New insights into the characterization of the mechanism of action of Hyoscine Butylbromide in the human colon ex vivo

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Abstract

Introduction: Hyoscine butylbromide (HBB) is one of the most used antispasmodics in clinical practice. Recent translational consensus has demonstrated a similarity between human colonic motor patterns studied *ex vivo* and *in vivo,* suggesting *ex vi*vo can predict *in vivo* results. It is unclear whether the mechanism of action of antispasmodics can predict different use in clinical practice. The aim of the present study is to bridge this gap dissecting HBB's role in excitatory and inhibitory neural pathways. Methods: 309 colon samples from 48 patients were studied in muscle bath experiments. HBB was tested on: 1-spontaneous phasic contractions (SPCs); 2-carbachol-induced contractility; electrical field stimulation (EFS)-induced selective stimulation of 3-excitatory and 4-inhibitory pathways and 5- SPCs and EFS-induced contractions enhanced by neostigmine. Atropine, AF-DX116 (M2 blocker) and DAU-5884 (M3 blocker) were used as comparators.

Results: In the presence of tetrodotoxin (TTX), HBB and atropine 1μM reduced SPCs. HBB and atropine concentration-dependently reduced carbachol- and EFS-induced contractions. Inhibitory effects of DAU-5884 on EFS-induced contractions were more potent than of AF-DX116. HBB did not affect the off-response associated to neural inhibitory responses. Neostigmine enhanced both SPCs and EFS-induced contractions. In the presence of TTX and ω-conotoxin (GVIA), neostigmine still enhanced SPCs. Addition of HBB and atropine reduced these responses.

Conclusions: This study demonstrates that HBB inhibits neural cholinergic contractions associated to muscarinic (mainly M3) receptors. HBB has a potential role in reducing colonic spasm induced by the release of acetylcholine from enteric motor neurons and from an atypical source including a potential non-neuronal origin.

Keywords: Hyoscine butylbromide, scopolamine butylbromide, antispasmodic, abdominal cramping, pain

1. Introduction

Hyoscine butylbromide (HBB) – also known as scopolamine butylbromide – is a tropane alkaloid and derivative of the tertiary ammonium compound hyoscine (Evangelista, 2004). HBB is a well-known antispasmodic drug (Corsetti et al., 2023; Fact.MR, 2019; Tytgat, 2007), available in both oral and parenteral pharmaceutical forms (Sanofi, 2020a, b; Tytgat, 2008). It is used worldwide to treat abdominal pain associated with spasm in both gastrointestinal (GI) and genitourinary tracts.

Studies conducted *ex vivo* on human colonic samples are used to understand the mechanism of action (MOA) of drugs (Heitmann et al., 2022). The enteric nervous system (ENS), together with the interstitial cells of Cajal (ICC), is responsible for the regulation of GI motor function. Spontaneous phasic contractions (SPCs) of about 2 to 6 contractions/min are the more common myogenic pattern in human colonic tissue (Auli et al., 2008; Carbone et al., 2013; Rae et al., 1998). This myogenic activity is probably originated in pacemaker cells and can be modulated by inhibitory and excitatory neural inputs (Mane et al., 2015). Inhibitory motor neurons co-release ATP (or a related purine) which act on $P2Y_1$ receptors and nitric oxide that exerts its inhibitory effects via activation of soluble guanylate cyclase (Gallego et al., 2008; Gallego et al., 2006). Excitatory motor neurons are immunoreactive for choline acetyltransferase and functional experiments demonstrate that acetylcholine is the main neurotransmitter acting on muscarinic receptors (Auli et al., 2008; Humenick et al., 2019; Ng et al., 2018). Accordingly, muscarinic receptors are pharmacological targets for spasmolytic drugs limiting smooth muscle contractility (Corsetti et al., 2023).

A recent translational consensus has revealed a similarity between colonic motor patterns studied *ex vivo* and *in vivo* in humans, suggesting *ex vivo* can predict *in vivo* results (Corsetti et al., 2019). HBB is known to target muscarinic receptors located on the smooth-muscle cells of the GI tract (Krueger et al., 2013) exerting a smooth-muscle relaxing/spasmolytic effect (Corsetti et al., 2023; Tytgat, 2007, 2008). Few previous studies have evaluated the MOA of HBB on human samples (Alvarez-Berdugo et al., 2015; Krueger et al., 2013; Zhang et al., 2016). However, none of these previous studies have assessed the effects of HBB using selective activation of excitatory and inhibitory pathways and,

therefore, could not selectively determine the MOA of HBB on these pathways. Moreover, none of these studies have neither compared the effect with the vehicle.

The present study aimed to fill this gap to guide its better use in clinical practice. Applying a systematic approach, the MOA of HBB was assessed on colonic strips, both circularly and longitudinally oriented from the right and left colon. We studied spontaneous myogenic contractions induced by isometric stretch in the presence of neural blockade tetrodotoxin (TTX); muscle contraction induced by the muscarinic agonist carbachol; selective activation of excitatory motor neurons; and selective activation of inhibitory motor neurons. Atropine and selective M2 and M3 antagonists were used as comparators (Alvarez-Berdugo et al., 2015; Drumm et al., 2020; Harrington et al., 2010; Zhang et al., 2016).

2. Materials and Methods

2.1 Human tissue collection and preparation

Human tissue samples of the right and the left colon were obtained from patients without elements of obstruction undergoing colon cancer surgery at Hospital Vall d' Hebron, Spain after they had provided their informed consent. HBB was not utilized at any stage of the anaesthetic protocol. Colon segments were taken from macroscopic normal marginal areas. After removal, specimens were transported in cold saline buffer to the laboratory at the Universitat Autònoma de Barcelona. Samples were placed in Krebs solution on a dissection dish, and the mucosal layer was carefully removed. Muscle strips (1 cm long $\times$ 0.4 cm wide) were cut in the circular and longitudinal direction. All experimental procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona, Spain (ethical approval code: 5604).

Circular and longitudinal oriented muscle strips were studied in 10 mL of organ bath filled with carbogenated (95% O_2–5% CO_2) Krebs solution at 37 ± 1°C. A tension of 4 g was applied, and tissues were allowed to equilibrate for 1 h. An isometric force transducer (Harvard VF-1) connected to an

amplifier was used to record the mechanical activity. Digitalised data (25 Hz) were collected using DATAWIN1 software (Panlab, Barcelona, Spain), which was coupled to an ISC-16 analogue-to-digital card installed on a computer. Electrical field stimulation (EFS) was applied through two platinum electrodes placed on the support holding the tissue to study the release of inhibitory and excitatory neurotransmitters.

2.2 Experimental design of the study

The design of the study was to evaluate the effect of HBB, and as such, 5 experiments were conducted. Atropine was used as a comparator in Experiments 1, 2, 3 and 5. Atropine was selected as it is considered the standard reference as anti-muscarinic compound even if it is not used as HBB in clinical practice as any clinical benefit as a GI antispasmodic is outweighed by atropine side-effects. AF-DX 116 (selective M2 blocker) and DAU-5884 (selective M3 blocker) were used as comparators in Experiments 2 and 3. Vehicle (distilled water), at the same volume used in each drug addition, was used as a comparator in all experiments reaching a maximum of 1.2% of the total volume.

2.2.1 Experiment 1: Effect of HBB on spontaneous phasic contractions (SPCs)

Tissue was incubated for 20 min with TTX (1 μM) to inhibit the neuronal activity. This was expected to reveal the spontaneous smooth muscle activity characterised by SPCs as demonstrated previously (Auli et al., 2008; Carbone et al., 2013; Rae et al., 1998). The samples were then incubated at increasing HBB concentrations, from 10^{-9} to 10^{-5} M. The experiment was repeated in the presence of the equivalent volume of the vehicle used in each concentration as a negative control. Thereafter, another subset of experiments was performed and samples pre-incubated with TTX (1μM) were then incubated with a single concentration of HBB or atropine 1 μM.

2.2.2 Experiment 2: Effect of HBB on carbachol-induced contractions

In this experiment, tissues were incubated 15-20 min with the muscarinic agonist carbachol (10^{-5} M), which caused an increase of the contractile activity. After stabilization, HBB, atropine, DAU-5884 (M3

antagonist) and AF-DX 116 (M2 antagonist) were then incubated at increasing concentrations from 10^{-9} to 10^{-5} M in intervals of 3–5 min until a stable response was reached. The experiment was also repeated in the presence of the equivalent volume of the vehicle (distilled water) used in each concentration to assess the effect of negative control.

2.2.3 Experiment 3: Effect of HBB on neural-mediated excitatory responses

To evaluate the effect of excitatory neurotransmitters released by neurons, the release of inhibitory neurotransmitters was inhibited incubating the muscle strips under non-nitrergic and non-purinergic (NNNP) conditions. Under these experimental conditions, EFS (voltage: 30 V; pulse duration: 0.4 ms; frequency: 50 Hz; and train duration: 300 ms) was applied once every 100 s and HBB, atropine, DAU-5884 (M3 antagonist) and AF-DX 116 (M2 antagonist) added at increasing concentrations from 10^{-9} to 10^{-5} M. The experiment was also repeated in the presence of the equivalent volume of the vehicle (distilled water) used in each concentration to assess the effect of negative control.

2.2.4 Experiment 4: Effect of HBB on neural-mediated inhibitory responses

To selectively isolate the response associated to inhibitory motoneurons, the effect of excitatory neurotransmitters was inhibited by incubating tissues in non-adrenergic and non-cholinergic (NANC) conditions. Thereafter, 3 EFS were applied (voltage: 30 V; pulse duration: 0.4 ms; frequency: 5Hz; and train duration: 2 min) in each strip. A first EFS was applied in control conditions, the second EFS was applied 30 min after adding HBB 10 µM (or its vehicle). Lastly, an EFS was performed 30 min after the incubation with the NO synthase inhibitor L-NNA (1 mM) and the $P2Y_1$ receptor blocker MRS2179 (10 µM). This combination blocks the inhibitory pathway (Gallego et al., 2008; Gallego et al., 2011; Gallego et al., 2006).

2.2.5 Experiment 5: Effect of HBB on contractions enhanced with neostigmine.

In this experiment, tissue was incubated with the Acetylcholinesterase blocker, neostigmine (10 µM), to inhibit the degradation of acetylcholine. The effect of HBB and atropine was studied under control

conditions (normal Krebs solution) and after either TTX 1µM or ω-conotoxin (GVIA) 0.1 µM to inhibit voltage-gated Na^+ channels and voltage-gated Ca^{2+} channels, respectively, in enteric neurons (Carbone et al., 2013; Rosli et al., 2020; Sanger et al., 2000). Some experiments were also performed with both toxins in the medium to make sure that the neuronal release of acetylcholine was completely blocked. HBB and atropine (10^{-7} to 10^{-5} M) were then added to evaluate their effect on neostigmine-enhanced muscle contractility and the neostigmine-enhanced EFS excitatory response.

2.3 Solutions and drugs

The Krebs solution for the experiments contained (in mM) 10.10 glucose, 115.48 NaCl, 21.90 $NaHCO_3$, 4.61 KCl, 1.14 NaH_2PO_4, 2.50 $CaCl_2$ and 1.16 $MgSO_4$, bubbled with a mixture of 5% CO_2: 95% O_2 (pH 7.4). The drugs used in the experiments were HBB, L-NNA, atropine sulphate, phentolamine, DAU-5884, AF-DX 116, TTX (Sigma Chemicals, the United States of America), 2′-deoxy-N6-methyl adenosine 3′,5′-diphosphate tetra-ammonium salt (MRS2179) (Merck Millipore, Germany), (2-hydroxyethyl) trimethylammonium chloride carbamate (carbachol), propranolol and ω-conotoxin (GVIA) (Tocris, the United Kingdom). Stock solutions were prepared by dissolving drugs in distilled water, except for L-NNA that required sonication to get dissolved in the Krebs solution and AF-DX 116 which was dissolved in DMSO.

2.4 Data analysis

The area under the curve (AUC) (mg x min), amplitude (mg), frequency (contractions/min) and tone (mg) were measured to estimate the drug effect on SPC in experiment 1. For the rest of the experiments the AUC was used as a general index of contractions to estimate the effect of drugs in different conditions. To normalise data, responses were expressed as a percentage of the basal AUC obtained before the first drug addition. The following formula was used: 100 x(AUC during EFS or after drug incubation/AUC before EFS or drug incubation). In this formula, 0% response represents the complete cessation of any activity, whereas 100% denotes no change compared with basal activity.

This procedure was used for Experiments 1, 2, 4 and 5 where the effect of drugs on myogenic activity, carbachol/neostigmine-induced contractions or inhibitory pathways was tested.

For EFS-induced excitatory responses and off-responses, the amplitude of the response was measured before and after drug addition, data were normalised (i.e. 100% represents amplitude before adding the drug) and the percentage of reduction was calculated for each drug. This procedure was used for Experiments 3, 4 and 5 where EFS-induced contractions and off-contractions were measured.

2.5 Statistical analysis

A nonlinear regression analysis was performed to assess the effect of drugs on carbachol- and EFS-induced contractions using the formula $Y = 100/(1 + 10^{\wedge}((\text{LogIC}_{50} - X)^{*}\text{Hill slope}))$. Graph data were expressed as the mean ± standard error of the mean (SEM) and considered significant when $p < 0.05$. Nonparametric Wilcoxon test or One-way ANOVA test followed by Dunnett post-hoc test were used to assess differences between two or more groups, respectively. Two-way ANOVA test followed by Sidak post-hoc test were used to compare the effect of drugs when different concentrations were tested. In each experimental condition, the '*n*' value represented the total number of muscle strips from different patients. From each patient, a mean of about eight strips was studied depending on the availability of the tissue. Statistical analysis was performed with GraphPad Prism version 6.01.

3. Results

A total of 309 colon samples were obtained from macroscopically normal regions of 48 patients (18 females and 30 males aged 35–92 years) undergoing colon cancer surgery (**Supplementary Table 1**).

3.1 Experiment 1: Effect of HBB on spontaneous phasic contractions

Details of the number of colonic samples studied in each experimental condition are presented in **Figure 1**; this figure shows the effect of HBB as compared with the vehicle on myogenic spontaneous contractions of circular and longitudinal smooth muscle. Consistent with a myogenic origin, SPCs were recorded in the presence of TTX. There was a progressive decrease in SPCs of both the circular and

longitudinal muscle with both HBB and the vehicle (rundown). However, at high concentrations a slight reduction in the AUC of the circular muscle incubated with HBB was observed. To differentiate the putative pharmacological effect from the time-dependent rundown, the effect of a single concentration of both HBB and atropine (10^{-6} M) was assessed. Atropine reduced the AUC by 54.6±8.8%, primarily through a reduction in the amplitude and frequency of SPC. Similar results were obtained with HBB, which reduced the AUC by 47±11.9%; in this case, the reduction was also attributable to the amplitude and frequency, although a slight decrease in tone was observed as well (**Figure 1**).

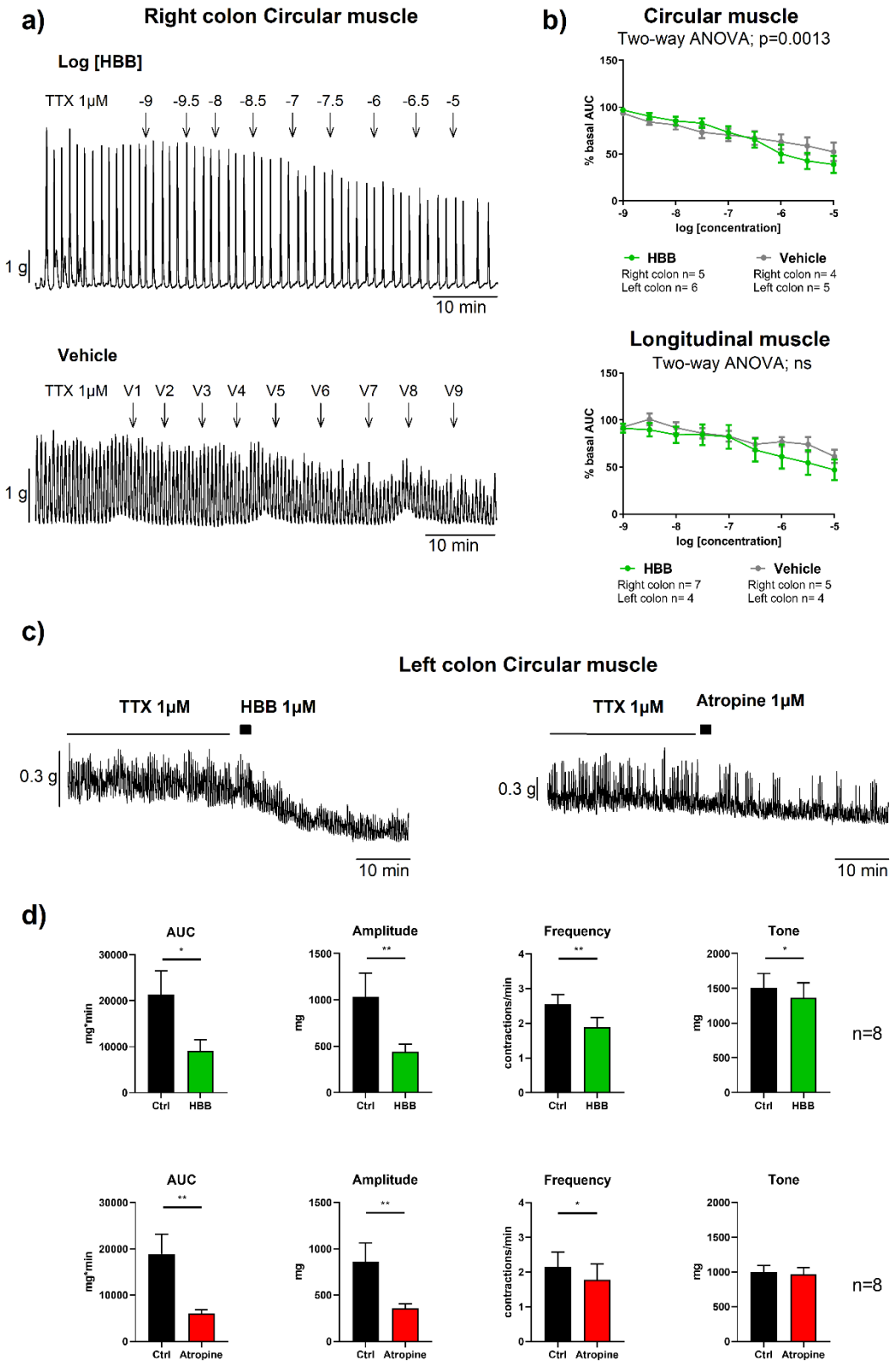

Figure 1. Effect of HBB, atropine and vehicle on spontaneous phasic contractions (SPCs) of circular and longitudinal colon smooth muscle.
(a) Representative mechanical recordings from the right colon, with circular muscle and (b) graphs showing the effect of accumulative concentrations of HBB (10^{-9} – 10^{-5}M) and vehicle on myogenic contractions. (c)

Representative mechanical recordings from the left colon, with circular muscle and (d) graphs showing the effect of a single concentration of HBB and atropine (10^{-6} M) on circular muscle myogenic contractions. AUC data from accumulative concentrations were normalised (i.e. 100%) to the basal AUC before adding the drug. Data expressed as mean ± SEM. Two-way ANOVA and non-parametric test (Wilcoxon test) were used to assess the effect of drugs. *p<0.05; ** p<0.01.

ANOVA, Analysis of Variance; AUC, area under the curve; HBB, hyoscine butylbromide; SEM, standard error of the mean; TTX, Tetrodotoxin

3.2 Experiment 2: Effect of HBB on carbachol-induced contractions

Details of the number of colonic samples studied in each experimental condition are presented in **Table 1**. The effect of vehicle, HBB, atropine, DAU-5884 and AF-DX 116 on carbachol-induced contractions in the circular and longitudinal smooth muscle is shown in **Figure 2**. Compared to vehicle, both HBB and atropine concentration-dependently reduced carbachol-induced contractions in both longitudinal and circular muscle layers of the right and the left colon (Two-way ANOVA p<0.001). EC_{50}s were in the sub-micromolar range, and complete abolition of contractions was achieved at 10 μM. Both atropine and HBB were more potent in the circular layer than in the longitudinal layer in both the right and left colonic regions (**Figure 2**). DAU-5884 was found to be more potent than AF-DX 116 in all colonic regions and layers. **Table 1** reports the key results of HBB and its comparators in each colon segment.

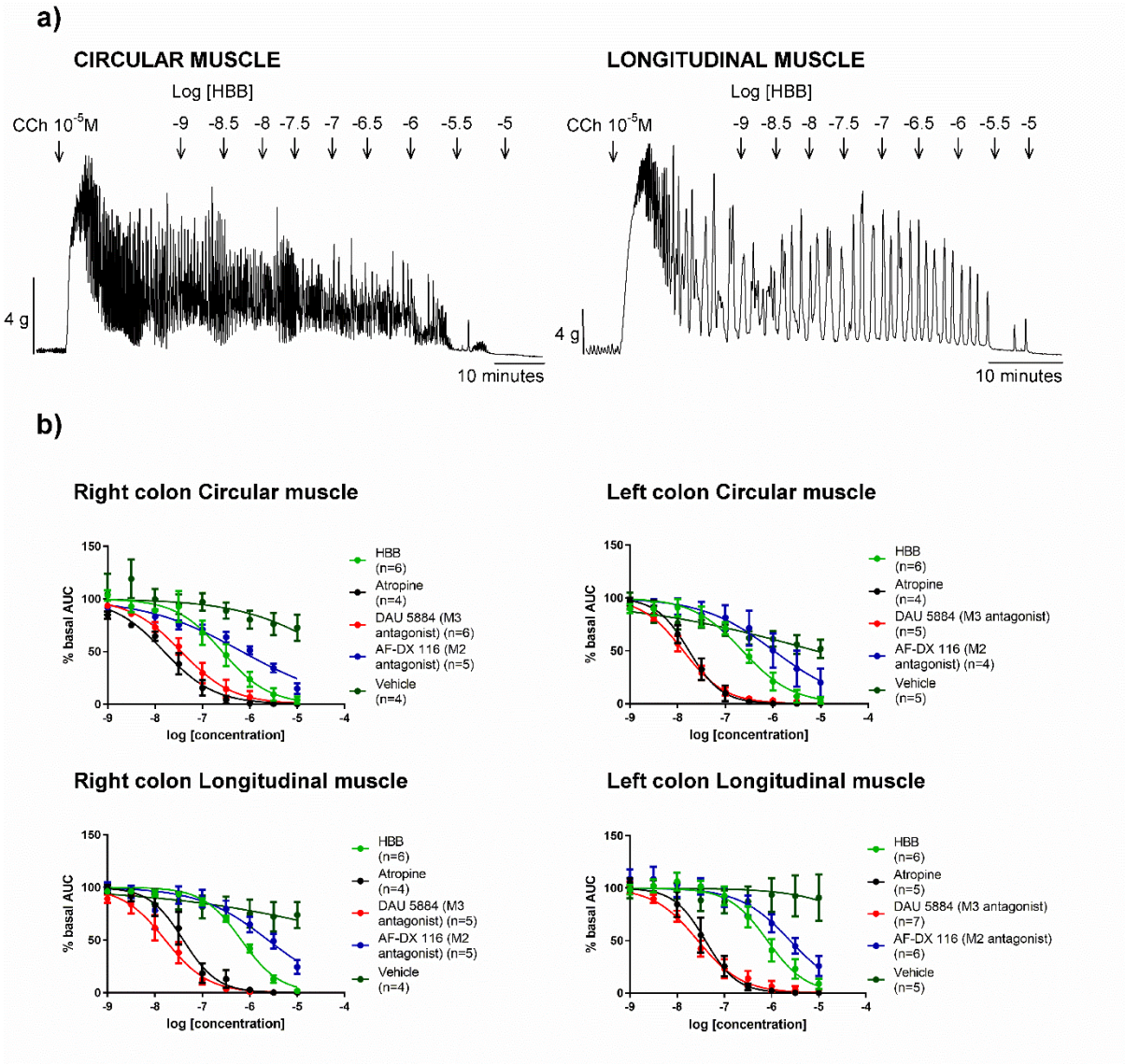

Figure 2. Effect of HBB, atropine, DAU-5884, AF-DX 116 and vehicle on carbachol-induced contractions in the circular and longitudinal colonic smooth muscle.

(a) Representative mechanical recording showing the effect of HBB on CCh-induced contractions in the circular (left) and longitudinal muscle (right). (b) Concentration–response curves of HBB, atropine, DAU-5884, AF-DX 116 and vehicle (same volume used in each concentration tested) in the circular (top) and longitudinal muscle (bottom) both in the right (left) and left colon (right). Data expressed as log of the concentration (data expressed as mean ± SEM).

CCh, carbachol; HBB, hyoscine butylbromide; SEM, standard error of the mean

3.3 Experiment 3: Effect of HBB on neural-mediated excitatory responses

In normal Krebs solution, EFS induces a complex response often consisting of a contraction or relaxation during the stimulus (on-contraction; on-relaxation) followed by a contraction after the

stimulus (off-contraction). In order to selectively stimulate excitatory motor neurons, tissue was incubated with NNNP conditions. Under these pharmacological conditions, the response shifted to a sharp and higher on-contraction (**Figure 3a**) that was concentration dependently reduced by muscarinic antagonists (see below). Moreover, EFS induced contractions were reduced by 92.4±1.6% by TTX 1µM (Control 1648±463 *vs* TTX 129.6±57.7 mg; $p<0.01$, n=8), 67±15.8% by ω-CTX GVIA 0.1µM (Control 988.2±235.5 *vs* ω-CTX GVIA 209.8±100.3 mg; $p<0.05$, n=5) and totally abolished with the combination of both toxins (n=4).

Details of the number of colonic samples studied in each experimental condition are presented in **Table 2**. The effect of vehicle, HBB, atropine, DAU-5884 and AF-DX 116 on neural-induced excitatory responses in the circular and longitudinal smooth muscle is shown in **Figure 3**. All muscarinic antagonists concentration-dependently reduced EFS-induced on-contractions. The rank of potency of the muscarinic antagonists was DAU-5884≥atropine>HBB>AF-DX 116 in both circular and longitudinal muscle layers. In contrast, the vehicle did not modify EFS-induced contractile responses (**Figure 3**). **Table 2** reports the results of HBB and its comparators on each colon segment.

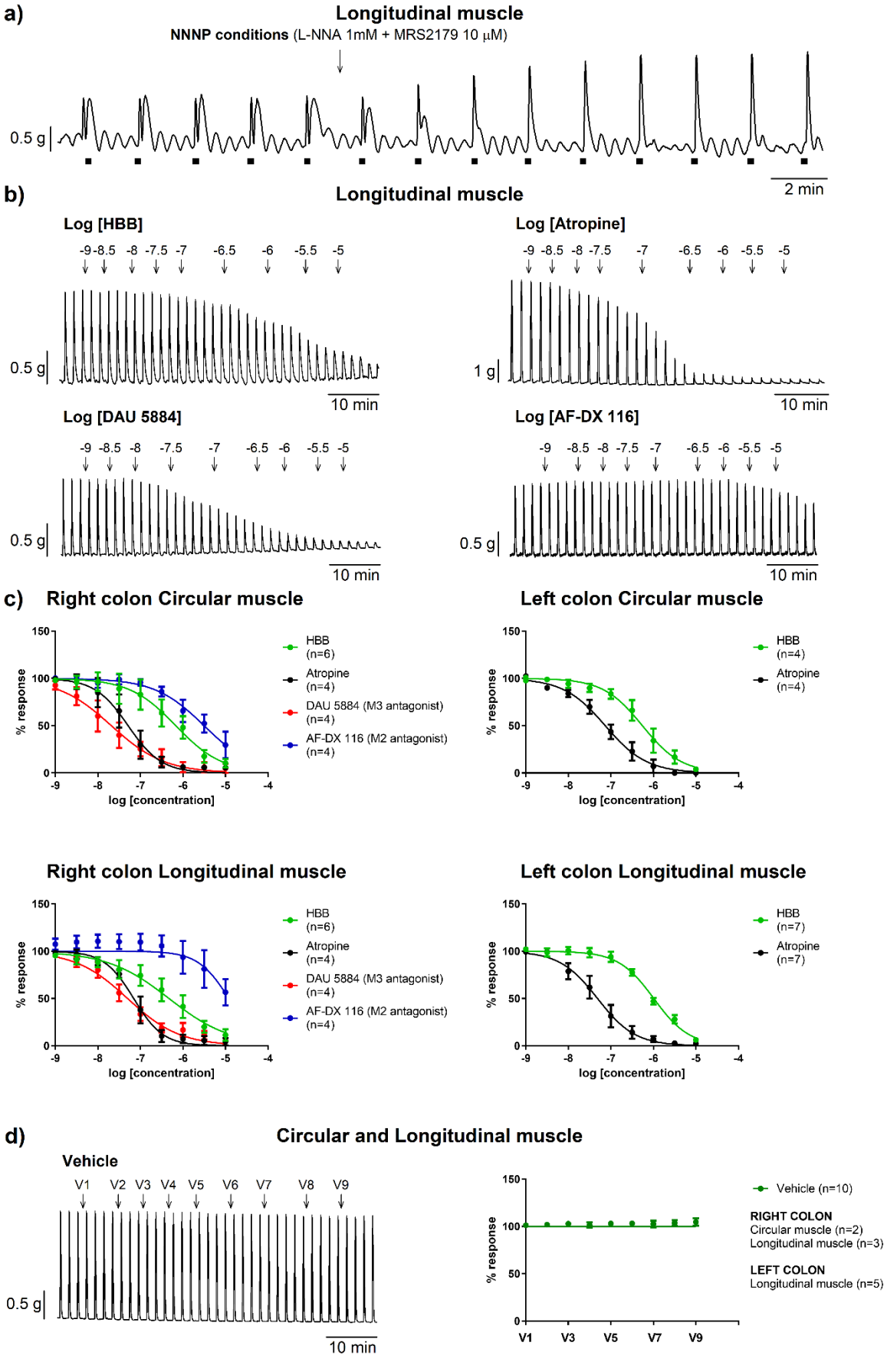
a)
Longitudinal muscle
NNNP conditions (L-NNA 1mM + MRS2179 10 μM)
0.5 g
2 min
b)
Longitudinal muscle
Log [HBB]
-9 -8.5 -8 -7.5 -7 -6.5 -6 -5.5 -5
0.5 g
10 min
Log [Atropine]
-9 -8.5 -8 -7.5 -7 -6.5 -6 -5.5 -5
1 g
10 min
Log [DAU 5884]
-9 -8.5 -8 -7.5 -7 -6.5 -6 -5.5 -5
0.5 g
10 min
Log [AF-DX 116]
-9 -8.5 -8 -7.5 -7 -6.5 -6 -5.5 -5
0.5 g
10 min
c)
Right colon Circular muscle
% response
log [concentration]
HBB (n=6)
Atropine (n=4)
DAU 5884 (M3 antagonist) (n=4)
AF-DX 116 (M2 antagonist) (n=4)
Left colon Circular muscle
% response
log [concentration]
HBB (n=4)
Atropine (n=4)
Right colon Longitudinal muscle
% response
log [concentration]
HBB (n=6)
Atropine (n=4)
DAU 5884 (M3 antagonist) (n=4)
AF-DX 116 (M2 antagonist) (n=4)
Left colon Longitudinal muscle
% response
log [concentration]
HBB (n=7)
Atropine (n=7)
d)
Circular and Longitudinal muscle
Vehicle
V1 V2 V3 V4 V5 V6 V7 V8 V9
0.5 g
10 min
% response
V1 V3 V5 V7 V9
Vehicle (n=10)
RIGHT COLON
Circular muscle (n=2)
Longitudinal muscle (n=3)
LEFT COLON
Longitudinal muscle (n=5)

Figure 3. Effect of HBB, atropine, DAU-5884, AF-DX 116 and vehicle on neural-induced excitatory responses in the circular and the longitudinal colon smooth muscle. (a) Representative mechanical recording from the right colon, longitudinal muscle showing a decrease in the off-response and an increase in the on-response after the incubation of L-NNA 1 mM and MRS2179 10 µM. Notice that each square at the bottom of the tracing represents an EFS (see methods). (b) Representative mechanical recordings from strips obtained from the right colon, with longitudinal muscle showing the effect of HBB, atropine, DAU-5884 and AF-DX 116 on the excitatory neural pathway. Each contraction is the consequence of an EFS. (c) Concentration–response curves of HBB, atropine, DAU-5884 and AF-DX 116 in the circular (top) and longitudinal muscle (bottom) in both the right (left) and left colon (right). (d) Representative mechanical recording from the right colon, longitudinal muscle (left) and the response (right) with vehicle (V1 to V9) used in each concentration. Notice that each contraction is the consequence of an EFS. Data expressed as log of the concentration (mean ± SEM).
HBB, hyoscine butylbromide; L-NNA, N^{ω}-nitro-L-arginine; NNNP, non-nitrergic and non-purinergic; SEM, standard error of the mean

3.4 Experiment 4: Effect of HBB on neural-mediated inhibitory responses

Details of the number of colonic samples studied in each experimental condition are shown in **Figure 4**. Each strip was incubated in NANC Krebs solution and 3 consecutive EFS were performed, in control conditions (before drug addition), 30 minutes after the incubation of the (1) vehicle, or (2) HBB 10µM and lastly, in the presence of (3) L-NNA 1mM + MRS2179 10µM. **Figure 4** shows that neither HBB nor its associated vehicle did not modify neither the on-relaxation nor the off-contraction in any muscle layer and colonic zone compared to the control. Incubation with L-NNA 1mM and MRS2179 10µM totally blocked the nerve-mediated on-relaxation and off-contractions, showing that the off-contraction is a consequence of the release of inhibitory neurotransmitters. Vehicle and HBB did not modify neither on-relaxation nor off-contractions in any muscle layer and colonic zone compared to control.

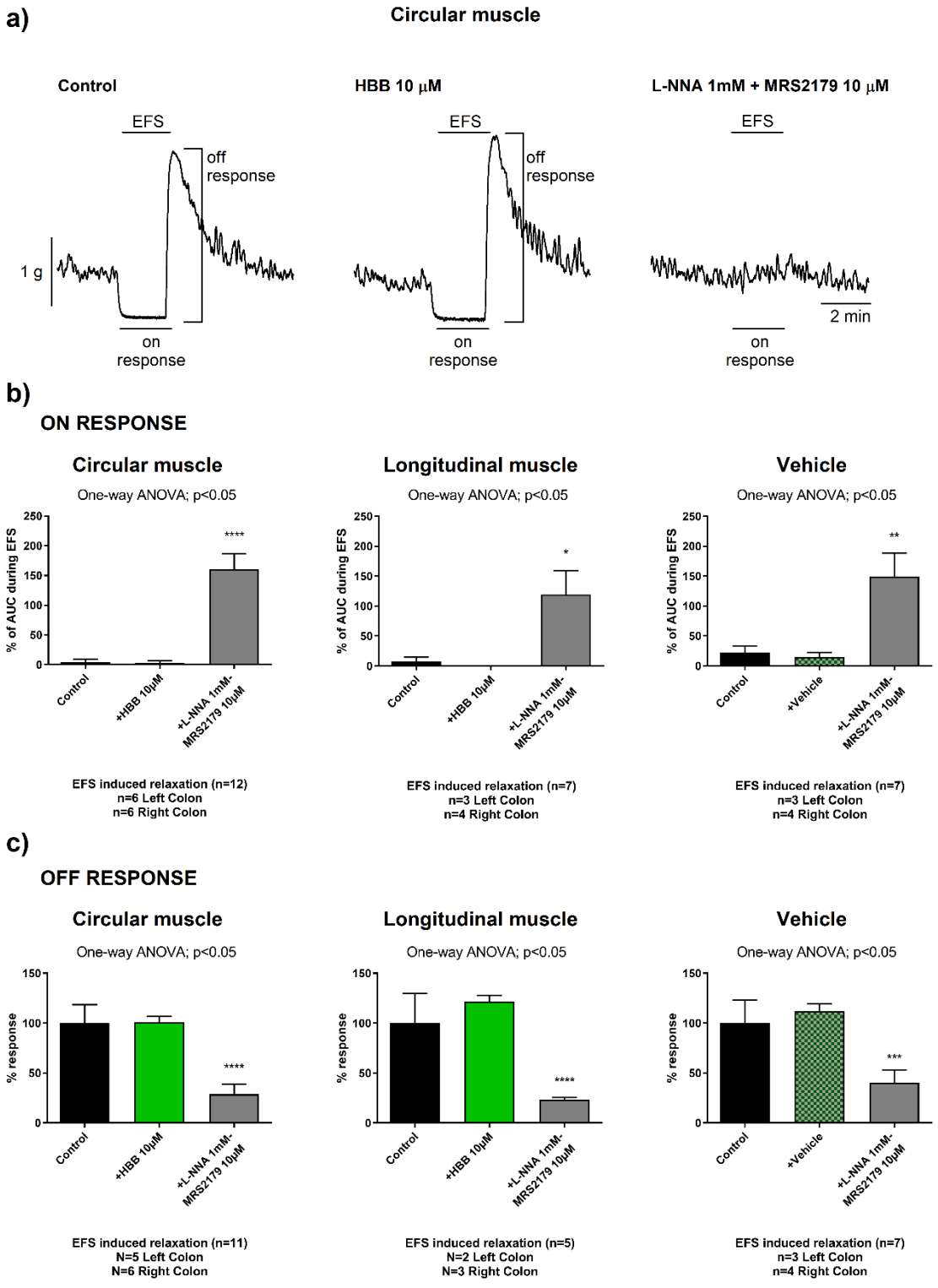

Figure 4. Effect of HBB on the neural-induced inhibitory responses in the circular and longitudinal muscle. (a) Representative mechanical recordings from the right colon, circular muscle showing the on-response (relaxation during the EFS) and the off-response (rebound contraction after the EFS) in control conditions, after HBB 10 µM and under NNNP conditions (L-NNA 1 mM and MRS2179 10 µM). (b) Histograms showing the % of the AUC during the on-response in control conditions, after HBB 10 µM, under NNNP conditions and after incubation with the vehicle. (c) Histograms showing the amplitude of the off-response in control conditions, after HBB 10 µM, under NNNP conditions and during incubation with the vehicle. On relaxation and off-contraction were normalised (i.e. 100%) to basal AUC before EFS or the amplitude of the response obtained in control conditions.

Dunnett post-hoc test after ANOVA was used to compare the effect of drugs and vehicle with control. * P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

ANOVA, analysis of variance; EFS, electrical field stimulation; HBB, hyoscine butylbromide; L-NNA, N^{ω}-nitro-L-arginine; NNNP, non-nitrergic and non-purinergic; SEM, standard error of the mean

3.5 Experiment 5: Effect of HBB on contractions enhanced by neostigmine

Details of the number of colonic samples studied in each experimental condition are shown in **Figures 5** and **6**. **Figure 5 (a-h)** shows the effect of HBB and atropine on neostigmine enhanced spontaneous contractions both in the absence and presence of TTX. In both cases, HBB and atropine concentration-dependently reduced the neostigmine enhanced spontaneous contraction. It is important to note that neostigmine was still able to increase contractions even though sodium channels in enteric neurons were blocked by TTX. Similar results were obtained by incubating the tissue with the N-type calcium channel blocker ω-conotoxin (GVIA), that blocks pre-junctional release of acetylcholine, **Figure 5(i-j)**. Neostigmine was able to increase contractility (100 *vs* 636±267%; n=8) when tissue was preincubated with the combination of TTX and ω-conotoxin (GVIA). This effect was concentration-dependently reduced (10^{-7} to 10^{-5}M) after adding atropine (n=4) or HBB (n=4). **Figure 6(a-d)** shows the effect of HBB and atropine on neostigmine enhanced EFS-induced excitatory responses, which both concentration-dependently reduced neostigmine enhanced EFS-induced excitatory responses.

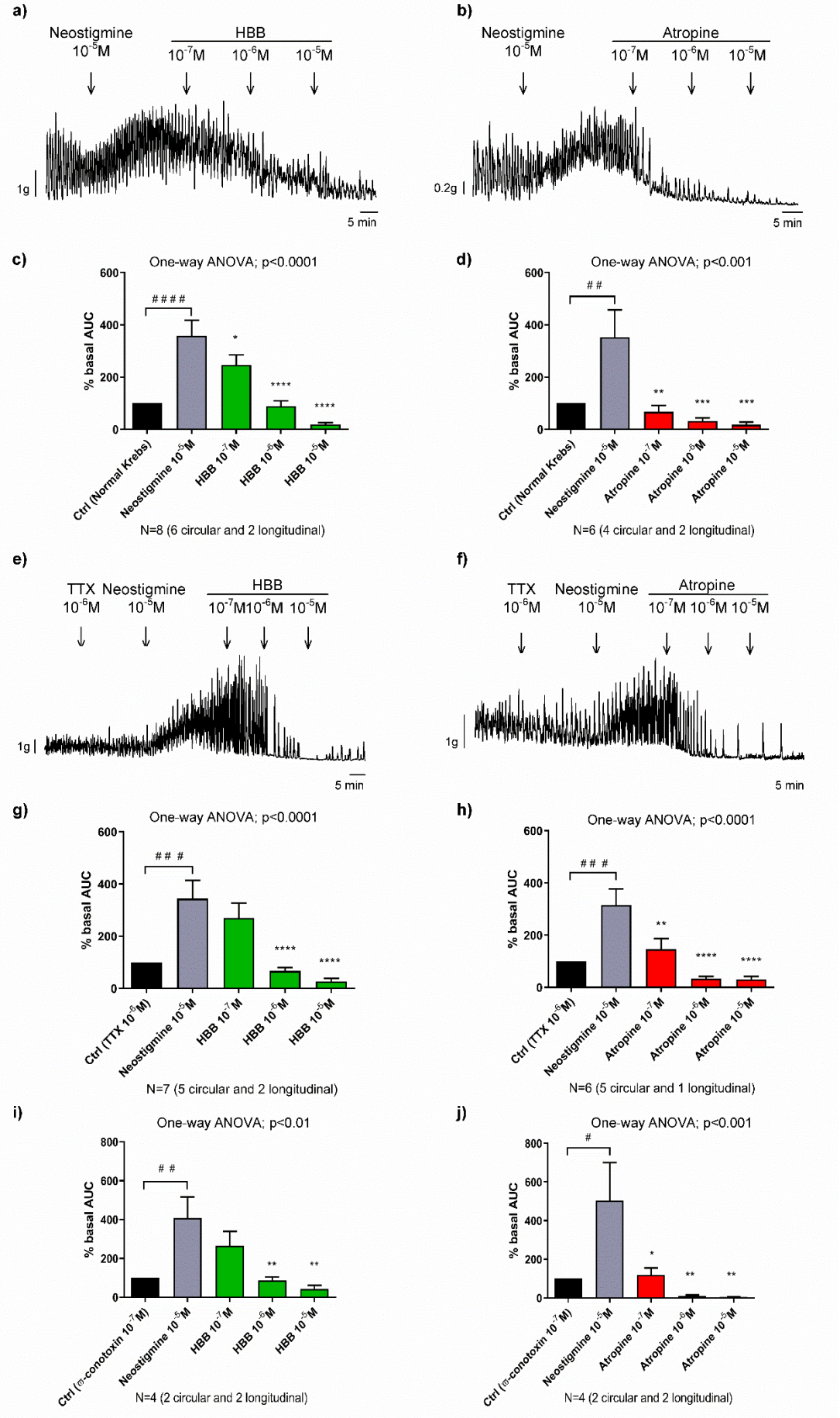
a)
Neostigmine
10-5M
HBB
10-7M 10-6M 10-5M
1g
5 min
b)
Neostigmine
10-5M
Atropine
10-7M 10-6M 10-5M
0.2g
5 min
c)
One-way ANOVA; p<0.0001
% basal AUC
* **** ****
Ctrl (Normal Krebs)
Neostigmine 10-5M
HBB 10-7M
HBB 10-6M
HBB 10-5M
N=8 (6 circular and 2 longitudinal)
d)
One-way ANOVA; p<0.001
% basal AUC
** *** ***
Ctrl (Normal Krebs)
Neostigmine 10-5M
Atropine 10-7M
Atropine 10-6M
Atropine 10-5M
N=6 (4 circular and 2 longitudinal)
e)
TTX
10-6M
Neostigmine
10-5M
HBB
10-7M 10-6M 10-5M
1g
5 min
f)
TTX
10-6M
Neostigmine
10-5M
Atropine
10-7M 10-6M 10-5M
1g
5 min
g)
One-way ANOVA; p<0.0001
% basal AUC
**** ****
Ctrl (TTX 10-6M)
Neostigmine 10-5M
HBB 10-7M
HBB 10-6M
HBB 10-5M
N=7 (5 circular and 2 longitudinal)
h)
One-way ANOVA; p<0.0001
% basal AUC
** **** ****
Ctrl (TTX 10-6M)
Neostigmine 10-5M
Atropine 10-7M
Atropine 10-6M
Atropine 10-5M
N=6 (5 circular and 1 longitudinal)
i)
One-way ANOVA; p<0.01
% basal AUC
** **
Ctrl (ω-conotoxin 10-7M)
Neostigmine 10-5M
HBB 10-7M
HBB 10-6M
HBB 10-5M
N=4 (2 circular and 2 longitudinal)
j)
One-way ANOVA; p<0.001
% basal AUC
# * ** **
Ctrl (ω-conotoxin 10-7M)
Neostigmine 10-5M
Atropine 10-7M
Atropine 10-6M
Atropine 10-5M
N=4 (2 circular and 2 longitudinal)

Figure 5. Effect of HBB and atropine on neostigmine-stimulated contractions in the absence ([a]–[d]) and presence of TTX ([e]–[h]) and ω-CTX GVIA ([i]–[J]) in the circular and longitudinal colonic smooth muscle.
(a)and (b) Representative mechanical recordings from the right colon, circular muscle and (c) and (d) histograms showing an increase in contractility after neostigmine 10^{-5} M and a concentration-dependent decrease in contractility after HBB and atropine incubation, respectively. (e) and (f) Representative mechanical recordings from the right colon, circular muscle and histograms showing an increase in motility after neostigmine 10^{-5} M in the presence of TTX 10^{-6} M ([g) and [h]) and ω-CTX (GVIA) 10^{-7} M, and ([I] and [J]) that was concentration-dependently decreased by HBB and atropine, respectively. Dunnett post hoc test after ANOVA #, $^{*}P$ <0.05; ##, $^{**}P$ <0.01; ###, $^{***}P$ <0.001; ####, $^{****}P$ <0.0001 compared with control and neostigmine 10^{-5} M, respectively. Data were normalised (i.e. 100%) to the basal AUC before adding the drug. Data expressed as mean ± SEM.

CTX, ω-conotoxin; HBB, hyoscine butylbromide; SEM, standard error of the mean; TTX, tetrodotoxin

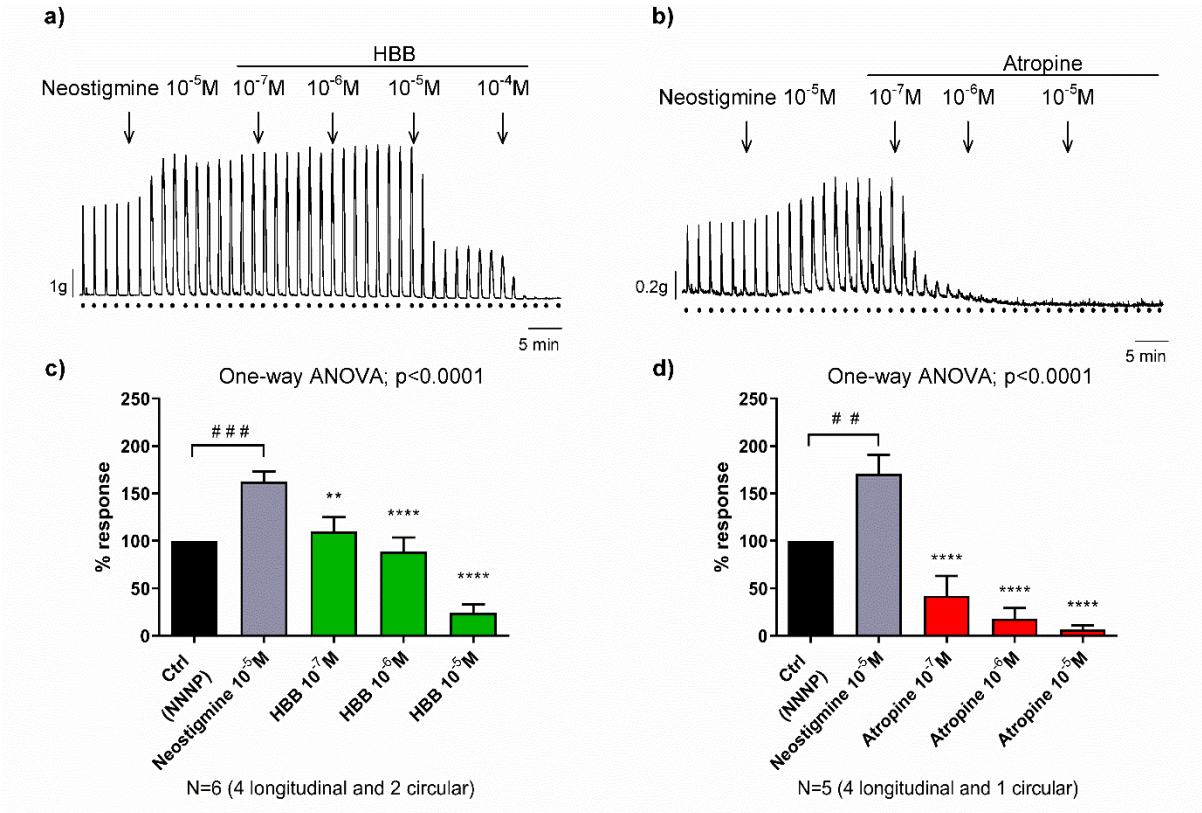

Figure 6. Effect of HBB and atropine on EFS-induced contractions enhanced by neostigmine in the circular and longitudinal colonic smooth muscle. (a) and (b) Representative mechanical recordings from the left colon, longitudinal muscle and (c) and (d) histograms showing an enhance of EFS-induced contractions after neostigmine 10^{-5}M and a concentration-dependent decrease in excitatory responses after HBB and atropine incubation, respectively. Dunnett post hoc test after ANOVA ##, **P <0.01; ###P <0.001; ****P <0.0001 compared with control and neostigmine 10^{-5}M respectively. Data expressed as mean ± SEM.
ANOVA, analysis of variance, EFS, electrical field stimulation; HBB, hyoscine butylbromide; NNNP, non-nitrergic and non-purinergic; SEM, standard error of the mean.

4. Discussion

This study provides a comprehensive assessment of the effect of HBB on neuromuscular response, including the selectively stimulated excitatory and inhibitory responses in the colon. HBB inhibits SPCs, carbachol-induced contractions and neural-mediated excitatory response. HBB has a greater effect on the circular than the longitudinal layer on carbachol-induced contractions. Moreover, the results indicate that HBB can inhibit the effect of atypical cholinergic mediated contractions.

Previously, Zhang et al., demonstrated that HBB concentration-dependently reduced colonic contractility. Our results demonstrate that vehicle and HBB concentration-dependently reduced SPCs possibly because of a rundown related to the duration of the experiment. To distinguish the putative pharmacological effect from the time-dependent rundown, a single concentration of both HBB and atropine was tested and a reduction in contractility was observed (Auli et al, 2008). In the presence of TTX, muscarinic antagonists reduced both the amplitude and frequency of myogenic contractions, while tone was only reduced by HBB. This suggests that this motility might be under the influence of muscarinic activation despite the blockade of neural activity (see below). The reduction in tone might also contribute to the spasmolytic activity of HBB.

Carbachol induced a strong contraction followed by large amplitude phasic contractions that were concentration-dependently inhibited with muscarinic antagonists. At high concentrations of antagonists, contractions were highly diminished or even abolished. This suggests a dependence on muscarinic receptors in this motor pattern. Both HBB and atropine were observed to be more potent in inhibiting carbachol-induced contractions in the circular than in the longitudinal muscle in both colonic regions, suggesting that the circular muscle layer is more susceptible of spasmolytic activity. In contrast, in previous published studies differences between layers were observed in the small intestine (Zhang et al., 2016) but not in the colon (Krueger et al., 2013; Zhang et al., 2016). It is unclear whether these differences are related to the region of the colon since previous studies did not specify

the colonic region assessed or to the use of a different muscarinic agonist (bethanechol, in previous studies).

In this study, it is compared the effect of HBB, DAU-5884 (preferred M3 antagonist), AF-DX 116 (preferred M2 antagonist) (Drumm et al., 2020), and atropine (non-selective antagonist) with vehicle on carbachol-induced contractions in humans. All muscarinic antagonists reduced carbachol-induced contractions in a concentration-dependent manner. The range of potency was DAU-5884 ≥atropine>HBB>AF-DX 116 in both muscle layers and regions of the colon. This suggests that M3 predominates over M2 in carbachol-induced responses. HBB binds to both M2 and M3 receptors with a slightly higher affinity for M2 (233nmol/l) than that for M3 (643nmol/l) receptors (Tytgat, 2008). The results of the present study are in line with these data and suggest that HBB and atropine blocks both receptors. M3 receptors are coupled to $G_{q/11}$ proteins that mediate phosphoinositide hydrolysis and Ca^{2+} mobilization whereas M2 receptors are coupled to $G_{i/o}$ proteins and mediate an inhibition of cAMP accumulation. This suggests that M3 receptors might directly contribute to the contractile response(Ehlert et al., 1999) and its associated spasm.

This study assesses the effect of HBB in excitatory responses. HBB concentration-dependently reduced neural-mediated contractions generated by the release of acetylcholine from excitatory motor neurons. The rank of potency was DAU-5884≥atropine>HBB>AF-DX 116, in both muscle layers. This indicates greater involvement of M3 receptors in EFS-induced contractile responses in the human colon. Our results are in line with previously reported results (Krueger et al., 2013) and show that HBB abolishes on-contractions which is probably associated to a total spasmolytic activity. In contrast, in the work of Zhang et al. the authors reported a reduction of 60% of the contractile response (Zhang et al., 2016). The discrepancy between these results is that in our work we selectively stimulate excitatory motor neurons and in the work of Zhang the authors do not discriminate between on and off-contractions and therefore, the pharmacological effect of HBB was probably underestimated.

In contrast, HBB did not affect neural-mediated inhibitory responses that are characterised by a relaxation during the stimulus followed by a rebound off-contraction due to the depolarisation of the tissue. The electrophysiological mechanism behind is the sustained inhibitory junction potential caused by ATP or a related purine and NO (Gallego et al., 2008; Gallego et al., 2011; Gallego et al., 2006). The results demonstrate that HBB does not have another MOA besides its anticholinergic properties since neither the on-relaxation nor the off-contraction was modified. If the drug has had an effect on L-type calcium channels (such as other spasmolytic drugs i.e. otilonium bromide), we would have obtained a reduction in the off-contraction.

EFS can activate receptors located at the neuromuscular junction whereas with carbachol all muscarinic receptors (junctional and extra-junctional) are stimulated (Bhetwal et al., 2013). In animal studies, it has recently been shown that M3 receptors are located in ICC and the signal is transduced to muscle cells (Drumm et al., 2020). If this is true in humans, the effect attributable to junctional muscarinic receptors is due to activation of M3 receptors in ICC causing a sharp contraction but limited in time since acetylcholinesterase limits the effect of neural released acetylcholine. When neostigmine is added, an increase in the amplitude and duration of the contraction is observed after EFS possibly attributable to the diffusion of acetylcholine to extra-junctional receptors (Bhetwal et al., 2013; Broad et al., 2013) and leading to a stronger contraction, possibly better mimicking a spasm. Accordingly, the present study suggests that the spasmolytic activity of HBB is due to the blockade of junctional and extra-junctional muscarinic receptors.

One important question that is not solved is how these extra-junctional muscarinic receptors can be endogenously activated. In our study, we blocked neural activity with TTX and ω-CTX (GVIA). Despite the presence of TTX-resistant action potentials (possibly due to Nav 1.8 activation) described in some afferent neurons(Miranda-Morales et al., 2010), TTX 1μM reduced the response to EFS by 92%. This confirms that most of the response was attributable to TTX-sensitive action potentials. When both toxins were applied in combination, no response to EFS was observed showing that conventional

neural release of acetylcholine was totally blocked. Under neural blockade, neostigmine was still able to increase contractions generating an atypical endogenous cholinergic response. Similar results have been reported in the rat and guinea-pig bladder (Zagorodnyuk et al., 2009) and in the stomach of mice(Cai et al., 2022). In the mouse stomach, authors used neostigmine, in the presence of TTX, and they attributed the response to a non-neural source of acetylcholine that might activate extra-junctional receptors (Cai et al., 2022). Acetylcholine is known to be released also by non-neuronal sources as part of the non-neuronal cholinergic system (NNCS)(Li et al., 2022; Wessler and Kirkpatrick, 2008). Similar to neurons, in some non-neuronal cells, acetylcholine is synthesised by the enzyme choline acetyltransferase (Beckmann and Lips, 2013) and might be released by organic cation transporters (Wessler et al., 2001). Evidence suggests that NNCS is distributed widely across the human organs, including intestinal epithelial cells (Tuft Cells), immune cells and even in some effector cells (Li et al., 2022; Wessler and Kirkpatrick, 2008; Huang et al., 2022). At present, the relative contribution of the NNCS in the muscle contractility is unknown since the potential cell has not been identified. However, it is reasonable to hypothesize that if there is a local increase in acetylcholine that might be increased in certain pathologies, muscarinic receptors will be activated. If the concentration of acetylcholine is enough, a muscle spasm will be developed which, according to our results, can be reversed by HBB. This might underly the mechanism responsible for the spasm associated with some organic GI diseases (Cheng et al., 2008; Kuol et al., 2022; Yu et al., 2017).

Another hypothesis that might also explain the atypical release of acetylcholine is the neuronal non-quantal release of acetylcholine that has been reported at the neuromuscular junction and in the autonomic nervous system (Chavez et al., 2011). Quantal release of acetylcholine might be blocked by TTX and ω-CTX (GVIA) whereas non-quantal release might be independent of nerve action potentials and calcium entrance. In airways it has been postulated that choline transporter might be involved in neuronal non-quantal release of acetylcholine (Chavez et al., 2011). Accordingly, it is possible that neuronal non-quantal release of acetylcholine might contribute to the observed response. Although at high concentrations HBB also blocks nicotinic receptors (Krueger et al., 2013), it would be important

to verify the role of pre-junctional nicotinic receptors in the effect of neostigmine. As far as we know neural non-quantal release of acetylcholine has not been demonstrated in the ENS.

5. Conclusions

The application of a systematic approach, including selective blockade of inhibitory and excitatory pathways, demonstrates that HBB inhibits colonic contractions induced by stimulation of muscarinic receptors at neuromuscular junction (enteric neurons) and extra-junctional level, where M3 receptors play a significant role. This study revealed a new effect of HBB inhibiting cholinergic-stimulated muscle contractions through an atypical source. Further research is needed to identify potentials non-neural cells that might be associated to the colonic spasm.

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Data Availability Statement

Qualified researchers may request access to data and related study documents, including the study report, study protocol with any amendments, statistical analysis plan and dataset specifications. Further details on Sanofi's data sharing criteria, eligible studies and process for requesting access can be found at: https://www.vivli.org/.

Conflict of Interest

Sara Traserra received salary from the Universitat Autònoma de Barcelona, which is funded by a Sanofi grant. Luis Gerardo Alcalá-González, Claudia Barber, Stefania Landolfi and Carolina Malagelada declared no conflict of interest. Maura Corsetti is a consultant for Sanofi and co-chief investigator of a Sanofi-sponsored research grant. Marcel Jimenez received a research grant from Sanofi. Robert Lange is currently a Sanofi employee and may hold shares and/or stock options in the company. Sylvie Forestier is the former employee at Sanofi.

Ethics Approval Statement

All experimental procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona, Spain (ethical approval code: 5604).

Author Contributions

All authors contributed to the conceptualization and methodology of the manuscript, study investigation, acquisition and curation and formal analysis of data for the manuscript. All authors contributed to the writing, critical review and approved the final version of the manuscript to be published.

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Tables

Table 1. Effect of HBB, atropine, DAU-5884 and AF-DX 116 on carbachol-induced contractions in the circular and longitudinal smooth muscle: EC_{50}, $logEC_{50}$ ± SEM and Hill slope ± SEM of each antagonist in the right and left colon samples. Results from nonlinear regression curve – Sidak's multiple comparisons test.

HBB					
Right colon	**Number of samples (N)**	**EC_{50}**	**$LogEC_{50}$ ± SEM**	**Hill slope ± SEM**	**R^2**
Circular	6	2.62×10^{-7} M	−6.58 ± 0.095	−0.88 ± 0.151	0.79
Longitudinal	6	6.64×10^{-7} M	−6.18 ± 0.033*	−1.07 ± 0.077	0.96
Left colon					
Circular	6	2.31×10^{-7} M	−6.64 ± 0.082	−0.83 ± 0.115	0.83
Longitudinal	6	8.16×10^{-7} M	−6.09 ± 0.084*	−1.07 ± 0.197	0.79
Atropine					
Right colon	**Number of samples (N)**	**EC_{50}**	**$LogEC_{50}$ ± SEM**	**Hill slope ± SEM**	**R^2**
Circular	4	1.56×10^{-8} M	−7.81 ± 0.059	−0.81 ± 0.082	0.93
Longitudinal	4	3.98×10^{-8} M	−7.40 ± 0.075*	−1.21 ± 0.223	0.89
Left colon					
Circular	4	1.73×10^{-8} M	−7.76 ± 0.051	−1.27 ± 0.166	0.94
Longitudinal	5	3.97×10^{-8} M	−7.40 ± 0.075*	−1.3 ± 0.258	0.87
DAU-5884 (Preferential M3 Antagonist)					
Right colon	**Number of samples (N)**	**EC_{50}**	**$LogEC_{50}$ ± SEM**	**Hill slope ± SEM**	**R^2**
Circular	6	3.58×10^{-8} M	−7.45 ± 0.070	−0.80 ± 0.092	0.88
Longitudinal	5	1.66×10^{-8} M	−7.78 ± 0.069	−0.96± 0.131	0.88
Left colon					
Circular	5	1.32×10^{-8} M	−7.88 ± 0.043	−0.99 ± 0.088	0.94
Longitudinal	7	2.75×10^{-8} M	−7.56 ± 0.067	−0.93 ± 0.119	0.86
AF-DX 116 (Preferential M2 Antagonist)					
Right colon	**Number of samples (N)**	**EC_{50}**	**$LogEC_{50}$ ± SEM**	**Hill slope ± SEM**	**R^2**
Circular	5	6.80×10^{-7} M	−6.17 ± 0.084**	−0.42 ± 0.038	0.86
Longitudinal	5	2.42×10^{-6} M	−5.62 ± 0.145**	−0.59 ± 0.125	0.59
Left colon					
Circular	4	1.05×10^{-6} M	−5.98 ± 0.178**	−0.58 ± 0.138	0.59
Longitudinal	6	2.46×10^{-6} M	−5.61 ± 0.121**	−0.82 ± 0.187	0.62

*P <0.01 circular vs. longitudinal muscle; **P <0.0001 DAU-5884 vs. AF-DX 116.

EFS, electrical field stimulation; HBB, hyoscine butylbromide; $logEC_{50}$, log of half maximal effective concentration; SEM, standard error of the mean

Table 2. Effect of HBB, atropine, DAU-5884 and AF-DX 116 on neural-induced excitatory responses in the circular and longitudinal smooth muscles: EC_{50}, $logEC_{50}$ ± SEM and Hill slope ± SEM of each antagonist in the right and left colonic samples. Results from nonlinear regression curve – Sidak's multiple comparisons test.

HBB					
Right colon	**Number of samples (N)**	**EC_{50}**	**$LogEC_{50}$ ± SEM**	**Hill slope ± SEM**	**R^2**
Circular	6	6.80×10^{-7} M	−6.17 ± 0.131	−0.80 ± 0.178	0.63
Longitudinal	6	4.72×10^{-7} M	−6.33 ± 0.119	−0.60 ± 0.095	0.70
Left colon					
Circular	4	5.52×10^{-7} M	−6.26 ± 0.071	−0.95 ± 0.131	0.90
Longitudinal	7	1.02×10^{-6} M	−5.99 ± 0.043	−1.05 ± 0.097	0.92
Atropine					
Right colon	**Number of samples (N)**	**EC_{50}**	**$LogEC_{50}$ ± SEM**	**Hill slope ± SEM**	**R^2**
Circular	4	5.16×10^{-8} M	−7.29 ± 0.098	−1.11 ± 0.244	0.83
Longitudinal	4	3.98×10^{-8} M	−7.16 ± 0.060	−1.26 ± 0.193	0.92
Left colon					
Circular	4	7.38×10^{-8} M	−7.13 ± 0.061	−0.89 ± 0.097	0.94
Longitudinal	7	4.87×10^{-8} M	−7.31 ± 0.075	−0.98 ± 0.146	0.84
DAU-5884 (Preferential M3 Antagonist)					
Right colon	**Number of samples (N)**	**EC_{50}**	**$LogEC_{50}$ ± SEM**	**Hill slope ± SEM**	**R^2**
Circular	4	2.08×10^{-8} M	−7.68 ± 0.117*	−0.69 ± 0.121	0.79
Longitudinal	4	1.66×10^{-8} M	−7.30 ± 0.078*	−0.71 ± 0.079	0.91
AF-DX 116 (Preferential M2 Antagonist)					
Right colon	**Number of samples (N)**	**EC_{50}**	**$LogEC_{50}$ ± SEM**	**Hill slope ± SEM**	**R^2**
Circular	4	3.14×10^{-6} M	−5.50 ± 0.101*	−0.75 ± 0.136	0.76
Longitudinal	4	1.21×10^{-5} M	−4.92 ± 0.190*	−1.20 ± 0.661	0.34

*P <0.0001 DAU-5884 vs. AF-DX 116.

EFS, electrical field stimulation; HBB, hyoscine butylbromide; $logEC_{50}$, log of half maximal effective concentration; SEM, standard error of the mean

Supplementary Table 1. Patient characteristics.

	Right colon	**Left colon**
Number of patients	26	22
Samples	160	149
Gender	10 women /16 men	8 women /14 men
Age range	43–91 years	35–92 years
(mean ± SD)	(73.0 years ± 12.62)	(64.4 years ± 15.39)

SD, standard deviation