ZO-1 via VIPergic pathways. American journal of physiology Gastrointestinal and liver physiology. 2003 Nov;285(5):G1028-36. PubMed PMID: 12881224. Epub 2003/07/26. eng.
12. Toumi F, Neunlist M, Cassagnau E, Parois S, Laboisse CL, Galmiche JP, et al. Human submucosal neurones regulate intestinal epithelial cell proliferation: evidence from a novel co-culture model. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society. 2003 Jun;15(3):239-42. PubMed PMID: 12787332. Epub 2003/06/06. eng.
13. Goyal RK, Hirano I. The enteric nervous system. The New England journal of medicine. 1996 Apr 25;334(17):1106-15. PubMed PMID: 8598871. Epub 1996/04/25. eng.
14. Furness JB. The enteric nervous system. Malden, Mass.: Blackwell Pub.; 2006. xiii, 274 p. p.
15. Schneider J, Jehle EC, Starlinger MJ, Neunlist M, Michel K, Hoppe S, et al. Neurotransmitter coding of enteric neurones in the submucous plexus is changed in non-inflamed rectum of patients with Crohn's disease. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society. 2001 Jun;13(3):255-64. PubMed PMID: 11437988. Epub 2001/07/05. eng.
16. Man AL, Gicheva N, Nicoletti C. The impact of ageing on the intestinal epithelial barrier and immune system. Cellular immunology. 2014 May-Jun;289(1-2):112-8. PubMed PMID: 24759078.
17. Camilleri M, Cowen T, Koch TR. Enteric neurodegeneration in ageing. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society. 2008 Apr;20(4):418-29. PubMed PMID: 18371012.
18. Bernard CE, Gibbons SJ, Gomez-Pinilla PJ, Lurken MS, Schmalz PF, Roeder JL, et al. Effect of age on the enteric nervous system of the human colon. Neurogastroenterol Motil. 2009 Jul;21(7):746-e46. PubMed PMID: 19220755. Pubmed Central PMCID: 2776702.

19. Krantis A. GABA in the Mammalian Enteric Nervous System. News Physiol Sci. 2000 Dec;15:284-90. PubMed PMID: 11390928. Epub 2001/06/08. Eng.
20. Farrant M, Nusser Z. Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. Nature reviews Neuroscience. 2005 Mar;6(3):215-29. PubMed PMID: 15738957. Epub 2005/03/02. eng.
21. Olsen RW, Sieghart W. GABA A receptors: subtypes provide diversity of function and pharmacology. Neuropharmacology. 2009 Jan;56(1):141-8. PubMed PMID: 18760291. Pubmed Central PMCID: 3525320. Epub 2008/09/02. eng.
22. Hortnagl H, Tasan RO, Wieselthaler A, Kirchmair E, Sieghart W, Sperk G. Patterns of mRNA and protein expression for 12 GABAA receptor subunits in the mouse brain. Neuroscience. 2013 Apr 16;236:345-72. PubMed PMID: 23337532. Pubmed Central PMCID: 3605588. Epub 2013/01/23. eng.
23. Rudolph U, Knoflach F. Beyond classical benzodiazepines: novel therapeutic potential of GABAA receptor subtypes. Nat Rev Drug Discov. 2011 Sep;10(9):685-97. PubMed PMID: 21799515. Epub 2011/07/30. eng.
24. Cherubini E, North RA. Actions of gamma-aminobutyric acid on neurones of guinea-pig myenteric plexus. British journal of pharmacology. 1984 May;82(1):93-100. PubMed PMID: 6733360. Pubmed Central PMCID: 1987234. Epub 1984/05/01. eng.
25. Bayer S, Crenner F, Aunis D, Angel F. Effects of GABA on circular smooth muscle spontaneous activities of rat distal colon. Life sciences. 2002 Jul 12;71(8):911-25. PubMed PMID: 12084388. Epub 2002/06/27. eng.
26. Bayer S, Jellali A, Crenner F, Aunis D, Angel F. Functional evidence for a role of GABA receptors in modulating nerve activities of circular smooth muscle from rat colon in vitro. Life sciences. 2003 Feb 14;72(13):1481-93. PubMed PMID: 12535716. Epub 2003/01/22. eng.
27. Seifi M, Brown JF, Mills J, Bhandari P, Belelli D, Lambert JJ, et al. Molecular and functional diversity of GABA-A receptors in the enteric nervous system of the mouse colon. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2014 Jul 30;34(31):10361-78. PubMed PMID: 25080596. Pubmed Central PMCID: 4115141. Epub 2014/08/01. eng.
28. Tonini M, Crema A, Frigo GM, Rizzi CA, Manzo L, Candura SM, et al. An in vitro study of the relationship between GABA receptor function and propulsive motility in the distal colon of the rabbit. British journal of pharmacology. 1989 Dec;98(4):1109-18. PubMed PMID: 2558756. Pubmed Central PMCID: 1854802. Epub 1989/12/01. eng.
29. Tonini M, De Petris G, Onori L, Manzo L, Rizzi CA, Crema A. The role of GABAA receptor function in peristaltic activity of the guinea-pig ileum: a comparative study with bicuculline, SR 95531 and picrotoxinin. British journal of pharmacology. 1989 Jun;97(2):556-62. PubMed PMID: 2547476. Pubmed Central PMCID: 1854512. Epub 1989/06/01. eng.
30. Seifi M, Rodaway S, Rudolph U, Swinny JD. GABAA Receptor Subtypes Regulate Stress-Induced Colon Inflammation in Mice. Gastroenterology. 2018 Sep;155(3):852-64 e3. PubMed PMID: 29802853.
31. Ogra PL. Ageing and its possible impact on mucosal immune responses. Ageing research reviews. 2010 Apr;9(2):101-6. PubMed PMID: 19664726.
32. Yee BK, Keist R, von Boehmer L, Studer R, Benke D, Hagenbuch N, et al. A schizophrenia-related sensorimotor deficit links alpha 3-containing GABAA receptors to a dopamine hyperfunction. Proceedings of the National Academy of Sciences of the United States of America. 2005 Nov 22;102(47):17154-9. PubMed PMID: 16284244. Pubmed Central PMCID: 1288020. Epub 2005/11/15. eng.
33. Seifi M, Swinny JD. Immunolocalization of AMPA receptor subunits within the enteric nervous system of the mouse colon and the effect of their activation on spontaneous colonic contractions. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society. 2016 May;28(5):705-20. PubMed PMID: 26867789.
34. Everington EA, Gibbard AG, Swinny JD, Seifi M. Molecular Characterization of GABA-A Receptor Subunit Diversity within Major Peripheral Organs and Their Plasticity in Response to Early

621 Life Psychosocial Stress. Frontiers in molecular neuroscience. 2018;11:18. PubMed PMID: 29467616.
622 Pubmed Central PMCID: 5807923.
623 35. Corteen NL, Carter JA, Rudolph U, Belelli D, Lambert JJ, Swinny JD. Localisation and stress-
624 induced plasticity of GABA receptor subunits within the cellular networks of the mouse dorsal raphe
625 nucleus. Brain structure & function. 2014 Jun 29. PubMed PMID: 24973971.
626 36. Seifi M, Rodaway S, Rudolph U, Swinny JD. GABAA Receptor Subtypes Regulate Stress-Induced
627 Colon Inflammation in Mice. Gastroenterology. 2018 May 23. PubMed PMID: 29802853.
628 37. Etzerodt A, Moestrup SK. CD163 and inflammation: biological, diagnostic, and therapeutic
629 aspects. Antioxidants & redox signaling. 2013 Jun 10;18(17):2352-63. PubMed PMID: 22900885.
630 Pubmed Central PMCID: 3638564. Epub 2012/08/21. eng.
631 38. Foong JP, Nguyen TV, Furness JB, Bornstein JC, Young HM. Myenteric neurons of the mouse
632 small intestine undergo significant electrophysiological and morphological changes during postnatal
633 development. The Journal of physiology. 2012 May 15;590(10):2375-90. PubMed PMID: 22371477.
634 Pubmed Central PMCID: 3424759.
635 39. Giaroni C, Knight GE, Zanetti E, Chiaravalli AM, Lecchini S, Frigo G, et al. Postnatal development
636 of P2 receptors in the murine gastrointestinal tract. Neuropharmacology. 2006 May;50(6):690-704.
637 PubMed PMID: 16434064.
638 40. Zizzo MG, Cavallaro G, Auteri M, Caldara G, Amodeo I, Mastropaolo M, et al. Postnatal
639 development of the dopaminergic signaling involved in the modulation of intestinal motility in mice.
640 Pediatric research. 2016 Sep;80(3):440-7. PubMed PMID: 27089499.
641 41. Ficara M, Pietrella E, Spada C, Della Casa Muttini E, Lucaccioni L, Iughetti L, et al. Changes of
642 intestinal microbiota in early life. The journal of maternal-fetal & neonatal medicine : the official
643 journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania
644 Perinatal Societies, the International Society of Perinatal Obstet. 2018 Sep 10:1-8. PubMed PMID:
645 30058404.
646 42. Nash MJ, Frank DN, Friedman JE. Early Microbes Modify Immune System Development and
647 Metabolic Homeostasis-The "Restaurant" Hypothesis Revisited. Frontiers in endocrinology.
648 2017;8:349. PubMed PMID: 29326657. Pubmed Central PMCID: 5733336.
649 43. Patel BA, Patel N, Fidalgo S, Wang C, Ranson RN, Saffrey MJ, et al. Impaired colonic motility
650 and reduction in tachykinin signalling in the aged mouse. Experimental gerontology. 2014 May;53:24-
651 30. PubMed PMID: 24560671.
652 44. Patel BA, Fidalgo S, Wang C, Parmar L, Mandona K, Panossian A, et al. The TNF-alpha
653 antagonist etanercept reverses age-related decreases in colonic SERT expression and faecal output in
654 mice. Scientific reports. 2017 Feb 15;7:42754. PubMed PMID: 28198447. Pubmed Central PMCID:
655 5309893.
656 45. Hermans L, Leunissen I, Pauwels L, Cuypers K, Peeters R, Puts NAJ, et al. Brain GABA Levels
657 Are Associated with Inhibitory Control Deficits in Older Adults. The Journal of neuroscience : the
658 official journal of the Society for Neuroscience. 2018 Sep 5;38(36):7844-51. PubMed PMID: 30064995.
659 Pubmed Central PMCID: 6125814.
660 46. Ben-Ari Y, Cherubini E, Corradetti R, Gaiarsa JL. Giant synaptic potentials in immature rat CA3
661 hippocampal neurones. J Physiol. 1989 Sep;416:303-25. PubMed PMID: 2575165. Pubmed Central
662 PMCID: 1189216.
663 47. Ben-Ari Y, Khalilov I, Kahle KT, Cherubini E. The GABA excitatory/inhibitory shift in brain
664 maturation and neurological disorders. Neuroscientist. 2012 Oct;18(5):467-86. PubMed PMID:
665 22547529.
666 48. Gilon P, Reusens-Billen B, Remacle C, Janssens de Varebeke P, Pauwels G, Hoet JJ. Localization
667 of high-affinity GABA uptake and GABA content in the rat duodenum during development. Cell Tissue
668 Res. 1987 Sep;249(3):593-600. PubMed PMID: 3664607.
669 49. Roberts RR, Murphy JF, Young HM, Bornstein JC. Development of colonic motility in the
670 neonatal mouse-studies using spatiotemporal maps. Am J Physiol Gastrointest Liver Physiol. 2007
671 Mar;292(3):G930-8. PubMed PMID: 17158255.

50. Heuckeroth RO. Hirschsprung disease - integrating basic science and clinical medicine to improve outcomes. Nature reviews Gastroenterology & hepatology. 2018 Mar;15(3):152-67. PubMed PMID: 29300049.
51. Rissman RA, Mobley WC. Implications for treatment: GABAA receptors in aging, Down syndrome and Alzheimer's disease. Journal of neurochemistry. 2011 May;117(4):613-22. PubMed PMID: 21388375. Pubmed Central PMCID: 3127285.
52. Vela J, Gutierrez A, Vitorica J, Ruano D. Rat hippocampal GABAergic molecular markers are differentially affected by ageing. Journal of neurochemistry. 2003 Apr;85(2):368-77. PubMed PMID: 12675913.
53. Limon A, Reyes-Ruiz JM, Miledi R. Loss of functional GABA(A) receptors in the Alzheimer diseased brain. Proceedings of the National Academy of Sciences of the United States of America. 2012 Jun 19;109(25):10071-6. PubMed PMID: 22691495. Pubmed Central PMCID: 3382476.
54. Miller SM, Kalanjati VP, Colditz PB, Bjorkman ST. Developmental Changes in Expression of GABAA Receptor Subunits alpha1, alpha2, and alpha3 in the Pig Brain. Developmental neuroscience. 2017;39(5):375-85. PubMed PMID: 28472809.
55. Ma X, Sun Q, Sun X, Chen D, Wei C, Yu X, et al. Activation of GABAA Receptors in Colon Epithelium Exacerbates Acute Colitis. Frontiers in immunology. 2018;9:987. PubMed PMID: 29867964. Pubmed Central PMCID: 5949344.
56. Firth M, Prather CM. Gastrointestinal motility problems in the elderly patient. Gastroenterology. 2002 May;122(6):1688-700. PubMed PMID: 12016432.

Figures

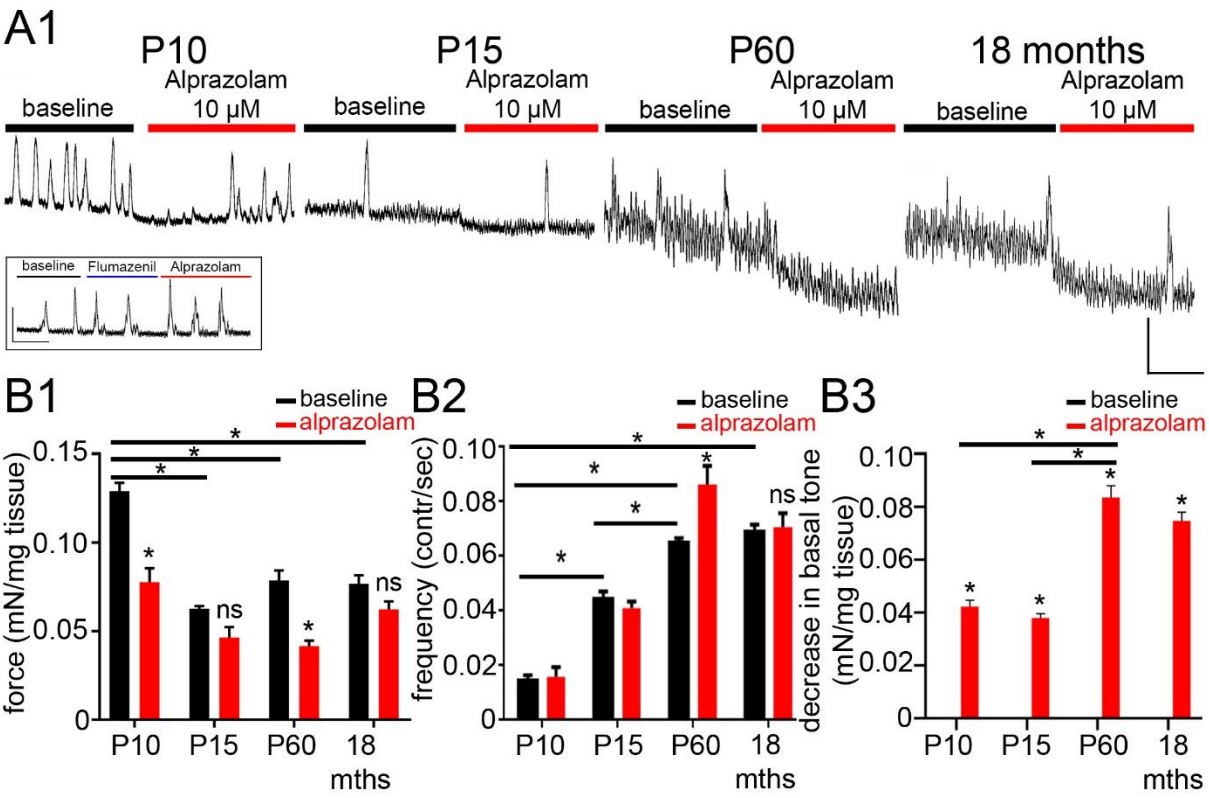

Figure 1

Spontaneous and $GABA_AR$-mediated colonic contractility is age dependent

(A) representative traces demonstrating changes in the spontaneous longitudinal muscle contractions of mouse colon at different ages, *in vitro*, and their responses to the application of the benzodiazepine alprazolam 10 µM. The pre-application of the benzodiazepine antagonist flumazenil abolished the effect of alprazolam (boxed trace).

(B) quantification of (B1) the force and (B2) the frequency of spontaneous longitudinal muscle contractions of mouse colon at different ages and how these parameters change in response to the application of 10 µM alprazolam. (B3) changes in the relaxant effect of alprazolam on colonic basal tone at different ages. Bars represent means and the lines represent the SEM. N = 8 animals, * $P < 0.05$, repeated measures ANOVA with Tukey's posthoc test. Scale bars, vertical 0.1 grams, horizontal 5 minutes.

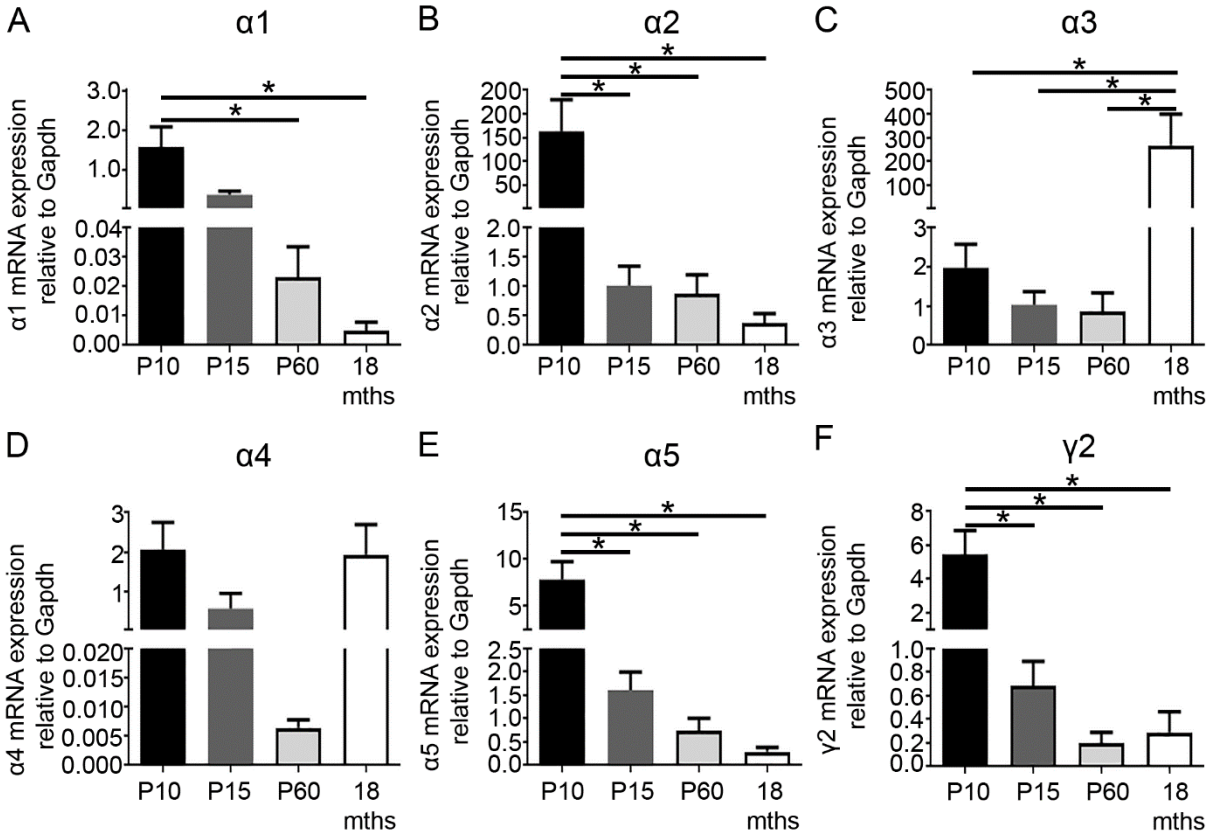

Figure 2

Colonic $GABA_AR$ subunit mRNA expression is age dependent

(A-F) quantification of the mRNA expression levels of the $GABA_AR$ α 1-5 and γ2 subunits respectively, in the mouse colon at different ages, relative to the housekeeping gene Gapdh, using qPCR. Bars represent means and the lines represent the SEM. N = 8 animals, * $P < 0.05$, ANOVA with Tukey's posthoc test.

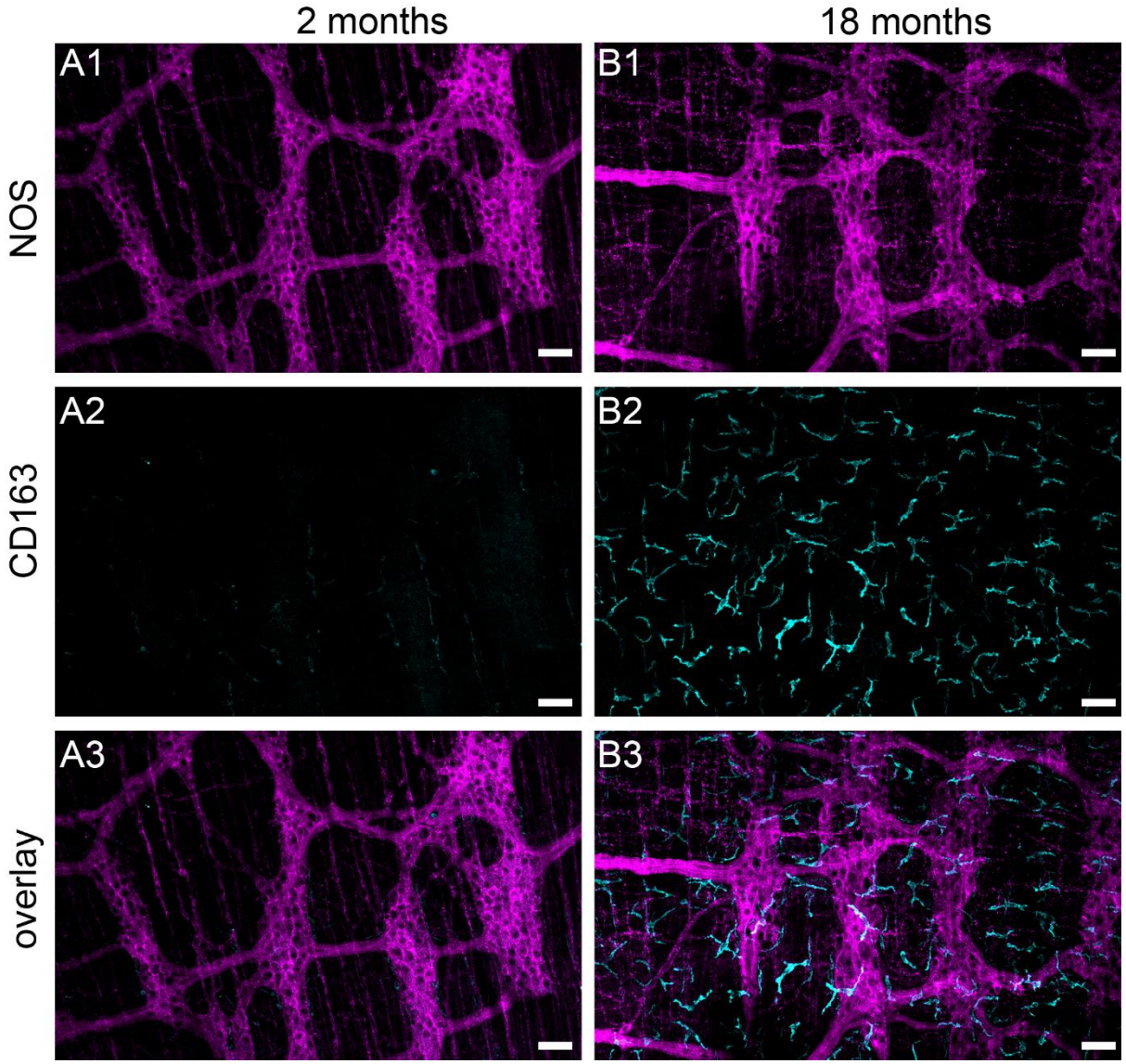

Figure 3

Native colonic inflammation increases with age

(A1) immunoreactivity for nitric oxide synthase (NOS), used to identify ENS plexuses in the colon of a mouse at 2 months of age. (A2) in the same field of view, immunoreactivity for CD163, a receptor expressed on activated monocytes and/or macrophages and thus a marker of inflammation. (A3) is an overlay of (A1 and A2). (B1) immunoreactivity for NOS in the colon of a mouse at 18 months of age, reacted and imaged under conditions identical to tissue from a 2 month old mouse. (B2) in the same field of view, immunoreactivity for CD163. Note the dramatic increase in the density of immunoreactive profiles indicating a significant age-dependent increase in colonic inflammation. (B3) is an overlay of (B1 and B2). Scale bars, 50 μm.

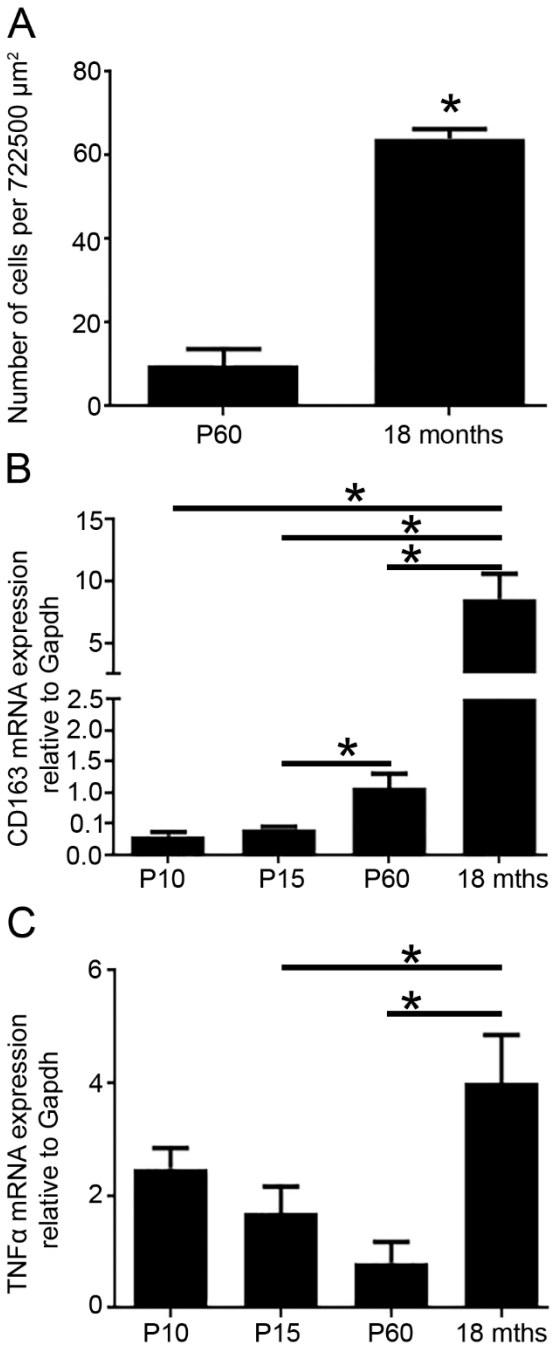

Figure 4

Quantification of the expression of inflammatory mediators in whole segments of the colon at different ages.

(A) quantification of the density of CD163-immunoreactive profiles in the ENS of mice 2 and 18 months of age. (B-C) quantification of the mRNA expression levels of CD163 and TNFα respectively, in the colon of mice 2 and 18 months of age, relative to the housekeeping gene Gapdh, using qPCR. Bars represent means and the lines represent the SEM. N = 6 animals, * $P < 0.05$, unpaired Student's *t* test and ANOVA with Tukey's posthoc test.

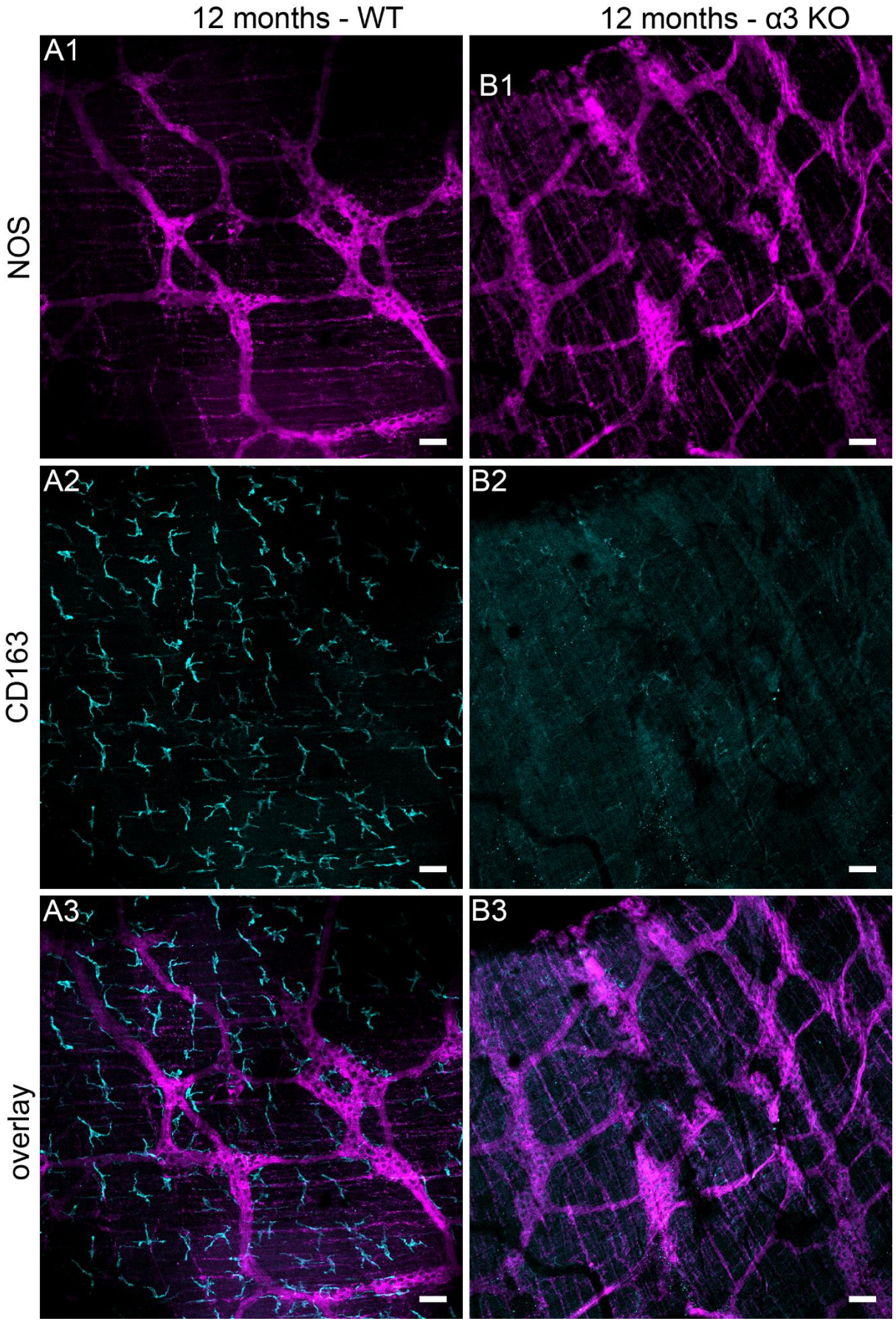

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Figure 5

$GABA_AR$ α3 subunit deletion prevents age-dependent colonic inflammation

(A1) immunoreactivity for NOS in the colon of a 12 month old wild type (WT) mouse. (A2) in the same field of view, immunoreactivity for CD163. (A3) is an overlay of (A1 and A2). (B1) immunoreactivity for NOS in the colon of a 12 month old $GABA_AR$ α3 subunit deleted (α3 KO) mouse, reacted and imaged under identical conditions to WT tissue. (B2) in the same field of view, immunoreactivity for CD163. Note the significant decrease in immunoreactive profiles indicating an absence of age-dependent colonic inflammation in the absence of the $GABA_AR$ α3 subunit. (B3) is an overlay of (B1 and B2). Scale bars, 50 μm.

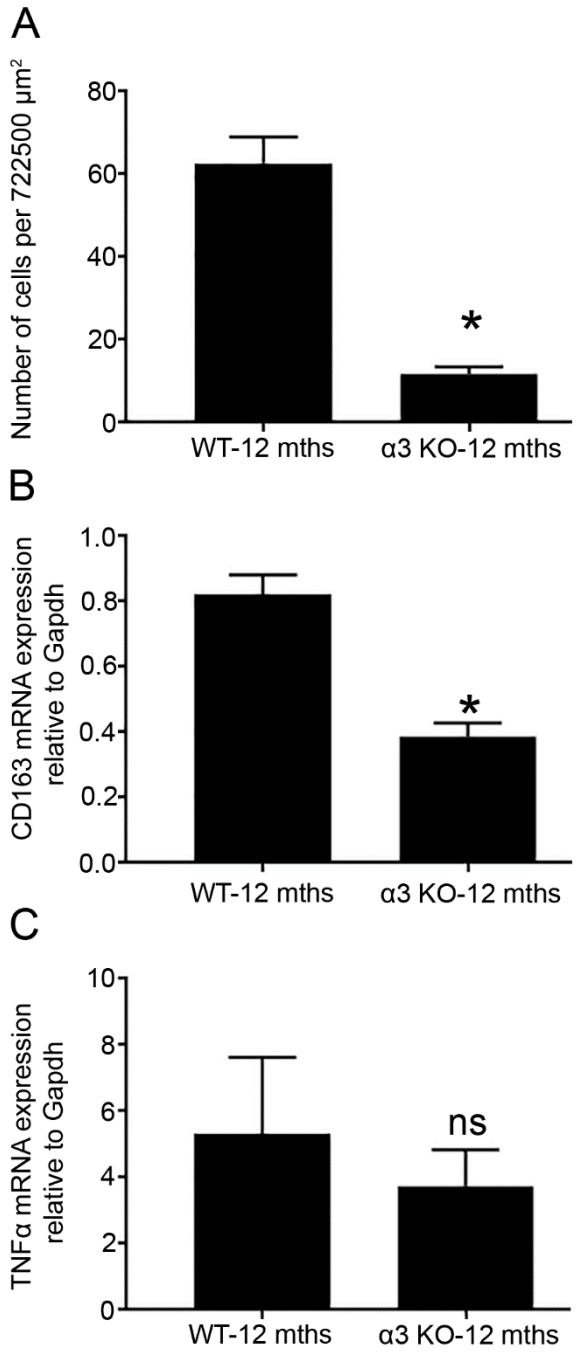

Figure 6

Quantification of the expression of inflammatory mediators in the colon of aged wild type and $GABA_AR$ α3 KO mice.

(A) quantification of the density of CD163-immunoreactive profiles in the ENS of WT and α3 KO mice at 12 months of age. (B-C) quantification of the mRNA expression levels of CD163 and TNFα respectively, in the colon of WT and α3 KO mice at 12 months of age, relative to the housekeeping gene Gapdh, using qPCR. Bars represent means and the lines represent the SEM. N = 6 animals, * $P < 0.05$, unpaired Student's *t* test.