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Age-related differences of γ-aminobutyric acid (GABA)ergic transmission in human colonic smooth muscle

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Abstract

Background: Enteric neurons undergo to functional changes during aging. We investigated the possible age-associated differences in enteric γ-aminobutyric acid (GABA) ergic transmission evaluating function and distribution of GABAergic receptors in human colon.

Methods: Mechanical responses to GABA and GABA receptor agonists on slow phasic contractions were examined in vitro as changes in isometric tension in colonic muscle strips from young (<65 years old) and aged patients (>65 years old). GABAergic receptor expression was assessed by quantitative RT-PCR.

Key Results: In both preparations GABA induced an excitatory effect, consisting in an increase in the basal tone, antagonized by the GABAA receptor antagonist, bicuculline, and potentiated by phaclofen, GABAB receptor antagonist.Tetrodotoxin (TTX) and atropine-sensitive contractile responses to GABA and GABAA receptor agonist, muscimol, were more pronounced in old compared to young subjects. Baclofen, GABAB receptor agonist, induced a TTX-sensitive reduction of the amplitude of the spontaneous. Nω−nitro-L-arginine methyl ester (L-NAME), nitric oxide (NO) synthase inhibitor abolished the inhibitory responses in old preparations, but a residual responses persisted in young preparations, which in turn was abolished by suramin, purinergic receptor antagonist. α3−GABAA receptor subunit expression tends to change in an age-dependent manner.

Conclusions and inferences: Our results reveal age-related differences in GABAergic transmission in human colon. At all the age tested GABA regulates muscular contractility modulating the activity of the intrinsic neurons. Activation of GABAA receptor, through acetylcholine release, induces contraction, which increases in amplitude with age. GABAB receptor activation leads to neural release of NO and purines, being a loss of purinergic-component in aged group.

KEYWORDS
Aging, GABA, GABAergic receptors; GABAA receptor subunit, intestinal motility

1 | INTRODUCTION

Aging is an inevitable process and, conventionally, "elderly" has been defined as a chronological age of 65 years old or older.[1] Functional impairment, and prominent physiologic changes associated with aging include alterations on the gastrointestinal (GI) tract, causing progressive deterioration of the physiological function and greater incidences of GI disorders. Specifically, the prevalence of motility disorders, such as fecal incontinence and constipation, substantially increases as a function of age; for instance, approximately 30%–40% of people aged 65 years old or older self-report constipation. Although the precise mechanism correlating enteric senescence and alterations of GI motility is unclear, dysmotility may be the result of degenerative neural mechanisms in the enteric nervous system (ENS) controlling GI smooth muscle contractility.

It is established that in the postnatal period the mammalian enteric nervous system undergoes developmental changes.[2-4] It is also clear that the cells of the adult ENS exhibit plasticity and continue to undergo changes later in life, due both to the highly dynamic nature of the GI system and to cellular changes occurring the aging process. A growing body of evidence on animal and human preparations reported that ENS in the human GI tract reveals significant progressive neuronal modifications throughout the lifetime in health and disease.[5-7] However to date, the reports on the enteric neuronal population affected by the aging process have been conflicting; several studies in aging rodents suggest loss of myenteric neurons[8] and a decline in the density of nNOS and ACh nerve fibers.[8,9] About humans, studies have generated contradictory findings likely due to small sample sizes with an unclear medical history and heterogeneous experimental approaches.[10] Recent evidence reveals functional changes unrelated to nitrergic system and explained by a decline in cholinergic function in ascending colon.[7]

γ-Aminobutyric acid (GABA), a well-known inhibitory neurotransmitter in the mammalian central nervous system (CNS),[11] is capable of mediating a rich variety of cellular communication patterns, by engaging a multitude of molecularly and functionally diverse GABA receptor subtypes: namely ionotropic GABAA (including GABAC) receptors or metabotropic GABAB receptors.[12] GABAA receptors (GABAAR), the major class of GABA receptors, are principally engaged in mediating the rapid effects of GABA. GABAARs are composed of subunits, which assemble in a heteropentameric structure to form an anion-permeable ion channel; so far 19 molecularly distinct subunits have been identified: α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3. As a result, GABAARs composed of various subunit combinations give rise to numerous receptor subtypes.

In the CNS molecularly distinct GABAAR subunits are diverse according to their cellular and subcellular expression patterns,[13] their activation and deactivation kinetics,[14] and their gating by different pharmacological ligands.[15] Moreover, the composition of the receptors varies with the development, but also with exposure to molecules such as steroids or benzodiazepines.[16] Because enteric neurons sustain elevated intracellular Cl− concentration,[17] maintained by a specific sodium-potassium-chloride symporter, activation of GABAA receptors in the ENS results in an excitatory effect, in contrast to the well-known inhibitory effect in the CNS.[18]

In the last few decades, several pieces of evidences have proven that GABAergic interneurons and GABA receptors are not restricted to CNS but are also distributed within the ENS.[19-21] In addition, GABA has been localized in enteric endocrine-like cells implicating GABA also as an endocrine mediator in the GI tract, with a multifunctional role in the regulation of GI activity. In particular, approximately 5–8% of myenteric neurons, which largely regulate GI motility, contain GABA,[22] although the precise role of GABA in gastrointestinal motility is still far to be clear. Seifi & Swinny,[23] recently reported a dynamic adaptation GABAergic system in mouse colon, over the course of lifetime, suggesting a role of the role of GABAAR α-subunit in mediating colonic contractility changes from the early postnatal period through to late adulthood.

However, the expression and functional plasticity of this neurotransmitter system at different ages in the human gut, and in particular in the colon is largely unexplored. Thus, this study aimed to determine, using pharmacological and molecular approaches, the effects of GABA on the spontaneous mechanical activity of the human colon and whether age-associated changes in GABAergic transmission can be identified in young *vs* aged subjects.

2 | MATERIALS AND METHODS

2.1 | Human tissue specimens

The study is conforming to Good Publishing Practice in Physiology.[24] Specimens of human sigmoid colon from patients of both sex, undergoing surgery for neoplastic conditions were subdivided in two distinct age groups, 37–65 years (young group $n = 10$; $n = 5$ for both males and females) and 66–93 years (old group, $n = 12$; $n = 6$ for both males and females). All the patients showed no symptoms of major clinical motility disorders. The experimental protocol was approved by the Institutional Ethics Committee of the Azienda di Rilievo Nazionale ad Alta Specializzazione (A.R.N.A.S.), Ospedale Civico Di Cristina Benfratelli-Palermo, and written informed consent was signed by the patients before surgery. Specimens were taken from a macroscopically normal region, at least 5 cm far from any visible lesion. Sigmoid colon tissues were stripped free of the mucosal layer, placed in pre-oxygenated Krebs solution and stored overnight at 4°C. Gastrointestinal tissue functionality in neuromuscular experiments is not modified by storage up to 24 h at 4°C.[25-27] Six samples were used for biomolecular analysis. Strips free of the mucosal layer were collected in sterile tubes and stored at −80°C.

2.2 | Preparation of circular muscle strips

Methods have been already described in a previous study.[28] Briefly, the colonic segments were opened along the mesenteric border, carefully cleaned to prevent soiling of the muscle layers with fecal

TABLE 1 Sequences of primers used for qRT-PCR[52]

Gene description	Forward primer sequence	Reverse primer sequence
β-actin	5'- TCCCTTGCCATCCTAAAGCCACC –3'	5'-CTGGGGCCATTCTCCTTAGAGAGAAG–3'
GABAAR α2	5'- GTTCAAGCTGAATGCCCAAT–3'	5'-ACCTAGAGCCATCAGGAGCA–3'
GABAARα3	5'- CAACTTGTTTCAGTTCATTCATCCTT–3'	5'-CTTGTTTGTGTGATTATCATCTTCTTAGG–3'
GABAAR γ2	5'-CACAGAAAATGACGGTGTGG–3'	5'-TCACCCTCAGGAACTTTTGG–3'

content, and pinned to the surface of a dissecting dish filled with oxygenated Krebs' Ringer solution to easily remove mucosa and submucosa. Muscle strips, approximately 4 mm wide and 10 mm long, were cut with the long axis lying in the direction of the circular muscle fibers in the inter-taenial region. Our attention was focused on circular muscle since, although data from humans to investigate age-related changes in the colon are limited, several evidence suggests that modifications occur with growth in this muscular layer[29] Strips were suspended in the organ baths containing 10 ml of oxygenated (95% O_2 and 5% CO_2) Krebs solution maintained at 37°C, subjected to an initial tension of 1 g and allowed to equilibrate for at least 2 h to allow the developing of rhythmic spontaneous contractions. At the end of the equilibration period, preparations were challenged with 1 μmol/L carbachol (CCh) or with 1 μmol/L Isoproterenol (Iso) until stable responses were obtained. 1 μmol/L Iso induced a relaxant response consisting in a decrease in tension below the basal tone with an amplitude of 2.30 ± 0.17 g ($n = 6$) and 2.42 ± 1.30 g ($n = 6$) in the young and in the old group respectively. There was no difference in the response to CCh or Iso between the two groups ($p > 0.05$).

In both groups, concentration-dependent cumulative curves were constructed by cumulative addition of GABA before and after treatment with selective GABAergic receptor antagonists. Moreover, selective GABAA, GABAB and GABAC receptor agonists were tested in both preparations. Agonists were applied for 6 min before adding the higher concentration. Time control experiments showed that a second curve to the agonists was reproducible. A submaximal dose of GABAA or GABAB agonists was tested in the presence of the neural blocker, tetrodotoxin, the muscarinic receptor antagonist, atropine, the purinergic receptor antagonist, suramin, or the nitric oxide synthase inhibitor, N(ω)-nitro-L-arginine-methyl ester (L-NAME).

Each preparation was tested with a single agonist/antagonist, except when otherwise stated. Concentrations of the drugs used were determined from literature.

2.3 | Solutions and drugs

The following drugs were used and stock solutions were prepared using distilled water or as indicated below. Atropine, baclofen, bicuculline, carbachol (CCh), gamma aminobutyric acid (GABA) isoproterenol (Iso), muscimol, N(ω)-nitro-L-arginine-methyl ester (L-NAME), 1,2,5,6-Tetrahydropyridin-4-yl methylphosphinic acid hydrate (TPMPA), 4-cis-aminocrotonic acid (CACA), suramin tetrodotoxin citrate (TTX) all purchased from Sigma (Sigma Aldrich, Inc., St. Louis, USA); phaclofen was from Tocris (Tocris Cookson Ltd.). Bicuculline was dissolved in dimethyl sulphoxide (DMSO) and phaclofen in 0.1 N NaOH. All the other drugs were dissolved in distilled water. The working solutions were prepared fresh on the day of the experiment by diluting the stock solutions in Krebs. Drugs were added to the organ bath in volumes of <1.0% of the bathing solution. Control experiments using DMSO or NaOH alone showed that both do not affect the spontaneous or evoked contractile activity.

2.4 | RNA preparation and real-time PCR analysis

Total RNA was extracted from colon tissues using Illustra™ RNAspin Mini RNA Isolation Kit (GE Healthcare, Little Chalfont) according to manufacturer's instructions. RNA was then reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem).

For quantitative SYBR Green Real-time PCR, the reaction was carried out in a total volume of 20 μl containing 2× SYBR Green I Master Mix (Applied Biosystems), 2 μl of cDNA, and 300 nM forward and reverse primers using the Step-One Real-Time PCR System (Applied Biosystems). The oligonucleotides used are reported in Table 1.

Relative changes in the target mRNA between young and old samples were determined using the ΔΔCt method. Levels of the target transcripts were normalized to β-actin, a housekeeping gene constantly expressed in all samples (ΔC_t). Final values were expressed as $2^{-(\Delta Ct)}$ versus β–actin.

2.5 | Statistical analysis

All data are means ± SEM: "*n*" indicates the number of patients. The excitatory responses induced by GABAergic drugs were estimated as increase in tension above the basal tone set as baseline and reported as a percentage of the increase of tone induced by 1 μmol/L CCh. Inhibitory response to Isoproterenol was calculated as the decrease in tension below the basal tone set as baseline. Baclofen did not induce a muscular relaxation but an inhibition of the amplitude of the slow phasic contractions and this effect was calculated as a decrease in the amplitude of the slow phasic contractions during 5 min after drug administration, normalized to the amplitude of the slow phasic contractions measured 5 min prior to the treatment, set as 100%.

Responses were fitted to sigmoid curves (Prism 5.0; Graph-PAD) and EC_{50} values with 95% confidence limits (CLs) were determined.

Statistically significant differences were calculated by Student's *t*-test or by means of analysis of variance, followed by Bonferroni's test, when appropriate. Spearman analysis was used for the correlation studies. A probability value less than.05 was regarded as significant.

3 | RESULTS

3.1 | Circular muscle contractility and GABA response in human colon

Circular muscular strips from the human colon, once mounted in the organ bath, developed a spontaneous activity characterized by rhythmic contractions with amplitude and frequency not significant different in the two age groups (young group ($n = 10$): amplitude of 1.67 ± 0.14 g in and frequency of 2.15 ± 0.56 c.p.m (contractions per minute); old group ($n = 12$): amplitude of 1.79 ± 0.27 g and frequency of 2.43 ± 0.50 c.p.m., $p > 0.05$) Correlation analysis revealed that neither in females nor in males of both groups the amplitude of spontaneous contractions was increased with age ($r = -0.2$, $p = 0.783$ in young males; $r = -0.5$, $p = 0.455$ in young females, $r = 0.14$, $p = 0.80$ in old males, $r = 0.08$, $p = 0.91$in old females).

1 μmol/L carbachol (CCh) induced a contractile response with an amplitude of 7.87 ± 0.67 g ($n = 8$) and of 8.49 ± 0.96 mg ($n = 10$) in the young and the old group respectively. Correlation analysis revealed that neither in females nor in males of both groups the CCh response was increased with age ($r = 0.10$, $p = 0.95$ in young males; $r = 0.31$, $p = 0.683$ in young females, $r = 0.18$, $p = 0.71$ in old males, $r = 0.1$, $p = 0.95$ in old females).

In the preparations of both groups cumulative addition of GABA (3 μmol/L – 3 mmol/L), induced a concentration-dependent contractile effect reaching the maximal amplitude at the dose of 3 mmol/L.

The GABA contractile response was significantly more pronounced n the preparations from the old group compared with the young one, suggesting an age difference effect, as shown in Figure 1A,B. In detail, E_{MAX} was about 44% and 64% of the amplitude of CCh 1 μmol/L induced contraction in the young and in the old group respectively (Figure 1C, $p < 0.05$). There was no difference in the increase of EMAX and EC_{50} for GABA response between gender in both groups (Figure 1C, $p > 0.05$).

Moreover, there is also a change in the agonist potency, which resulted to be increased in old compared young group (Table 2).

The contractile response to GABA in the preparations from both groups was significantly antagonized by bicuculline (10 μmol/L), a competitive antagonist of GABAA receptors (Figure 2; Table 2), which *per se* did not modify the amplitude or the frequency of spontaneous activity.

Moreover, phaclofen (10 μmol/L), selective competitive antagonist of GABAB receptor antagonist, increased the contractile response to GABA, whilst TPMPA (10 μmol/L), a selective GABAC receptor antagonist, was ineffective in both groups (Figure 2; Table 2).

Although preparations from both groups were able to relax to Isoproterenol 1 μmol/L, a relaxation to GABA was never observed, even in preparation precontracted with CCh (data not shown).

3.2 | Pharmacological characterization of GABAergic agonist effects

Muscimol (3 μmol/L–1 mmol/L), selective GABAA receptor agonist, mimicked the contractile GABA response, in both groups. Also the

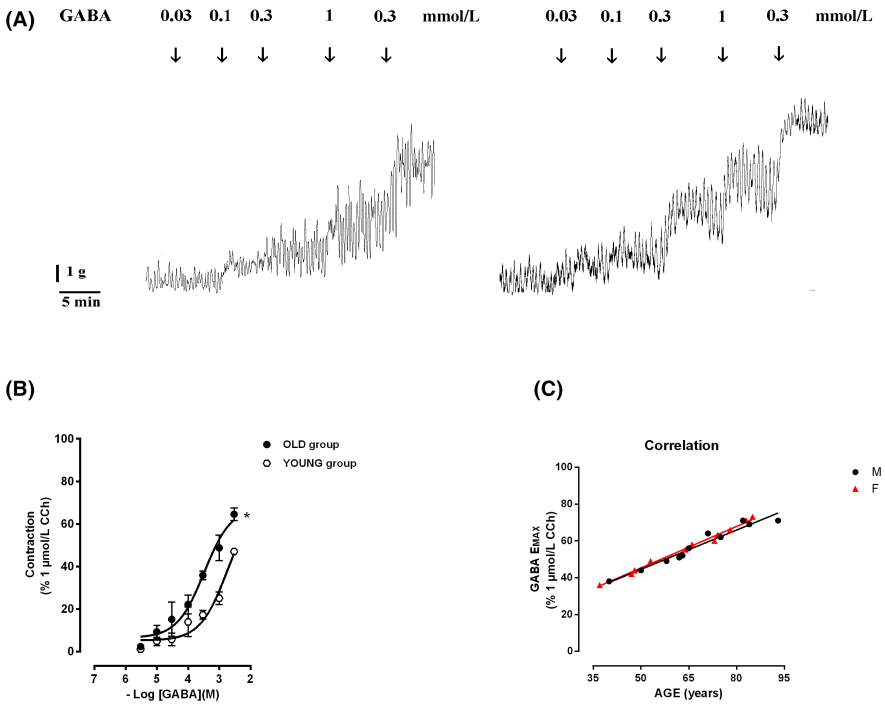

FIGURE 1 Age-related differences in GABA response. A, Original recordings showing the responses evoked by GABA in young and old human colonic circular smooth muscle strips. B, Concentration-response curves to GABA in young ($n = 10$) and old ($n = 12$) human colonic circular smooth muscle strips. C, Spearman correlation analysis of GABA EMAX and age in females ($r = 1$, $p < 0.01$) and males ($r = 0.97$, $p < 0.05$). Data are means ± SEM. Contractile responses are expressed as a percentage of response to 1 μmol/L carbachol (CCh). *$p < 0.05$ when compared to the young group

TABLE 2 Effects of GABAergic agonists before and after treatments with antagonists

	Young group				Old group			
	EC_{50}	95% CI	E_{MAX}	*n*	EC_{50}	95% CI	EMAX	*n*
GABA	1.7 mmol/L	0.6–5.7 mmol/L	3.47 ± 0.32g	10	0.3 mmol/L*	0.1–0.7 mmol/L	5.2 ± 0.16 g*	12
GABA+BICUCULLINE	7.0 mmol/L§	0.7–98 mmol/L	3.18 ± 0.4 g	5	3.5 mmol/L§	0.9–3.8 mmol/L	4.6 ± 0.26g*	5
GABA+PHACLOFEN	0.6 mmol/L§	0.3–0.9 mmol/L	4.68 ± 0.24 g	5	0.2 mmol/L§	0.01–0.3 mmol/L	6.5 ± 0.18 g	5
MUSCIMOL	0.3 mmol/L	0.1–0.6 mmol/L	3.98 ± 0.18 g	5	0.06 mmol/L*	0.04–0.1 mmol/L	6.0 ± 0.1g*	5
MUSCIMOL+BICUCULLINE	1.8 mmol/L	0.9–2.9 mmol/L	3.41 ± 0.34 g	5	0.36 mmol/L	0.1–0.91 mmol/L	5.5 ± 0.38	5
BACLOFEN	0.04 mmol/L	0.02–0.06 mmol/L	−96 ± 3% spontaneous contraction	5	0.07 mmol/L	0.04 −0.10 mol/L	−97 ± 2% spontaneous contraction	5
BACLOFEN+PHACLOFEN	0.21 mmol/L§	0.08–0.28 mmol/L	−91 ± 3% spontaneous contraction	5	0.28 mmol/L§	0.06–0.36 mmol/L	−92 ± 2% spontaneous contraction	5

*$p < 0.05$ when compared to young group. §$p < 0.05$ when compared to the respective own control condition.

contractile response to muscimol was significantly increased in preparations from old group compared with young group (Table 2, Figure 3A). The maximal response to muscimol was about 46% and about 76% of the response to CCh 1 μmol/L in young in old group, respectively. E_{MAX} value of muscimol in old compared with young group, was significantly different, confirming an age difference effect (Table 2, Figure 3A). Muscimol-induced effects were antagonized in a competitive manner by the selective GABAA receptor antagonist, bicuculline (10 μmol/L) (Table 2, Figure 3B). Phaclofen (10 μmol/L), GABAB receptor antagonist, or TPMPA (10 μmol/L), GABAC receptor antagonist, were unable to modify the response induced by muscimol (Figure 3B).

In the preparations of both groups the contractile response to submaximal dose of muscimol (0.3 mmol/L), was antagonized by TTX (1 μmol/L), blocker of neuronal voltage-dependent sodium channels, which *per se* did not modify the amplitude of the spontaneous contractions and abolished by atropine (1 μmol/L), a muscarinic receptor antagonist (Figure 3B).

Baclofen, a selective GABAB receptor agonist, in the range from 3 to 500 μmol/L, induced a concentration-dependent reduction of the phasic spontaneous activity (Table 2) No marked differences in the inhibitory response to baclofen have been observed between the groups (Figure 4A, Table 2). Baclofen effect was antagonized in a competitive manner by phaclofen (10 μmol/L), selective GABAB receptor antagonist (Figure 4B, Table 2), but unaffected by GABAA and GABAC receptor antagonist. Also the inhibitory response to baclofen was antagonized by TTX (1 μM) (Figure 4B).

Lastly, we tested the possibility that GABAB receptor activation may lead to the release of NO from nitrergic neurons. L-NAME (100 μmol/L), an inhibitor of the NO synthase, partially reduced the inhibitory response induced by the submaximal dose of baclofen (300 μmol/L) in young preparations, whilst the baclofen-inhibitory effect was almost abolished in old preparations. The residual inhibitory effect in the young group was abolished by pretreatment with suramin (0.1 μmol/L), a purinergic receptor antagonist, Figure 4B). L-NAME, or suramin *per se* did not modify the amplitude or the frequency of spontaneous activity.

CACA (0.1–100 μmol/L), a selective GABAC receptor agonist, failed to induce any changes in the mechanical activity in both groups (data not show).

3.3 | GABAA receptor subunit mRNA expression within age

Due to the difference in the responses induced by GABAA receptor activation, we next explored, using qPCR, the possible changes with the age of the expression of GABAAR subunits in our preparations.

We focused our attention on GABAAR α2, α3 and γ2 subunits, given the observations of Seifi[30] that reveals a pivotal role of these subunits in the modulation of mechanical activity in the mouse colon, showing also an expression profile difference in late adulthood.

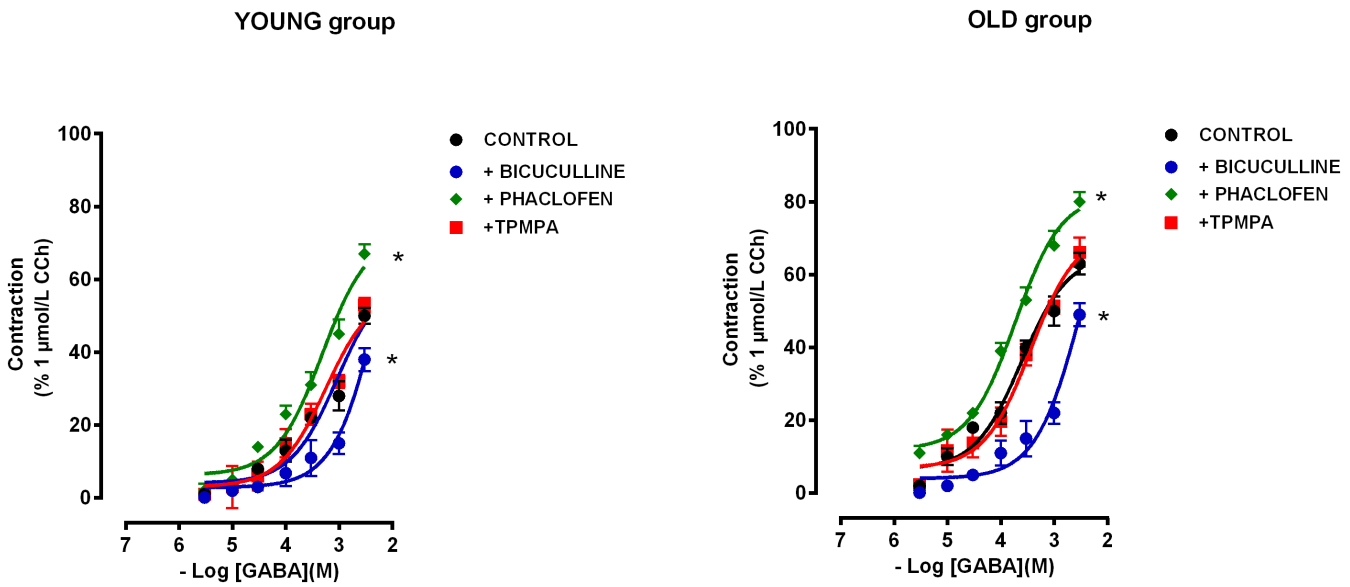

FIGURE 2 Concentration-response curves to GABA in the absence or in the presence of bicuculline, competitive antagonist of GABAA receptors (10 µmol/L, $n = 5$) or Phaclofen, competitive antagonist of GABAB receptors (10 µmol/L, $n = 5$) and TPMPA GABAC receptor antagonist (10 µmol/L, $n = 5$) in young and in old human colonic circular smooth muscle strips. Data are means ± SEM. Contractile responses are expressed as a percentage of response to 1 µmol/L carbachol (CCh). $^*p < 0.05$ compared to the respective own control condition

mRNA expression of the investigated GABAA subunits was present in the human colon at all ages, being α2 subunits more relevant. Our data may suggest an age- and subunit-specific changes in the GABAAR expression. In the old group, there is a decrease in the mRNA expression of the GABAARα3 subunit, although this trend did not reach statistical significance. There is a slight, but not statistically significant, increase of α α2 and γ2 subunit mRNA expression in the old group (Figure 5).

4 | DISCUSSION

Our study provides the first evidence that in human colon GABA participates in the regulation of muscular contractility by modulating the activity of the intrinsic neurons, mainly via activation of GABAA receptors mediating excitatory effects. Age-related differences in GABAA receptor-mediated contraction are revealed, being the response more pronounced in the old compared to the young subjects. This is accompanied by a greater potency and efficacy of GABAAR agonist. A dynamic age-dependent change in the expression of GABAA receptor subunits might be suggested.

Growing evidence indicates that, in the human intestine, changes in GABAergic system are associated with pathological conditions such as IBD,[31] IBS[32] or colon cancer,[33] although its role in physiological condition is less known.

Indeed, the current knowledge on GABA effects, concerning an involvement of GABA in the modulation of gastrointestinal motility[18] are mainly based on animal studies, but so far data about GABAergic transmission in the human gut, and particularly in the large intestine are lacking.

Our results indicate that GABA in the human colon evokes contractile effects mediated by activation of GABAA receptor subtype, as suggested by the effects of GABAA receptor agonist, muscimol and by GABAA receptor antagonist, bicuculline. One of the most interesting finding of this study is the observation that the contractile response via GABAA receptors appears to be significantly powerful in the old group compared to the young group, suggesting an age-related change in GABAergic response.

The elderly, conventionally referred to person aged 65 years old or more,[1] is known to markedly affect the function of most organs and tissues and no exception is for the gastrointestinal system. Gastrointestinal disorders, such as constipation[34,35] fecal incontinence[36] and gastric reflux[37] are very common among the aged population, impacting seriously on the quality of life and healthcare costs.

Although colonic motility has been shown to be impaired in aged patients,[37] in the current study, we did not detect any significant changes in the force or in frequency of spontaneous colonic contractions in the old group in comparison to the young group.

However, the proceeding of GI motility disorders could be the result of different factors not directly associated with the muscle layer functionality, as the modifications of the control mechanism. In particular, age-related changes in enteric neural control due to a degeneration of neurochemically-distinct cell-types have been suggested,[7,38-41] but few studies investigated the modifications in the neurotransmitter receptor systems.[5,42,43]

Despite a number of studies on CNS reporting age-dependent modifications in the GABAergic system, only few previous evidence in animal gut reported age-specific changes in the GABAergic receptor expression,[44] but the lack of human data warrants further research in this area.

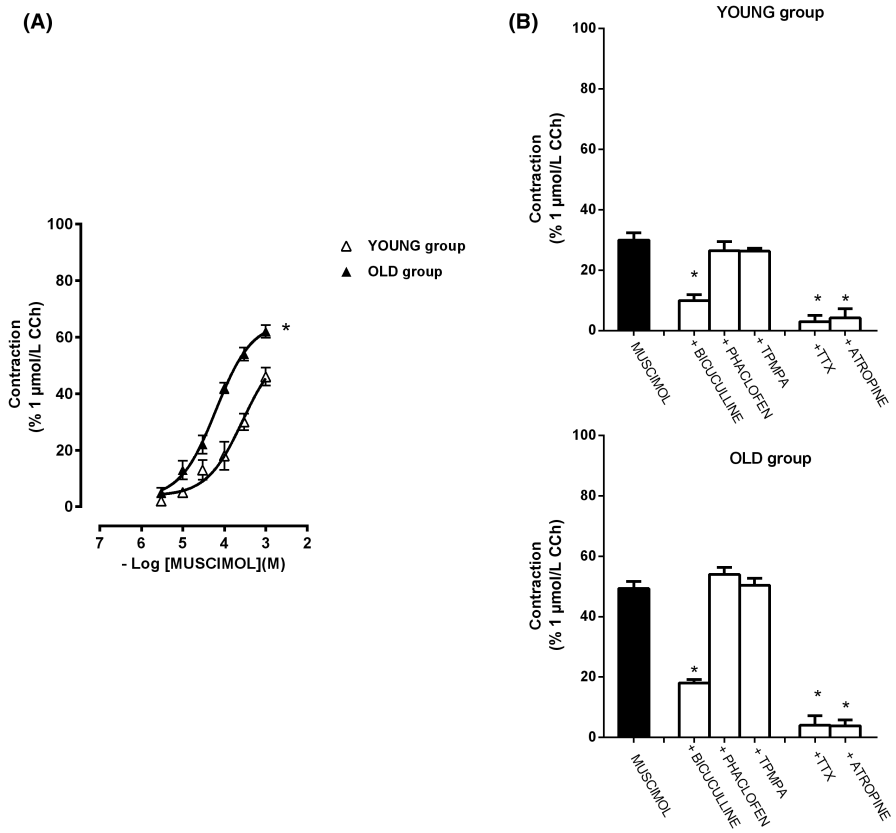

FIGURE 3 A, Concentration-response curves to muscimol, GABAA receptor agonist in young and old human colonic circular smooth muscle strips. Data are means ± SEM. Contractile responses are expressed as percentage of response to 1 µmol/L carbachol (CCh). *$p < 0.05$ when compared to the young group. B, Histogram showing the effects of muscimol (300 µmol/L) in young and old human colonic circular smooth muscle strips in the absence or in the presence of bicuculline, competitive antagonist of GABAA receptors (10 µmol/L) or Phaclofen, competitive antagonist of GABAB receptors (10 µmol/L) and TPMPA, GABAC receptor antagonist (10 µmol/L), TTX, Na^+ voltage-gated neural channel blocker (1 µmol/L), atropine, muscarinic receptor blocker (1 µmol/L), Data are means ± SEM ($n = 5$ each group). Contractile responses are expressed as a percentage of response to 1 µmol/L carbachol (CCh). *$p < 0.05$ compared to the respective own control condition

In our study we observed an increased GABAA-mediated contractile response in tissues from the old group compared to the young one, which prompts us to investigate the nature of GABAergic response in order to identify the mechanism responsible for such a difference.

GABAA-mediated contractile responses involved a neurogenic cholinergic mechanism in both groups since the responses were abolished by the treatment with the nerve toxin, TTX, or with atropine, muscarinic receptor antagonist. Our data are consistent with the data on the pharmacology of GABA receptor in the gut where the activation of GABAA can induce contraction via ENS in the isolate gastrointestinal preparations as antral longitudinal strips from guinea pig[45] or duodenal longitudinal strips from mice.[46] The observation that atropine abolished the responses even in the preparation from old groups, indicates the major role played by acetylcholine also in the elderly. It is possible to exclude an age-related difference in the sensibility to Ach in the colonic tissues since the contractile response to the cholinergic agonist, CCh, was not different between the two experimental groups.

Several studies suggests that there is considerable reserve of neurons in the enteric nervous system, thereafter even if a decline in cholinergic neurons occurs at older ages this may not translate into a change in function until a critical mass is lost[10]

We can speculate that an increase in the number of GABAA receptors or a change in the subunit composition of these receptor subtype can be responsible of an increased in Ach release and in turn in the contractile response.

Interestingly, in both groups, GABA-mediated contractile responses were significantly increased in the presence of phaclofen, GABAB receptor antagonist. These observations could be explained assuming that GABA acts also on GABAB receptors, subserving inhibitory effects on smooth muscle activity. The possibility that in the elderly there was a removal of this negative control leading to a more evident contraction, can be excluded by the observation that specific activation of GABAA receptors by muscimol led *per se* to an enhanced contraction in the old group.

In the brain region-specific age-related GABAAR subunit diversity expression has been observed, either increase or decrease,[47-50]

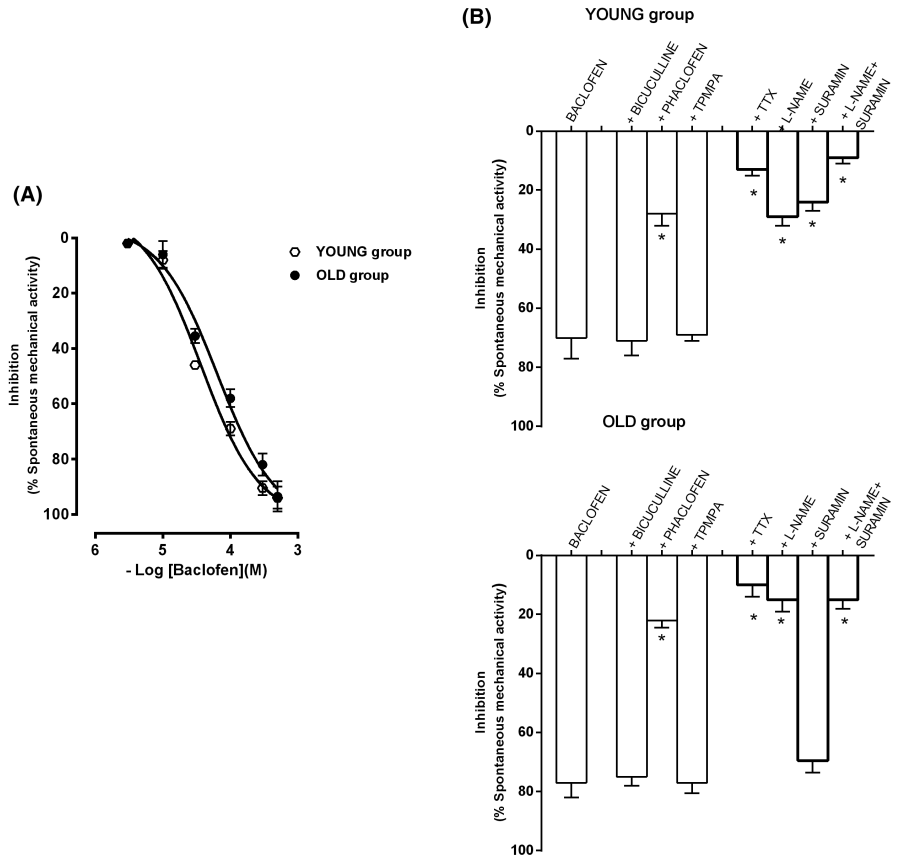

FIGURE 4 A, Concentration-response curves to baclofen, GABAB receptor agonist in young and old human colonic circular smooth muscle strips. Data are means ± SEM. The inhibitory response was expressed as the percentage of the inhibition of spontaneous activity, 100% inhibition corresponding to total suppression of spontaneous contractions. $^*p < 0.05$ when compared to the young group. B, Histogram showing the effects of baclofen (300 μmol/L) in young and old human colonic circular smooth muscle strips in the absence or in the presence of bicuculline, competitive antagonist of GABAA receptors (10 μmol/L) or Phaclofen, competitive antagonist of GABAB receptors(10 μmol/L) and TPMPA GABAC receptor antagonist (10 μmol/L), TTX, Na^+ voltage-gated neural channel blocker (1 μmol/L), L-NAME nitric oxide synthase (NOS) inhibitor (100 μmol/L), suramin, non selective P2 receptor antagonist (100 nmol/L), and L-NAME+suramin. Data are means ±SEM ($n = 4$–5 each group). Inhibitory response was expressed as the percentage of the inhibition of spontaneous activity, 100% inhibition corresponding to total suppression of spontaneous contractions. $^*p < 0.05$ compared to the respective own control condition

and Seifi[44] demonstrated, in mouse colon, a molecular and functional plasticity of the GABAA receptor system over the course of a lifetime, showing a change in the α3 subunit expression in late adulthood (18 months old). Thus, we aimed to evaluate possible differences in the expression of GABAA subunit receptors in our preparations. We focused our attention on GABAA α2, α3 and γ2 receptor subunits, given the observations of Seifi et al[29] that reveals a pivotal role of these subunits in the modulation of mechanical activity in the mouse colon. Our molecular evidences suggest that α2 subunit is predominant in GABAA receptors and there is a likely age-related differences in the expression of GABAA receptor subunits. In particular, a decrease, with a tendency toward statistical significance, was observed in the level of the α3 GABAA receptor subunits, being almost unaltered the mRNA expression of α2 and γ2 subunits. Indeed, reported an opposite tendency in the modification of α3 GABAA receptor subunit in mouse colon.[44] The α subunits, thought to be essential components of "classic" GABAA receptors and their role of in receptor function have been well studied in central nervous system. Moreover, in the brain difference in the α subunits has been found accordingly with the different subcellular localization of GABAA receptors, either synaptic or extrasynaptic localization.[14,51] Whether or not these differences in the contractile responses to GABA in our preparations could be correlate with the different pharmacological responses to GABAA receptor activation or to a different subcellular localization needed further analyses.

Activation of GABAB receptors induced an inhibitory effect, which was also due to the release of inhibitory transmitters from enteric nerves. There was not substantially difference in the potency and efficacy of GABAB receptor agonist between preparations from young and old groups. Surprisingly, we observed a difference in the nature of inhibitory components involved in the GABAergic response in young compared to old preparations. In the young group, the inhibitory effects of baclofen were abolished by co-administration of L-NAME and suramin suggesting that GABA could elicit a neural release of NO together with a purinergic compound, as already observed in mouse gastric preparations.[52] In the old group, GABAB receptor-activation

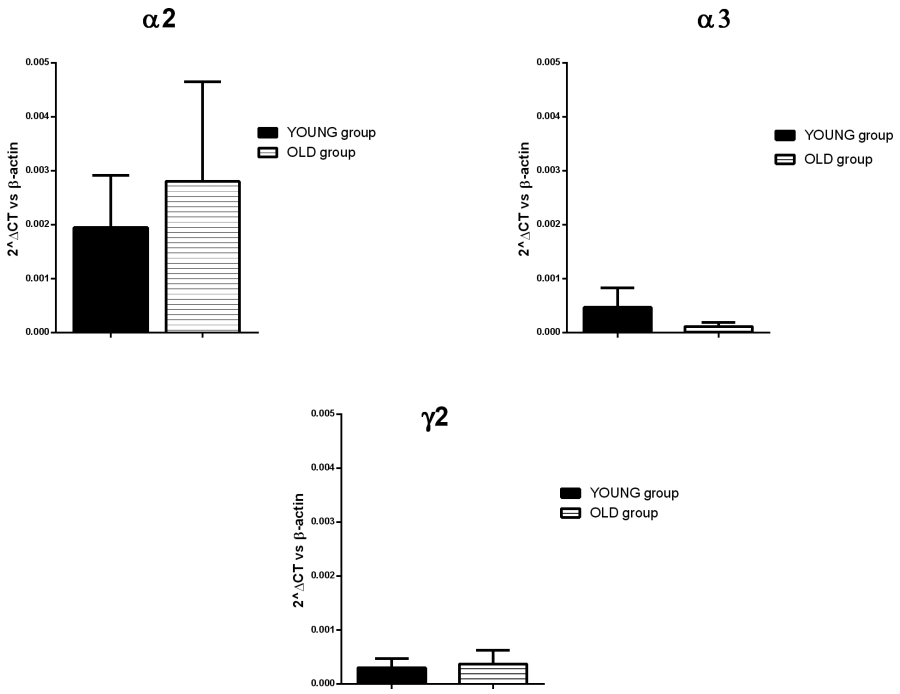

FIGURE 5 mRNA expression of α2, α3 and γ2 GABAAR subunit evaluated by qRT PCR in young and old human colonic muscle strips. Values are plotted as $2^{-(\Delta Ct)}$ *versus* β−actin. Data are means ± SEM. $n = 3$ for each group

seems to be able to induce only release of nitric oxide from enteric neurons. Our study does not allow us to discriminate whether loss of purinergic-component in the aged group is due to a change of localization of GABAB receptors during the elderly or, most likely, to age-related neuronal functionality changes of the purinergic system.

We are aware that the limitation of our study is the lack of validation of the direct association between GABAAR subunit changes and the difference in the response to GABA. In conclusion, the present study has revealed age-related variations in GABAA receptor- mediated responses in the human colon. Since GABAergic system has been identified as a therapeutic target for the treatment of different bowel dysfunctions, our findings highlight that age differences should be considered in the therapeutic development of GABA receptor agents.

A deeper investigation of GABA-mediated enteric neural signalling in the large intestine could give a scientific rationale for the study of new drugs, targeting both motor and non-motor disorders affecting the aging colon, since GABA influence on enteric neurons leads to a modulation of the different functions as the peristaltic reflex. For instance, the development of drugs potentiating the GABAAergic neurotransmission can be usefully exploited for the improvement of propulsive activity, ameliorating colonic motor function in gut motor disturbances, frequent in old patients,[34,35] as acute colonic pseudo-obstruction. Hence, future researches about the use of more specific GABA receptor agonist should be deeply addressed to give rise to novel clinical applications to restore the colonic motor activity in age-related GI pathologies characterized by either colonic hypomotility or hypermotility.

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CONFLICT OF INTEREST

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

MGZ designed and performed the study, analyzed and interpreted the data, prepared figures, and drafted and edited the manuscript; AC performed the experiments; SR performed the biomolecular experiments, prepared figures and interpreted the data; RA, participated to supervision, experimental design, result analysis and manuscript revision.RS designed the study, interpreted the data, edited and revised the manuscript. All the authors contributed to the critical revision and approved the final version of the manuscript.

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