JPET Fast Forward. Published on August 6, 2020 as DOI: 10.1124/jpet.120.265967
This article has not been copyedited and formatted. The final version may differ from this version.

Gender-related differences of tachykinin NK_2 receptor expression and activity in human colonic smooth muscle

Stelina Drimousis[1], Irit Markus[1], Tim V Murphy[2], D Shevy Perera[3], Kim-Chi Phan-Thien[3], Li Zhang[4], Lu Liu[1]

[1]Department of Pharmacology, School of Medical Sciences, UNSW Sydney, NSW, Australia

[2]Department of Physiology, School of Medical Sciences, UNSW Sydney, NSW, Australia

[3]Sydney Colorectal Associates, Hurstville, NSW, Australia

[4]School of Biotechnology and Biomolecular Sciences, UNSW Sydney, NSW, Australia

JPET Fast Forward. Published on August 6, 2020 as DOI: 10.1124/jpet.120.265967
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Tachykinin NK_2 receptors in human colonic smooth muscle

Corresponding author:
Associate Professor Lu Liu
UNSW Sydney, High St, Kensington, NSW 2052, Australia
Phone: +61 2 9385 8762
Fax: +61 2 9385 0023
Lu.Liu@unsw.edu.au

Number of text pages: 35
Number of tables: 1
Number of figures: 9
Number of references: 54
Number of words in abstract: 250
Number of words in introduction: 695
Number of words in discussion: 1500

Abbreviations

2-APB	2-aminoethoxydiphenyl borate
α-SMA	α-smooth muscle actin
ACh	Acetylcholine
DAPI	4', 6'-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
EC_{50}	Half maximal effective concentration
E_{max}	Maximal contractile responses
EFS	Electrical field stimulation
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IBS	Irritable bowel syndrome
ICCs	Interstitial cells of Cajal
IP_3	Inositol 1,4,5-triphosphate
LMN-NKA	[Lys^5,$MeLeu^9$,Nle^{10}]NKA(4-10)
NKA	Neurokinin A
PBS	Phosphate buffered saline
pK_B	Dissociation constant
PKC	Protein kinase C
PLC	Phospholipase C
SP	Substance P
sscDNA	single strand cDNA
TBS-TX	Tris-buffered saline with 0.05% Triton

Recommended section: Gastrointestinal, Hepatic, Pulmonary and Renal

ABSTRACT

The tachykinin NK_2 receptor plays a key role in gastrointestinal motor function. Enteric neurons release neurokinin A (NKA) which activates NK_2 receptors on gastrointestinal smooth muscle, leading to contraction and increased motility. In patients with diarrhoea-predominant irritable bowel syndrome, the NK_2 receptor antagonist ibodutant had a greater therapeutic effect in females than males. The present study aimed to determine whether gender influences the expression and activity of NK_2 receptors in human colonic smooth muscle. *In vitro* functional studies were performed to examine the contractile responses of colonic muscle strips to NKA and the selective NK_2 receptor agonist [Lys^5,$MeLeu^9$,Nle^{10}]NKA(4-10). Contractions were also measured in the presence of ibodutant to determine its antagonistic potency. The signal transduction pathways coupled to NK_2 receptor activation were investigated using second messenger inhibitors. Western blot and fluorescent immunohistochemistry were conducted to determine the protein expression and localization of NK_2 receptors. NK_2 receptor-mediated contractility was greater in females compared to males. When against NKA, ibodutant was more potent in females. NK_2 receptor expression increased with age in females, but not in males. Phospholipase C-mediated signalling was less prominent in females compared to males, while Ca^{2+} sensitization via Rho kinase and protein kinase C appeared to be the dominant pathway in both genders. The distribution of NK_2 receptors in the human colon did not differ between the genders. Overall, gender differences exist in the expression and activity of NK_2 receptors in colonic smooth muscle. These gender distinctions should be considered in the therapeutic development of NK_2 receptor agents.

JPET Fast Forward. Published on August 6, 2020 as DOI: 10.1124/jpet.120.265967
This article has not been copyedited and formatted. The final version may differ from this version.

SIGNIFICANCE STATEMENT

The tachykinin NK_2 receptor has been identified as a therapeutic target for the treatment of bowel and bladder dysfunctions. The present study has revealed gender-related variations in NK_2 receptor activity, signalling transduction pathways, antagonist potency and changes in expression with age. These factors may underlie the gender differences in the treatment of diarrhoea-predominant irritable bowel syndrome with NK_2 receptor antagonists. Our findings highlight that gender differences should be considered in the therapeutic development of NK_2 receptor agents.

INTRODUCTION

Tachykinins are a family of peptides that regulate a broad range of biological activities, including smooth muscle contraction, vasodilation, inflammation and nociception. The major naturally occurring tachykinins are substance P (SP), neurokinin A (NKA) and neurokinin B, and they exhibit preferential affinity for tachykinin NK_1, NK_2 and NK_3 receptors, respectively (Nakamura et al., 2011). Tachykinin receptors primarily couple to the $G\alpha_{q/11}$ class of G proteins which activate phospholipase C (PLC), leading to the formation of inositol 1,4,5-triphosphate (IP_3) and the subsequent release of Ca^{2+} from intracellular stores (Regoli et al., 1994; Mizuta et al., 2008). These receptors may also activate $G\alpha_s$ proteins, leading to increased cAMP levels (Nakajima et al., 1992; Regoli et al., 1994; Perdona et al., 2019).

In the gastrointestinal tract, SP and NKA are primarily released from myenteric and submucosal neurons and are involved in the modulation of motor and secretory events (Shimizu et al., 2008; Steinhoff et al., 2014). Among the three tachykinin receptor subtypes, NK_2 receptors have an important role in regulating human colonic motility. Numerous pharmacological studies using human colonic smooth muscle strips have revealed that the contractile effects of endogenous tachykinins are mediated by NK_2 receptors (Croci et al., 1998; Cao et al., 2000; Liu et al., 2002; Liu et al., 2009; Nakamura et al., 2011; Tanaka et al., 2012). This is in line with the high mRNA expression levels of the TACR2 gene (encoding NK_2 receptors) in human colonic smooth muscle (Liu et al., 2011). Furthermore, NK_2 receptor activation has been shown to be responsible for the tachykinergic component of excitatory neurotransmission, and altered NK_2 receptor function has been implicated in colonic

motility disorders, such as inflammatory bowel disease, diverticular disease and slow transit constipation in both adults and children (Menzies et al., 2001; Mitolo-Chieppa et al., 2001; Liu et al., 2002; Stanton et al., 2003; Maselli et al., 2004; Auli et al., 2008; Burcher et al., 2008; Alvarez-Berdugo et al., 2015).

Given their key involvement in human colonic motor function, NK_2 receptors have been considered as possible drug targets for the development of effective therapies for diarrhoea-predominant irritable bowel syndrome (IBS) (Lecci et al., 2004; Corsetti et al., 2015; Szymaszkiewicz et al., 2019). Specifically, the NK_2 receptor antagonist ibodutant emerged as a promising candidate due to its high selectivity and potency for human NK_2 receptors, long duration of action and good oral bioavailability (Cialdai et al., 2006; Meini et al., 2009; Santicioli et al., 2013). However, clinical trials uncovered a gender distinction in the effectiveness of ibodutant, whereby dose-dependent improvements in symptoms reached statistical significance over placebo in female IBS patients only (Tack et al., 2017).

The gender difference observed in the therapeutic outcome of ibodutant in diarrhoea-predominant IBS sparks an interest in the sex-specific variations surrounding colonic NK_2 receptors. Gender differences in the efficacy of ibodutant have also been reported in a guinea pig colitis model, in which colonic contractions induced by colorectal distension were inhibited by ibodutant at lower doses in females than those required in males (Bellucci et al., 2016). Previously, our findings revealed a gender-related distinction in the density of [^{125}I]NKA binding sites (corresponding to the number of NK_2 receptors) in human colonic smooth muscle, with a significantly higher binding capacity observed in males compared to females (Burcher et al., 2008). However, [^{125}I]NKA binding affinities and the expression levels of NK_2 receptor mRNA did not differ between the genders (Burcher et al., 2008).

Further investigation is required to explain the existence of gender distinctions in the efficacy of ibodutant and the abundance of NK_2 receptor binding sites. The present study aims to clarify these observations by uncovering any further sex-related differences in the expression and activity of NK_2 receptors in human colonic smooth muscle. Although NK_2 receptors primarily couple to the $G\alpha_{q/11}$-PLC-IP_3-Ca^{2+} transduction cascade to mediate smooth muscle contraction, such as in the bladder and airway (Mizuta et al., 2008; Der et al., 2019), this signal transduction does not appear to be the main pathway used by NK_2 receptors in human colonic smooth muscle (Warner et al., 2000; O'Riordan et al., 2001). Therefore, this study also aims to uncover the signal transduction pathways coupled to NK_2 receptor activation in the human colon.

MATERIALS AND METHODS

Human colon specimens

Ascending and sigmoid colon segments were taken 10 - 20 cm away from the tumour site of colorectal carcinoma patients undergoing surgical resection. Patients with obstructions or those who had received radiation therapy or chemotherapy were excluded from the study. The mean age of the specimens used was 58.5 years for females (age range: 31 - 85 years, n = 55) and 60.7 years for males (age range: 32 - 84, n = 43). There were no significant differences in the age of the female and male specimens used for the functional and expression studies. Specimens were collected from Hurstville Private, St George Private and Prince of Wales Public and Private Hospitals. The research was performed in accordance with the Declaration of Helsinki and approved by the South Eastern Sydney Local Health District Human Research Ethics Committee, Sydney, NSW, Australia. The specimens were transported to the laboratory in ice-cold, carbogenated (95% O_2 and 5% CO_2) Krebs-Henseleit solution (NaCl 118 mM, KCl 4.7 mM, $NaHCO_3$ 25 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 2.5 mM, D-glucose 11.7 mM). Specimens were gently washed to remove faecal matter and fat was trimmed. For the functional experiments, a portion of smooth muscle was dissected away from the other layers and stored in Krebs-Henseleit solution overnight at 4 ºC. The remaining tissue was processed for the expression studies as described below.

Functional organ bath set-up

Colonic smooth muscle strips (each approximately 3 x 6 mm) were cut along the circular axis. Muscle strips were mounted under 1 g tension in 3.5 ml organ baths containing carbogenated Krebs-Henseleit solution at 37 ºC, as previously described

(Liu et al., 2002). One end of each strip was fixed to a hook at the bottom of the bath, and the other end was connected to an isometric force transducer (Grass FTO3C, Warwick, RI, USA). The change of muscle tension was recorded using the Polygraph computer program (Mr. E. Crawford, UNSW Sydney, NSW, Australia). Preparations were allowed to equilibrate for 30 min before exposure to antagonists and inhibitors, and 60 min before exposure to tachykinin agonists.

Contractile responses to NK_2 receptor agents

During optimizing experimental conditions, it was shown that NKA and [Lys^5,$MeLeu^9$,Nle^{10}]NKA(4-10) (LMN-NKA) induced human colonic contractile responses remained unaffected in the presence of the NK_1 receptor antagonist CP9994 (100 nM) but were greatly reduced by the NK_2 receptor antagonist SR 48968 (100 nM) (Supplemental Fig. 1, A and B). Furthermore, the selective NK_1 receptor agonist [Pro^9] SP (up to 10 μM), was virtually ineffective in contracting human colonic smooth muscle (Supplemental Fig. 1, C). These results suggest that the influence of NK_1 receptors is negligible. There was no evidence for the involvement of enteric neuron transmission as atropine (1 μM) showed no effect to NKA and LMN-NKA (Supplemental Fig. 2). Thus, the subsequent experiments were conducted without the presence of an NK_1 antagonist nor atropine.

A single concentration-response curve to NKA or the selective NK_2 receptor agonist LMN-NKA (0.001 - 10 μM) was obtained from each strip, using 60 min intervals to avoid desensitization. Washing occurred twice between each concentration of agonist. Acetylcholine (ACh, 10 mM) was used as an internal standard and applied at the start and end of each concentration-response curve, as per previously published protocols (Warner et al., 2000; Liu et al., 2002; Burcher et al., 2008). In parallel

experiments, contractile responses were measured in the presence of the selective NK_2 receptor antagonist ibodutant (either 0.01, 0.1 or 1 μM) pre-incubated for 30 min before the addition of agonist.

Contractile responses in the presence of second messenger inhibitors

NKA and LMN-NKA were fixed at the submaximal concentration of 0.3 μM and added at 60 min intervals. Muscle strips were washed twice between each application of agonist. Female muscle strip desensitization was not observed under control conditions, while slight desensitization occurred in male strips. For both control and treated muscle strips, results were expressed as a percentage of the average of two initial contractile responses under control conditions (before inhibitors were applied). In treated muscle strips, preincubation with second messenger inhibitors occurred for 30 min using increasing concentrations (3, 30, 100, 300 μM) of either U73122 hydrate (PLC inhibitor), 2-aminoethoxydiphenyl borate (2-APB, IP_3 receptor inhibitor), GF109203X (protein kinase C (PKC) inhibitor) or Y-27632 dihydrochloride (Rho kinase inhibitor). The inactive analogue of U73122 hydrate, U-73343 (100 μM), did not produce an effect on the agonist-induced contractions.

Neuronal tachykinin release by EFS

Muscle strips were suspended between two platinum rings at 1 g tension, and preparations were allowed to equilibrate for 30 min. Electrical field stimulation (EFS) was conducted using cumulative frequencies (0.5, 5, 10, 50 Hz) of 1 ms duration, in 80 V trains lasting 10 s (Liu et al., 2009). Each frequency of EFS was given 10 min apart. Immediately after each stimulation, 300 μl bath fluid was collected and stored at -80 ºC, and an equal volume of Krebs-Henseleit solution was added back to each

bath. The amount of released NKA and SP was later analyzed using radioimmunoassay kits (RK-046-15 for NKA and RK-061-05 for SP, Phoenix Pharmaceuticals, Burlingame, CA, USA).

Protein expression of NK_2 receptors by Western blot

Total protein was extracted from frozen human sigmoid colonic smooth muscle using lysis buffer containing 1 mM EGTA, 10 mM Tris-HCl and protease inhibitor cocktail tablets (Sigma-Aldrich, St Louis, MO, USA). Protein samples (20 µg) were denatured at 95 ºC for 8 min and loaded into 50 µl wells of 10% Mini-PROTEAN precast gels (Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis was conducted at 100 V for 1.5 h. Protein transfer onto polyvinylidene difluoride membranes was carried out using the Trans-Blot Turbo Transfer Pack and System (Bio-Rad Laboratories) at 25 V for 7 min. Immunodetection was achieved by incubating with the anti-NK_2 receptor antibody (ATR-002, Alomone Labs, Jerusalem, Israel) at a dilution of 1:1000 for 1 h at room temperature. The specificity of the NK_2 antibody was verified using corresponding control peptides, same as previously confirmed (Delvalle et al., 2018), as well as by conducting positive and negative control Western blots. The anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (G9545, Sigma-Aldrich, 1:50000) was used as the internal control. After washing, the membranes were incubated with anti-rabbit IgG (whole molecule)-peroxidase secondary antibody (A4914, Sigma-Aldrich, 1:5000) for 1 h at room temperature. Visualization of protein bands was performed using SuperSignal West Pico PLUS Chemiluminescent Substrate (34577, Thermo Scientific, Waltham, MA, USA) and densitometry analysis was carried out using Image Studio Lite 5.2 (LI-COR Biosciences, Lincoln, NE, USA).

Localization of NK_2 receptors by immunofluorescence

Full-thickness human sigmoid colon samples were fixed in Zamboni's solution, embedded in paraffin, sectioned and mounted on poly-L-lysine coated slides. The slides were deparaffinized with xylene, rehydrated with graded ethanol concentrations (100%, 70%) and washed in phosphate buffered saline (PBS, 0.1 M, pH = 7.4). Antigen retrieval was performed at 110 ºC for 5 min using EnVision FLEX Target Retrieval Solution Low pH (Dako Omnis, Santa Clara, CA, USA). Non-specific binding sites were blocked by PBS containing 10% goat serum (G9023, Sigma-Aldrich) for 30 min. Slides were incubated overnight with primary antibodies diluted in Tris-buffered saline with 0.05% Triton (TBS-TX, 0.1 M, pH = 7.6) containing 2% serum. The anti-NK_2 receptor antibody (ATR-002, Alomone Labs, 1:50) was double labelled with either the anti-α-smooth muscle actin (α-SMA) antibody (ab7817, Abcam, Melbourne, VIC, Australia, 1:50), the anti-cD117/c-kit antibody (AF332, R&D Systems, Minneapolis, MN, USA, 1:50), the anti-Hu C/D antibody (A21271, Invitrogen, Carlsbad, CA, USA, 1:200), the anti-β tubulin antibody (MMS435P, Covance, Princeton, NJ, USA, 1:100), the anti-S100 antibody (ab4066, Abcam, 1:50) or the anti-GFAP antibody (ab4674, Abcam, 1:100). The next day, the slides were incubated with secondary antibodies diluted in TBS-TX for 2 h at room temperature. The secondary antibodies used were goat anti-rabbit IgG Alexa Fluor 488 (ab150077, Abcam), goat anti-mouse IgG Alexa Fluor 594 (ab150116, Abcam) and goat anti-chicken IgG Alexa Fluor 488 (BA-9010, Vector Laboratories, Burlingame, Ca, USA). Slides were mounted in 4', 6'-diamidino-2-phenylindole (DAPI, Invitrogen). Images were captured by the Neurolucida/Stereoinvestigator system (MBF Bioscience). Obvious visual differences were noted, but no quantitative statistical analysis was performed. Negative controls

were conducted under the same conditions without primary antibodies and did not produce a signal.

Gene expression of second messenger molecules by real-time PCR

Human ascending and sigmoid colonic smooth muscle samples were frozen in RNAlater (R0901, Sigma-Aldrich). Total RNA was extracted using the Trizol method (Invitrogen) and purified by DNase treatment (3 U at 37 ºC, 20 min). Single strand cDNA (sscDNA) was synthesized using the SuperScript™ III First-Strand Synthesis Supermix (Invitrogen) in a reaction mixture containing 2 µg RNA and 50 ng μl^{-1} random hexamers, at 1 cycle of 25 ºC for 10 min, 50 ºC for 50 min and 85 ºC for 5 min. RNase H treatment occurred (2 U μl^{-1}) at 37 ºC for 20 min. sscDNA was diluted to a working concentration of 500 ng μL^{-1}.

Gene expression was determined by real-time PCR using the SYBR Select Master Mix (Applied Biosystems, Austin, TX, USA). The primers were designed with Primer3, and primer sequences and PCR amplification efficiencies are listed in Supplementary Table 1. Each PCR reaction was performed in a volume of 10 µl containing 5 µl Master Mix, 10 µM forward and reverse primers and 80 ng μl^{-1} sscDNA. The solutions were dispensed into 384 well plates by the Eppendorf epMotion 5075 Automated Pipetting System. GAPDH was used as a housekeeping gene. Inter-run comparisons were achieved using a designated calibrator (one colonic smooth muscle sample) in each real-time PCR assay. PCR amplification occurred for 1 cycle at 95 ºC (2 min), 40 cycles at 95 ºC (15 s), 60 ºC (15 s) and 68 ºC (20 s), followed by 10 min of gradual temperature elevation from 60 ºC to 95 ºC for the melting curve analysis.

Data analysis

Data were analyzed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) and statistical significance was achieved when $P < 0.05$. The results obtained from ascending and sigmoid colonic muscle strips did not differ, so data from both regions were combined in the analyses.

For the functional studies, data were expressed as tension (g). Similar results were seen when the data were expressed as tension (g)/ strip weight (g). However, ACh could not be used as a reliable internal standard because contractile responses varied between the experimental groups. Results are presented as mean ± SEM unless otherwise stated. Concentration-response curves were generated using non-linear regression analysis to determine maximal contractile responses (E_{max}) and half maximal effective concentration (EC_{50}) values. The data were analyzed by two-way ANOVA followed by with Bonferroni multiple comparisons test. For the antagonist ibodutant, Schild plots were created and pA_2 values were taken as the x-intercept of the Schild regression analysis. The apparent pK_B values of each individual antagonist concentration were also calculated using the equation: pK_B = log (dose ratio - 1) - log [B], where [B] is the antagonist concentration (Arunlakshana and Schild, 1959; Kenakin, 2009). Data were analyzed using one-way ANOVA with Bonferroni's multiple comparisons test.

For the NKA and SP release study, basal levels of NKA and SP were expressed as nanograms/strip weight (ng/g), and comparisons of median values were made using Mann-Whitney analysis. EFS-induced release was expressed as a percentage of the basal level and presented as mean ± SEM. Gender differences were assessed by two-way ANOVA with Bonferroni's multiple comparisons test, whereas

JPET Fast Forward. Published on August 6, 2020 as DOI: 10.1124/jpet.120.265967
This article has not been copyedited and formatted. The final version may differ from this version.

comparisons between basal and EFS-induced tachykinin levels were made at each EFS frequency using one sample t-test analysis.

For the expression studies, NK_2 receptor protein levels were expressed as a percentage of GAPDH. The mRNA level of genes encoding second messengers was expressed as fold change normalized to GAPDH and relative to the calibrator, using the equation: Fold change = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = [Ct_{(target)} - Ct_{(GAPDH)}]_{sample} - [Ct_{(target)} - Ct_{(GAPDH)}]_{calibrator}$ (Pfaffl, 2001). Median values were compared using Mann-Whitney analysis or the Kruskal-Wallis test for multiple comparisons. Spearman analysis was used for the correlation studies.

Drugs

NKA was purchased from Abcam (ab120185) and LMN-NKA was custom synthesized by China Peptides (Pudong, Shanghai, China). Both were reconstituted in 0.01 M acetic acid with β-mercaptoethanol. ACh was purchased from Sigma-Aldrich (A6625) and reconstituted in water. Ibodutant was a gift from our collaborators at The Menarini Group (Florence, Italy) and was prepared in dimethyl sulfoxide (DMSO). The second messenger inhibitors 2-APB (1224), GF109203X (0741) and Y-27632 dihydrochloride (1254) were purchased from Tocris Bioscience (Noble Park, Victoria, Australia), while the inhibitor U73122 hydrate (U6756) was obtained from Sigma-Aldrich. The second messenger inhibitors were dissolved in DMSO. Aliquots of all reagents were stored at -20 ºC.

RESULTS

Contractile responses to NK_2 receptor agonists

The endogenous NK_2 receptor ligand NKA and the selective NK_2 receptor agonist LMN-NKA evoked prominent contractile responses in human colonic smooth muscle strips (Fig. 1). There were no gender differences in contractions produced by NKA (n = 11 females, 10 males) (Fig. 1, A). On the other hand, LMN-NKA elicited contractile responses that were significantly greater in females (n = 11) compared to males (n = 11, $^{**}P = 0.0023$, two-way ANOVA) (Fig. 1, B). In line with this, the E_{max} of NKA did not vary between the genders (1.8 ± 0.27), while the E_{max} of LMN-NKA was significantly greater in females (2.0 ± 0.18) compared to males (1.2 ± 0.17, $^{**}P = 0.0029$, unpaired t-test). When comparing the E_{max} of NKA and LMN-NKA in males, values were close to statistical significance ($P = 0.0742$), suggesting that suppressed responses to LMN-NKA in male strips underlie the observed gender difference. There was no correlation between age and E_{max} for either agonist, in either gender. There were no gender differences in EC_{50} values. For NKA, the EC_{50} values and 95% confidence intervals were 65.63 nM (25 - 167) in females and 75.31 nM (28 - 200) in males. For LMN-NKA, the EC_{50} values and 95% confidence intervals were 87.06 nM (45 - 166) in females and 28.76 nM (10 - 78) in males.

Potency of NK_2 receptor antagonist ibodutant

Ibodutant antagonized the contractile responses elicited by NKA and LMN-NKA in the human colonic smooth muscle strips of females (n = 11) and males (n = 9 - 11), and produced a rightward shift of the agonist concentration-response curves (Fig. 2). Schild plots were generated to calculate pA_2 values for ibodutant against both agonists, and its competitive antagonism property was confirmed, since ibodutant did

not change the maximum contractilities of the agonists, and the slopes of Schild plots are not different from unity (Fig. 2 and Fig. 3, Table 1). The apparent pK_B values of each individual antagonist concentration were also calculated and presented as mean values in Table 1.

When against NKA, ibodutant displayed a significantly higher degree of antagonism in females (pA_2 = 8.51 ± 0.38) compared to males (pA_2 = 7.23 ± 0.21, *P < 0.05, one-way ANOVA) (Table 1). On the other hand, no gender differences were observed in the potency of ibodutant when against LMN-NKA (pA_2 = 8.30 ± 0.36 for females and 8.26 ± 0.22 for males) (Table 1). Surprisingly, ibodutant appeared agonist-dependent in the male colon, being less potent against NKA compared to LMN-NKA (*P < 0.05, one-way ANOVA). These results were similar to the apparent pK_B values calculated from the individual concentration of ibodutant (Table 1). There was no significant difference in the potency of ibodutant against the different agonists in female colonic muscle strips. The pA_2 values of ibodutant obtained from our study were lower than that previously reported in the human colon (pK_B = 9.1), and gender-related differences were not described (Santicioli et al., 2013). It is noted that in their study, the concentration-response curves to the NK_2 receptor agonist [βAla^8]NKA(4-10) were cumulatively constructed (Santicioli et al., 2013). Nevertheless, a 60 min interval between each addition of the agonists was applied in our study to avoid desensitization. The different protocols may contribute to the discrepancies between the two studies.

Basal and EFS-induced release of NKA and SP

NKA and SP released from human colonic smooth muscle strips were measured to uncover any gender differences that could be contributing to the

observed distinctions in contractility (Fig. 4). The basal level of NKA did not vary between females (4.7 ± 2.0, n = 14) and males (2.8 ± 0.8, n = 15) (Fig. 4, A). On the other hand, a higher amount of SP was spontaneously released under resting conditions from muscle strips of females (5.5 ± 1.0, n = 10) compared to those of males (2.6 ± 0.4, n = 9, *P = 0.0279, Mann-Whitney test) (Fig. 4, B).

The neuronal release of NKA and SP elicited by cumulative frequencies of EFS was then recorded and expressed as a percentage of the basal level. EFS-induced release of NKA (Fig. 4, C) and SP (Fig. 4, D) was significantly greater than basal amounts at 5 Hz and 10 Hz for both genders (one sample t-test). At 50 Hz, there was a significant increase in the amount of NKA released from male smooth muscle only (*P = 0.0467, one sample t-test) (Fig. 4, C). Interestingly, SP release in response to 0.5 Hz was significantly lower than basal levels in male strips (****P < 0.0001, one sample t-test) and a trend of decrease was also observed in female strips (P = 0.0934) (Fig. 4, D). Overall, there were no significant differences in EFS-induced tachykinin release between the genders.

NK_2 receptor protein expression

Western blot and densitometry analysis of human colonic sigmoid smooth muscle were performed to uncover any gender differences in the protein expression of NK_2 receptors (Fig. 5). The NK_2 receptor protein band was consistent with an ~45 kDa protein size as indicated by the manufacturer (Fig. 5, A). Data are expressed as a percentage of GAPDH levels (~36 kDa protein size). There was no difference in NK_2 receptor protein expression between females (n = 25) and males (n = 15) (Fig. 5, B). There was a noticeable trend increase in the expression of the NK_2 receptor in specimens older than 50 years of age compared to younger ones (Fig. 5, C).

Correlation analysis revealed an increased NK_2 receptor expression with age in females (r = 0.5023, *P = 0.0105, Spearman correlation test), but not in males (r = 0.127, P = 0.6499) (Fig. 5, D).

NK_2 receptor localization throughout the smooth muscle layers

Strong NK_2 receptor immunoreactivity was detected throughout the circular and longitudinal smooth muscle layers of the human sigmoid colon (n = 6) (Fig. 6). Extensive colocalization with α-SMA (n = 2) indicates that NK_2 receptors are present on smooth muscle cells (Fig. 6, A - C). On the other hand, NK_2 receptors did not appear to be expressed on interstitial cells of Cajal (ICCs, n = 2), as evidenced by double labelling with c-kit (Fig. 6, D - I). NK_2 receptor staining was colocalized with the neuronal marker β-tubulin on some neuronal processes throughout the smooth muscle (Fig. 6, J - L). No obvious gender differences were observed in the distribution of NK_2 receptors throughout the smooth muscle layers.

NK_2 receptor localization within the myenteric ganglia

Dense NK_2 receptor immunoreactivity was expressed within the myenteric ganglia of the human sigmoid colon (n = 6) (Fig. 7). While weak colocalization with the neuronal marker HuC/D was observed, strong NK_2 receptor staining was detected on the structures surrounding the HuC/D positive cells (n = 2) (Fig. 7, A - C). Similarly, double labelling with β-tubulin (n = 6) suggests that NK_2 receptors are present on a limited number of enteric neurons (Fig. 7, D - F). On the other hand, NK_2 receptor staining was extensively colocalized with the glial cell markers S100 (Fig. 7, G - I, n = 6) and GFAP (Fig. 7, J - L, n = 2) within the myenteric ganglia, as well as on glial

processes throughout the muscle layers. There were no obvious gender differences in the localization of NK_2 receptor expression within the myenteric ganglia.

NK_2 receptor-mediated signalling pathways

Contractile responses of human colonic smooth muscle strips to NKA and LMN-NKA were examined in the presence of various second messenger inhibitors, to determine if the gender differences observed in contractility could be attributed to receptor signalling (Fig. 8). Following the blockade of PLC by U73122, contractile responses produced by NKA in male strips (n = 11) were significantly attenuated (****P* = 0.0005, two-way ANOVA) (Fig. 8, A). NKA-induced contractions in female strips (n = 9) were also reduced, but to a lesser extent (***P* = 0.0058), and a gender difference was observed at the highest concentration (300 μM) of inhibitor (**P* = 0.0138, two-way ANOVA) (Fig. 8, A). Similar observations were made using LMN-NKA as the agonist (Fig. 8, B). Contractions elicited by LMN-NKA in both male (n = 10) and female (n = 9) strips were reduced by PLC inhibition (*****P* < 0.0001 and ***P* = 0.0078, respectively, two-way ANOVA), and overall, this inhibitory effect was more pronounced in males compared to females (****P* < 0.0008, two-way ANOVA) (Fig. 8, B).

Since activation of PLC triggers separate transduction pathways involving IP_3 receptors and PKC, the effect of inhibiting these signalling proteins was also examined. Blockade of IP_3 receptors by 2-APB did not affect contractions elicited by NK_2 receptor activation in either gender (n = 6 females, 5 males) (Fig. 8, C and D). On the other hand, PKC inhibition by GF109203X produced concentration-dependent reductions of NK_2 receptor-mediated contractile responses in both female (n = 6) and male (n = 6) strips *(*****P* < 0.0001 for both genders and agonists, two-way ANOVA) (Fig. 8, E and F). During smooth muscle contraction, the $G\alpha_{q/11}$-PLC cascade may

JPET Fast Forward. Published on August 6, 2020 as DOI: 10.1124/jpet.120.265967
This article has not been copyedited and formatted. The final version may differ from this version.

occur alongside $G\alpha_{12/13}$-Rho kinase signalling, so the involvement of this latter pathway was also examined. In both genders (n = 9 for each), contractile responses evoked by NKA were significantly diminished by Rho kinase blockade with Y-27632 in a concentration-dependent manner (*****P* < 0.0001, two-way ANOVA) (Fig. 8, G). Similarly, inhibition of Rho kinase caused a concentration-dependent attenuation of LMN-NKA-induced contractions in both female (n = 9) and male (n = 10) strips (*****P* < 0.0001, ****P* < 0.0004, respectively, two-way ANOVA) (Fig. 8, H). No sex-related distinctions were observed for PKC and Rho kinase inhibition (Fig. 8, E - H).

Since gender differences were observed for PLC inhibition, real-time PCR was conducted to uncover any gender variations in the expression of genes encoding the PLCβ family of isoenzymes, as well as the $G\alpha_q$ family of G proteins (Fig. 9). There were no observed differences in the mRNA expression of G proteins $G\alpha_q$, $G\alpha_{11}$ and $G\alpha_{14}$ (Fig. 9, A - C) or PLCβ 1-4 isoenzymes (Fig. 9, D - G) between male (n = 25 - 27) and female (n = 26 - 27) human colonic smooth muscle samples.

DISCUSSION

The present study has uncovered several gender differences in NK_2 receptor activity and expression in the human colon. NK_2 receptor-mediated contractility is more pronounced in females compared to males, and this is accompanied by a greater potency of the NK_2 receptor antagonist ibodutant, a higher basal release of SP, and an increased NK_2 receptor protein expression with age. In addition, the involvement of PLC-mediated signalling appears to be less prominent in females compared to males. The present findings also appreciate the importance of NK_2 receptor-mediated Ca^{2+} sensitization pathways via PKC and, for the first time, Rho kinase in the human colon.

Male colonic smooth muscle exhibited reduced responsiveness to the selective NK_2 receptor agonist LMN-NKA. This finding is in contrast to our previous report, in which contractile responses induced by LMN-NKA in human colonic muscle strips were similar in both genders (Burcher et al., 2008). However, in that earlier study, the data were expressed as a percentage of the maximum response to ACh, so this may underlie the discrepancy. On the other hand, there were no gender differences in contractility produced by the natural NK_2 receptor ligand NKA in the current study, and this is consistent with our previous findings (Burcher et al., 2008). The differences observed for NKA and LMN-NKA could be due to the activation of NK_1 receptors by NKA, since natural tachykinins preferentially but not exclusively bind to all three tachykinin receptor subtypes (Nakamura et al., 2011).

The NK_2 receptor antagonist ibodutant displays a significantly higher degree of antagonism in females compared to males when against NKA. This is in line with the results obtained from human clinical trials and may underlie the gender discrepancies reported for the efficacy of ibodutant in diarrhoea-predominant IBS patients (Tack et

al., 2017). Conversely, the potency of ibodutant against LMN-NKA did not vary between genders, and this is consistent with previous findings using [β-Ala^8]NKA(4-10) as the selective NK_2 receptor agonist (Santicioli et al., 2013). Ibodutant has been shown to display a high affinity for human NK_2 receptors in [^{125}I]NKA binding studies using transfected cells (pK_i =10.1) (Cialdai et al., 2006) and colonic smooth muscle (pK_i = 9.9) (Santicioli et al., 2013). Its affinity values at NK_1 (pK_i = 6.1) and NK_3 (pK_i = 6.2) receptors were shown to be four orders of magnitude lower, confirming the selectivity of ibodutant for NK_2 receptors (Cialdai et al., 2006). While the mechanisms underlying agonist-dependent differences in the gender-specific antagonistic activity of ibodutant remain unclear, they may be occurring to different extents in males and females. Nevertheless, the potency of ibodutant against NKA, which we have observed to be greater in females, would more closely reflect the physiological setting.

Interestingly, the antagonistic activity of ibodutant at the NK_2 receptor appears agonist-dependent in males with a higher potency against LMN-NKA compared to NKA. NK_2 receptors can couple to both $G\alpha_{q/11}$ and $G\alpha_s$ proteins, and different ligands can stabilise distinct NK_2 receptor states, leading to the activation of different downstream pathways (Palanche et al., 2001; Maillet et al., 2007; Valant et al., 2009). Thus, NKA- and LMN-NKA-dependent conformations of the NK_2 receptor may be responsible for the agonist-specific effect of ibodutant in the male colon.

In terms of tachykinin peptide release, basal levels of NKA were similar in both genders, whereas a higher amount of SP was released from female colonic smooth muscle. There were no sex-related variations in EFS-induced tachykinin release. In a previous study, KCl-induced NKA and SP levels were higher in colonic smooth muscle from female guinea pigs subjected to colitis compared to controls, while this difference was not seen in males (Bellucci et al., 2016). The overall amount of SP released was

lower compared to NKA in guinea pig colonic smooth muscle (Bellucci et al., 2016), and this is consistent with the current findings. It appears that alternative splicing of the TAC1 gene (encoding SP and NKA) favours NKA production in colonic enteric neurons. The reduced level of SP at lower frequency of EFS (0.5 Hz) may be due to an autoregulatory mechanism, whereby SP exerts a feedback inhibition of its own release by activating NK_1 receptors on enteric nerve terminals (Lomax et al., 1998).

NK_2 receptor protein expression increases with age in females, supporting the evidence that changes in sex hormone levels influence tachykinin peptide and receptor expression (Villablanca and Hanley, 1997; Pinto et al., 2009). Sex hormones have also been shown to influence intestinal smooth muscle contractions. In human colonic circular muscle strips, carbachol-evoked contractions were reduced in the presence of estrogen, and this effect was prevented by estrogen receptor antagonism (Hogan et al., 2009). Similarly, progesterone inhibited spontaneous and EFS-induced contractions in mice colonic circular muscle strips, and decreased fecal output in two diarrheal mouse models (Li et al., 2012). Since sex hormones affect tachykinin expression and colonic contractile responses, they may also be contributing to the age-related changes of NK_2 receptor protein expression in females. In other words, NK_2 receptor expression and activity may be regulated by sex hormones.

NK_2 receptor immunoreactivity was densely localized on colonic circular and longitudinal smooth muscle, but was absent on ICCs, reinforcing previous immunofluorescence findings in the human colon (Renzi et al., 2000; Warner et al., 2000; Jaafari et al., 2007; Nakamura et al., 2011). This suggests that NK_2 receptors mediate colonic contractions by direct activation of smooth muscle cells, without regulating intestinal pacemaker cell activity.

Strong NK_2 receptor staining was also detected throughout the myenteric ganglia, suggesting that apart from mediating direct myogenic effects, NK_2 receptors also play a neuroregulatory role in colonic motor pathways. While weak NK_2 receptor colocalization with neuronal markers was observed, intense NK_2 receptor labelling was found on myenteric glial cells. Glial cells were traditionally considered to provide passive support to neurons, but an abundance of evidence now exists linking impaired glial function to altered intestinal motility (Aube et al., 2006; Nasser et al., 2006; McClain et al., 2014). Interestingly, tachykinin receptor agonists can induce glial cell activation in the mouse colon (Broadhead et al., 2012). Furthermore, NKA has been shown to activate neuronal and glial networks during neuroinflammation and neurodegeneration (Delvalle et al., 2018). However, this effect is via NK_2 receptors on surrounding sensory neurons and nerve varicosities, since NK_2 receptors were undetectable on mouse enteric glial cells (Delvalle et al., 2018). This earlier study used the same NK_2 receptor antibody as in the current study, indicating a species difference in the expression of NK_2 receptors on enteric glial cells.

U73122 showed a more prominent effect in males compared to females. This does not appear to be associated with the gene expression of PLC, since the mRNA expression levels of all PLCβ isoenzymes were similar in both genders. The mechanism underlying the gender distinction is unclear but may involve differences in the protein levels of phosphorylated PLCβ isoenzymes, which have not been determined in the current study. In addition to PLC inhibition, U73122 has been shown to inhibit the Ca^{2+} pump on the sarcoplasmic reticulum to deplete Ca^{2+} (MacMillan and McCarron 2010).

Interestingly, NK_2 receptor-mediated contractile responses in the human colon do not appear to be related to IP_3 receptor activation. In accordance, inositol

phospholipid hydrolysis stimulated by LMN-NKA has been previously shown to be much weaker in human colonic smooth muscle compared to rat bladder positive controls (Warner et al., 2000). In addition, NKA-evoked contractions of human colonic smooth muscle were unaffected by intracellular Ca^{2+} depletion (O'Riordan et al., 2001). Thus, NK_2 receptor induced intracellular Ca^{2+} release by IP_3 receptor activation is a minor pathway in mediating human colon contractility.

NKA and LMN-NKA evoked contractile responses that were significantly diminished by PKC and Rho kinase inhibitors, suggesting that Ca^{2+} sensitization contributes to NK_2 receptor-mediated contractility of the human colon. Ca^{2+} sensitization is the phenomenon by which smooth muscle contractions can be sustained independently of Ca^{2+}, by PKC- and Rho kinase-mediated inhibition of myosin light chain phosphatase, which prevents the dephosphorylation of myosin light chain (Puetz et al., 2009; Perrino, 2016). Previously, the contribution of PKC and Rho kinase to Ca^{2+} sensitization evoked by muscarinic receptor activation has been demonstrated in membrane-permeabilized rat colonic muscle strips (Takeuchi et al., 2004; Takeuchi et al., 2007). This is the first report demonstrating the role of PKC and Rho kinase signalling in NK_2 receptor activated-smooth muscle contractions of the human colon.

In conclusion, the present study has revealed an increased NK_2 receptor activity in females, which may underlie the gender differences in the treatment of diarrhoea-predominant IBS. These sex-related differences may be due to the influence of ovarian hormones, since the expression of NK_2 receptors increases with age in females. These findings highlight that gender differences should be considered in the therapeutic development of NK_2 receptor agents. The pronounced localization of NK_2 receptors on enteric glial cells in the human colon suggests that aside from direct

JPET Fast Forward. Published on August 6, 2020 as DOI: 10.1124/jpet.120.265967
This article has not been copyedited and formatted. The final version may differ from this version.

myogenic activation, NK_2 receptors may also have an important neuroregulatory role. Furthermore, the current study indicates the importance of both $G\alpha_{q/11}$-PKC and $G\alpha_{12/13}$-Rho kinase signalling pathways in NK_2 receptor-mediated contractile responses in the human colon.

Acknowledgements

We would like to thank Dr Maria Altamura and Dr Stefania Meini of Menarini Ricerche S.p.A., Firenze, Italy for the supply of ibodutant and the discussion of ibodutant results. We also thank Professor Elizabeth Burcher for providing intellectual input and Ms Haylee Guy for assistance with specimen collections.

Authorship Contributions

Participated in research design: Drimousis and Liu

Conducted experiments: Drimousis and Markus

Contributed to new reagents or analytic tools: Perera and Phan-Thien

Performed data analysis: Drimousis and Liu

Wrote or contributed to the writing of the manuscript: Drimousis, Murphy, Zhang and Liu

References

Alvarez-Berdugo D, Espin F, Arenas C, Lopez I, Clave P and Gallego D (2015) Changes in the response to excitatory antagonists, agonists, and spasmolytic agents in circular colonic smooth muscle strips from patients with diverticulosis. Neurogastroenterol Motil 27:1600-1612.

Arunlakshana O and Schild HO (1959) Some quantitative uses of drug antagonists. Br J pharmacol Chemother 14:48-58.

Aube AC, Cabarrocas J, Bauer J, Philippe D, Aubert P, Doulay F, Liblau R, Galmiche JP and Neunlist M (2006) Changes in enteric neurone phenotype and intestinal functions in a transgenic mouse model of enteric glia disruption. Gut 55:630-637.

Auli M, Martinez E, Gallego D, Opazo A, Espin F, Marti-Gallostra M, Jimenez M and Clave P (2008) Effects of excitatory and inhibitory neurotransmission on motor patterns of human sigmoid colon in vitro. Br J Pharmacol 155:1043-1055.

Bellucci F, Bueno L, Bugianesi R, Crea A, D'Aranno V, Meini S, Santicioli P, Tramontana M and Maggi CA (2016) Gender-related differential effect of tachykinin NK2 receptor-mediated visceral hyperalgesia in guinea pig colon. Br J Pharmacol 173:1329-1338.

Broadhead MJ, Bayguinov PO, Okamoto T, Heredia DJ and Smith TK (2012) Ca2+ transients in myenteric glial cells during the colonic migrating motor complex in the isolated murine large intestine. J Physiol 590:335-350.

Burcher E, Shang F, Warner FJ, Du Q, Lubowski DZ, King DW and Liu L (2008) Tachykinin NK2 receptor and functional mechanisms in human colon: changes with indomethacin and in diverticular disease and ulcerative colitis. J Pharmacol Exp Ther 324:170-178.

Cao W, Pricolo VE, Zhang L, Behar J, Biancani P and Kirber MT (2000) Gq-linked NK2 receptors mediate neurally induced contraction of human sigmoid circular smooth muscle. Gastroenterology 119:51-61.

Cialdai C, Tramontana M, Patacchini R, Lecci A, Catalani C, Catalioto RM, Meini S, Valenti C, Altamura M, Giuliani S and Maggi CA (2006) MEN15596, a novel nonpeptide tachykinin NK2 receptor antagonist. Eur J Pharmacol 549:140-148.

Corsetti M, Akyuz F and Tack J (2015) Targeting tachykinin receptors for the treatment of functional gastrointestinal disorders with a focus on irritable bowel syndrome. Neurogastroenterol Motil 27:1354-1370.

Croci T, Aureggi G, Manara L, Emonds-Alt X, Le Fur G, Maffrand JP, Mukenge S and Ferla G (1998) In vitro characterization of tachykinin NK2-receptors modulating motor responses of human colonic muscle strips. Br J Pharmacol 124:1321-1327.

Delvalle NM, Dharshika C, Morales-Soto W, Fried DE, Gaudette L and Gulbransen BD (2018) Communication Between Enteric Neurons, Glia, and Nociceptors Underlies the Effects of Tachykinins on Neuroinflammation. Cell Mol Gastroenterol Hepatol 6:321-344.

Der B, Molnar PJ, Ruisanchez E, Orsy P, Kerek M, Farago B, Nyirady P, Offermanns S and Benyo Z (2019) NK2 receptor-mediated detrusor muscle contraction involves Gq/11-dependent activation of voltage-dependent Ca(2+) channels and the RhoA-Rho kinase pathway. Am J Physiol Renal Physiol 317:F1154-F1163.

Hogan AM, Kennelly R, Collins D, Baird AW and Winter DC (2009) Oestrogen inhibits human colonic motility by a non-genomic cell membrane receptor-dependent mechanism. Br J Surg 96:817-822.

Jaafari N, Khomitch-Baud A, Christen MO and Jule Y (2007) Distribution pattern of tachykinin NK2 receptors in human colon: involvement in the regulation of intestinal motility. J Comp Neurol 503:381-391.

Kenakin TP (2009) A pharmacology primer : theory, applications, and methods, Academic Press/Elsevier, Amsterdam ; Boston.

Lecci A, Capriati A and Maggi CA (2004) Tachykinin NK2 receptor antagonists for the treatment of irritable bowel syndrome. Br J Pharmacol 141:1249-1263.

Li CP, Ling C, Biancani P and Behar J (2012) Effect of progesterone on colonic motility and fecal output in mice with diarrhea. Neurogastroenterol Motil 24:392-e174.

Liu L, Markus I, Saghire HE, Perera DS, King DW and Burcher E (2011) Distinct differences in tachykinin gene expression in ulcerative colitis, Crohn's disease and diverticular disease: a role for hemokinin-1? Neurogastroenterol Motil 23:475-483, e179-480.

Liu L, Shang F, Markus I and Burcher E (2002) Roles of substance P receptors in human colon circular muscle: alterations in diverticular disease. J Pharmacol Exp Ther 302:627-635.

Liu L, Shang F, Morgan MJ, King DW, Lubowski DZ and Burcher E (2009) Cyclooxygenase-dependent alterations in substance P-mediated contractility and tachykinin NK1 receptor expression in the colonic circular muscle of patients with slow transit constipation. J Pharmacol Exp Ther 329:282-289.

Lomax AE, Bertrand PP and Furness JB (1998) Identification of the populations of enteric neurons that have NK1 tachykinin receptors in the guinea-pig small intestine. Cell Tissue Res 294:27-33.

Macmillan D and McCarron J G (2010) The phospholipase C inhibitor U-73122 inhibits Ca(2+) release from the intracellular sarcoplasmic reticulum Ca(2+) store by inhibiting Ca(2+) pumps in smooth muscle. Br J Pharmacol 160:1295-1301.

Maillet EL, Pellegrini N, Valant C, Bucher B, Hibert M, Bourguignon JJ and Galzi JL (2007) A novel, conformation-specific allosteric inhibitor of the tachykinin NK2 receptor (NK2R) with functionally selective properties. FASEB J 21:2124-2134.

Maselli MA, Piepoli AL, Guerra V, Caruso ML, Pezzolla F, Lorusso D, Demma I and De Ponti F (2004) Colonic smooth muscle responses in patients with diverticular disease of the colon: effect of the NK2 receptor antagonist SR48968. Dig Liver Dis 36:348-354.

McClain J, Grubisic V, Fried D, Gomez-Suarez RA, Leinninger GM, Sevigny J, Parpura V and Gulbransen BD (2014) Ca2+ responses in enteric glia are mediated by connexin-43 hemichannels and modulate colonic transit in mice. Gastroenterology 146:497-507 e491.

Meini S, Bellucci F, Catalani C, Cucchi P, Giolitti A, Santicioli P and Giuliani S (2009) Multifaceted approach to determine the antagonist molecular mechanism and interaction of ibodutant ([1-(2-phenyl-1R-[[1-(tetrahydropyran-4-ylmethyl)-piperidin-4-ylmethyl]-carbamoyl]-ethylcarbamoyl)-cyclopentyl]-amide) at the human tachykinin NK2 receptor. J Pharmacol Exp Ther 329:486-495.

Menzies JR, McKee R and Corbett AD (2001) Differential alterations in tachykinin NK2 receptors in isolated colonic circular smooth muscle in inflammatory bowel disease and idiopathic chronic constipation. Regul Pept 99:151-156.

Mitolo-Chieppa D, Mansi G, Nacci C, De Salvia MA, Montagnani M, Potenza MA, Rinaldi R, Lerro G, Siro-Brigiani G, Mitolo CI, Rinaldi M, Altomare DF and Memeo V (2001) Idiopathic chronic constipation: tachykinins as cotransmitters in colonic contraction. Eur J Clin Invest 31:349-355.

Mizuta K, Gallos G, Zhu D, Mizuta F, Goubaeva F, Xu D, Panettieri RA, Jr., Yang J and Emala CW, Sr. (2008) Expression and coupling of neurokinin receptor subtypes to inositol phosphate and calcium signaling pathways in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 294:L523-534.

Nakajima Y, Tsuchida K, Negishi M, Ito S and Nakanishi S (1992) Direct linkage of three tachykinin receptors to stimulation of both phosphatidylinositol hydrolysis and cyclic AMP cascades in transfected Chinese hamster ovary cells. J Biol Chem 267:2437-2442.

Nakamura A, Tanaka T, Imanishi A, Kawamoto M, Toyoda M, Mizojiri G and Tsukimi Y (2011) Bidirectional Regulation of Human Colonic Smooth Muscle Contractility by Tachykinin NK2 Receptors. J Pharmacol Sci 117:106-115.

Nasser Y, Fernandez E, Keenan CM, Ho W, Oland LD, Tibbles LA, Schemann M, MacNaughton WK, Ruhl A and Sharkey KA (2006) Role of enteric glia in intestinal physiology: effects of the gliotoxin fluorocitrate on motor and secretory function. Am J Physiol Gastrointest Liver Physiol 291:G912-927.

O'Riordan AM, Quinn T, Hyland JM, O'Donoghue DP and Baird AW (2001) Sources of calcium in neurokinin A-induced contractions of human colonic smooth muscle in vitro. Am J Gastroenterol 96:3117-3121.

Palanche T, Ilien B, Zoffmann S, Reck MP, Bucher B, Edelstein SJ and Galzi JL (2001) The neurokinin A receptor activates calcium and cAMP responses through distinct conformational states. J Biol Chem 276:34853-34861.

Perdona E, Cavallini P, Sava A, Griffante C, Ricca DJ, Thor KB, Rupniak NMJ and Corsi M (2019) Potency, efficacy, and selectivity of GR64349 at human recombinant neurokinin NK2 and NK1 receptors. Neurosci Lett 711:134456.

Perrino BA (2016) Calcium Sensitization Mechanisms in Gastrointestinal Smooth Muscles. J Neurogastroenterol Motil 22:213-225.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45.

Pinto FM, Pintado CO, Pennefather JN, Patak E and Candenas L (2009) Ovarian steroids regulate tachykinin and tachykinin receptor gene expression in the mouse uterus. Reprod Biol Endocrinol 7:77.

Puetz S, Lubomirov LT and Pfitzer G (2009) Regulation of smooth muscle contraction by small GTPases. Physiology (Bethesda) 24:342-356.

Regoli D, Boudon A and Fauchere JL (1994) Receptors and antagonists for substance P and related peptides. Pharmacol Rev 46:551-599.

Renzi D, Pellegrini B, Tonelli F, Surrenti C and Calabro A (2000) Substance P (neurokinin-1) and neurokinin A (neurokinin-2) receptor gene and protein expression in the healthy and inflamed human intestine. Am J Clin Pathol 157:1511-1522.

Santicioli P, Meini S, Giuliani S, Catalani C, Bechi P, Riccadonna S, Ringressi MN and Maggi CA (2013) Characterization of ibodutant at NK(2) receptor in human colon. Eur J Pharmacol 702:32-37.

Shimizu Y, Matsuyama H, Shiina T, Takewaki T and Furness JB (2008) Tachykinins and their functions in the gastrointestinal tract. Cell Mol Life Sci 65:295-311.

Stanton MP, Hengel PT, Southwell BR, Chow CW, Keck J, Hutson JM and Bornstein JC (2003) Cholinergic transmission to colonic circular muscle of children with slow-transit constipation is unimpaired, but transmission via NK2 receptors is lacking. Neurogastroenterol Motil 15:669-678.

Steinhoff MS, von Mentzer B, Geppetti P, Pothoulakis C and Bunnett NW (2014) Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. Physiol Rev 94:265-301.

Szymaszkiewicz A, Malkiewicz A, Storr M, Fichna J and Zielinska M (2019) The place of tachykinin NK2 receptor antagonists in the treatment diarrhea-predominant irritable bowel syndrome. J Physiol Pharmacol 1:08.

Tack J, Schumacher K, Tonini G, Scartoni S, Capriati A, Maggi CA and Iris i (2017) The neurokinin-2 receptor antagonist ibodutant improves overall symptoms, abdominal pain and stool pattern in female patients in a phase II study of diarrhoea-predominant IBS. Gut 66:1403-1413.

Takeuchi T, Kushida M, Hirayama N, Kitayama M, Fujita A and Hata F (2004) Mechanisms involved in carbachol-induced Ca(2+) sensitization of contractile elements in rat proximal and distal colon. Br J Pharmacol 142:657-666.

Takeuchi T, Nakajima H, Hata F and Azuma YT (2007) A minor role for Ca2+ sensitization in sustained contraction through activation of muscarinic receptor in circular muscle of rat distal colon. Pflugers Arch 454:565-574.

Tanaka T, Tanaka A, Nakamura A, Matsushita K, Imanishi A, Matsumoto-Okano S, Inatomi N, Miura K, Toyoda M, Mizojiri G and Tsukimi Y (2012). Effects of TAK-480, a novel tachykinin NK2–receptor antagonist, on visceral hypersensitivity in rabbits and ricinoleic acid-induced defecation in guinea pigs. J Pharmacol Sci 120:15-25.

Valant C, Maillet E, Bourguignon JJ, Bucher B, Utard V, Galzi JL and Hibert M (2009) Allosteric functional switch of neurokinin A-mediated signaling at the neurokinin NK2 receptor: structural exploration. J Med Chem 52:5999-6011.

Villablanca AC and Hanley MR (1997) 17β-estradiol stimulates substance P receptor gene expression. Mol Cell Endocrinol 135:109-117.

Warner FJ, Liu L, Lubowski DZ and Burcher E (2000) Circular muscle contraction, messenger signalling and localization of binding sites for neurokinin A in human sigmoid colon. Clin Exp Pharmacol Physiol 27:928-933.

This research received no specific grant from any funding agency

Legends for Figures

Fig. 1. Gender-related differences in NK_2 receptor-mediated contractility. Contractile responses of female (n = 11) and male (n = 10 - 11) human colonic circular smooth muscle strips to A) the endogenous NK_2 receptor ligand neurokinin A (NKA) and B) the selective NK_2 receptor agonist [Lys^5,$MeLeu^9$, Nle^{10}]NKA(4-10) (LMN-NKA). Data are expressed as mean tension (g) ± SEM. Significance by two-way ANOVA is indicated.

Fig. 2. Sex-specific distinctions in the potency of the selective NK_2 receptor antagonist ibodutant. The effect of different concentrations of ibodutant on contractile responses induced by the endogenous NK_2 receptor ligand neurokinin A (NKA, left panel) and the selective NK_2 receptor agonist [Lys^5,$MeLeu^9$, Nle^{10}]NKA(4-10) (LMN-NKA, right panel) in female (A and B) and male (C and D) human colonic circular smooth muscle strips. Data are expressed as a tension (g), and curves are presented as a percentage of the mean maximum response for each concentration of ibodutant. n = 9 - 11 for each curve, and closed circles indicate mean ± SEM.

Fig. 3. The Schild plots generated to calculate pA_2 values for ibodutant against the endogenous NK_2 receptor ligand neurokinin A (NKA, left panel) and the selective NK_2 receptor agonist [Lys^5,$MeLeu^9$, Nle^{10}]NKA(4-10) (LMN-NKA, right panel) in female (A and B) and male (C and D) human colonic circular muscle strips (n=11).

Fig. 4. Gender-related differences in the neuronal release of tachykinin peptides. A and B) Basal release of neurokinin A (NKA) and substance P (SP) from female (n = 14, 10 respectively) and male (n = 15, 9 respectively) human colonic smooth muscle strips. Horizontal lines denote medians. Significance by Mann-Whitney test is indicated, where **P* = 0.0279. C and D) The release of NKA and SP in response to increasing frequencies of electric field stimulation (EFS). Data are expressed as a percentage of the basal amount released, and presented as mean ± SEM. One sample t-test analysis of EFS-induced tachykinin release compared to basal level is indicated by **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001.

Fig. 5. Western blot and densitometry analysis of NK_2 receptor protein expression in human colonic sigmoid smooth muscle. Results are expressed as a percentage of the loading control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A) NK_2 receptor and GAPDH protein blots with specific bands corresponding to the expected molecular weight (MW) of ~45 kDa and ~36 kDa, respectively. B) NK_2 receptor protein expression in female (n = 25) and male (n = 15) samples. Horizontal lines denote medians. C) NK_2 receptor protein expression in samples from patients younger (closed circles) or older (open circles) than 50 years of age. D) Spearman correlation analysis of NK_2 receptor expression and age in females (r = 0.5023, **P* = 0.0105) and males (r = 0.127, *P* = 0.6499).

Fig. 6. Fluorescent immunoreactivity of the NK_2 receptor (left panel) in the smooth muscle layers of the human sigmoid colon. The cell markers used (middle panel) are α-smooth muscle actin (α-SMA) (B, n = 2), the Interstitial cells of Cajal marker c-kit (E and H, n = 2), and the neuronal marker β-tubulin (K, n = 6). The merged images (right panel) contain the nuclear marker 4', 6'-diamidino-2-phenylindole (DAPI, blue). Staining is present on myenteric ganglia (mg), circular muscle (cm) and longitudinal (lm) muscle. The scale bar indicates 20 μm. Representative images were taken from female specimens aged 62 yo (A - C, J - L) and 46 yo (G - I), and a male specimen aged 49 yo (D - F).

Fig. 7. Fluorescent staining of the NK_2 receptor (left panel) within the myenteric ganglia of the human sigmoid colon. The cell markers used (middle panel) are the nerve cell body marker Hu C/D (B, n = 2), the neuronal marker β-tubulin (E, n = 6), and the glial cell markers S100 (H, n = 6) and GFAP (K, n = 2). The merged images (right panel) contain the nuclear marker 4', 6'-diamidino-2-phenylindole (DAPI, blue). Staining is present on myenteric ganglia (mg), circular muscle (cm) and longitudinal (lm) muscle. The scale bar indicates 20 μm. Representative images were taken from a female specimen aged 62 yo (A - F) and a male specimen aged 49 yo (G - L).

Fig. 8. Gender-related variations in NK_2 receptor-mediated second messenger signalling pathways. Contractile responses of female (red) and male (blue) human colonic circular smooth muscle strips to neurokinin A (NKA, left panel) and [Lys^5,$MeLeu^9$, Nle^{10}]NKA(4-10) (LMN-NKA, right panel) at 0.3 μM, either alone (dashed lines) or in the presence of the phospholipase C (PLC) inhibitor U73122 (A - B), the inositol 1,4,5-triphosphate (IP_3) receptor inhibitor 2-aminoethoxydiphenyl borate (2-APB, C - D), the protein kinase C (PKC) inhibitor GF109203X (E - F), or the Rho kinase inhibitor Y-27632 dihydrochloride (G - H) at 3, 30, 100 and 300 μM. Data are expressed as a percentage of the contractile response before pharmacological inhibition, and presented as mean ± SEM (n = 5 - 11 for each curve). Significance by two-way ANOVA is indicated by a single symbol for $P < 0.05$, two for $P < 0.01$, three for $P < 0.001$ and four for $P < 0.0001$ (* for before vs. after inhibition within each gender; † for females vs. males

Fig. 9. Gene expression of the $G\alpha_q$ family of G proteins (A - C) and the phospholipase C (PLC) β isoenzymes (D - G) in female (n = 26 - 27) and male (n = 25 - 27) human colonic smooth muscle. Results are normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and presented as fold change relative to the inter-run calibrator. Horizontal lines denote medians.

Table 1. Summary of Schild plot results for ibodutant against NKA and the selective NK_2 receptor agonist LMN-NKA in human colonic circular smooth muscle.

Parameters	Ibodutant against NKA		Ibodutant against LMN-NKA	
	Female	Male	Female	Male
pA_2 ± SEM	8.36 ± 0.38*	7.26 ± 0.21	8.14 ± 0.36	8.33 ± 0.22**
(Apparent pK_B)	(8.22 ± 0.17)**	(7.10 ± 0.14)	(7.99 ± 0.19)	(8.11 ± 0.08)**
Schild slope ± SEM	0.76 ± 0.14	0.81 ± 0.055	0.72 ± 0.17	0.90 ± 0.12
Slope different from 1	No	No	No	No
	P = 0.24	*P* = 0.077	*P* = 0.24	*P* = 0.51

**P < 0.05, ** P < 0.01, compared to male against NKA (One-way ANOVA with Bonferroni's test).*

pA_2 values and slopes were generated from Schild plot and expressed as mean ± SEM. All slopes were not significantly different from 1, and there was no different between the slopes.

Values in parentheses are the mean apparent pK_B of individual ibodutant concentration calculated using the equation pK_B = log (dose ratio - 1) - log [B], where [B] is the antagonist concentration (Arunlakshana and Schild, 1959; Kenakin, 2009).

JPET Fast Forward. Published on August 6, 2020 as DOI: 10.1124/jpet.120.265967
This article has not been copyedited and formatted. The final version may differ from this version.

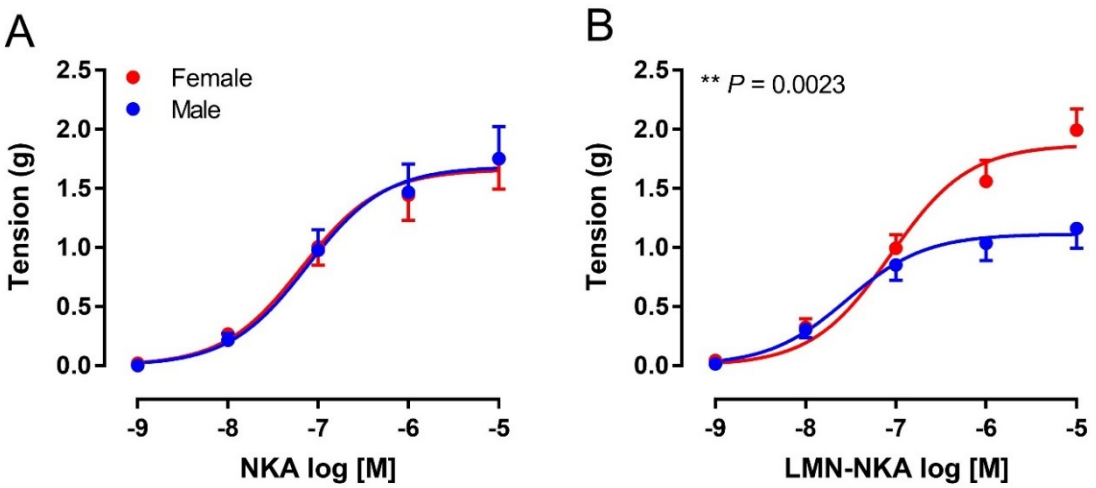


Figure 1

JPET Fast Forward. Published on August 6, 2020 as DOI: 10.1124/jpet.120.265967
This article has not been copyedited and formatted. The final version may differ from this version.

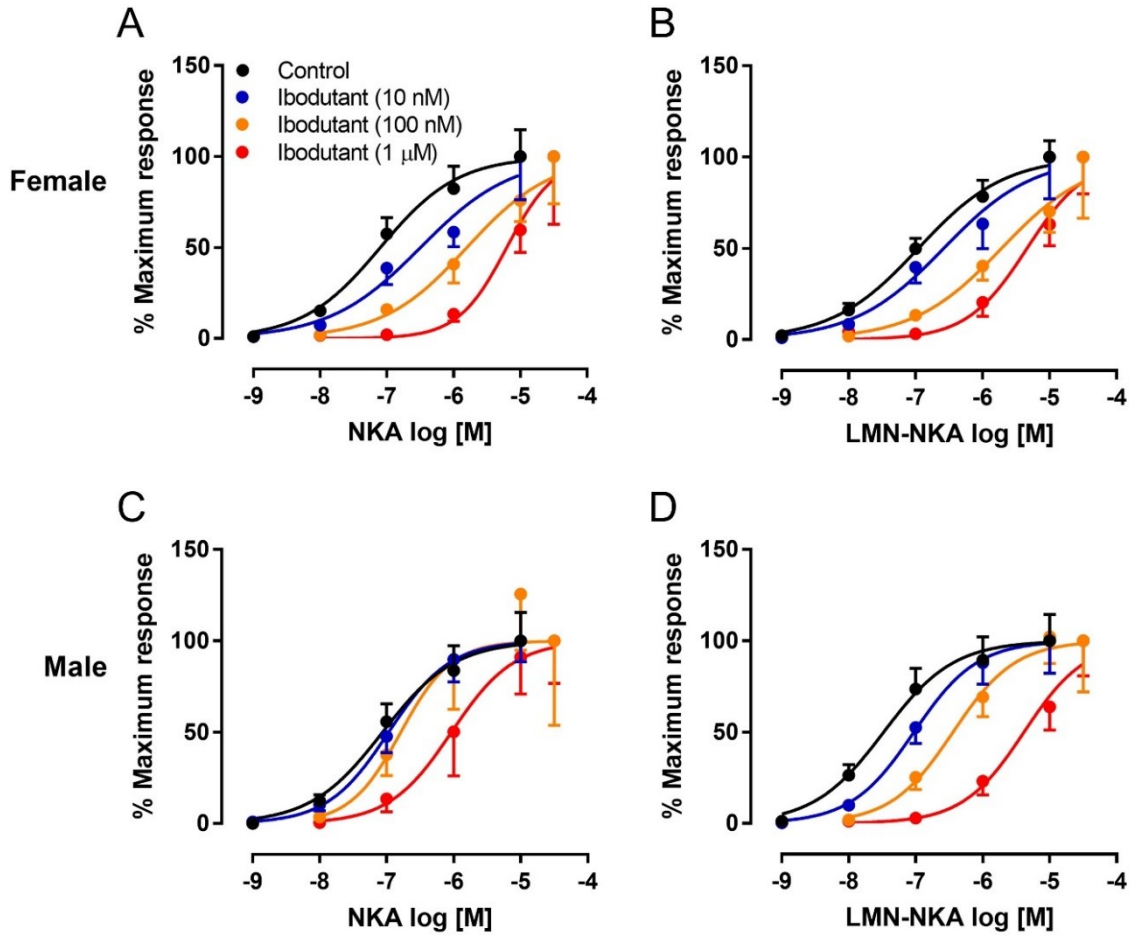


Figure 2

JPET Fast Forward. Published on August 6, 2020 as DOI: 10.1124/jpet.120.265967
This article has not been copyedited and formatted. The final version may differ from this version.

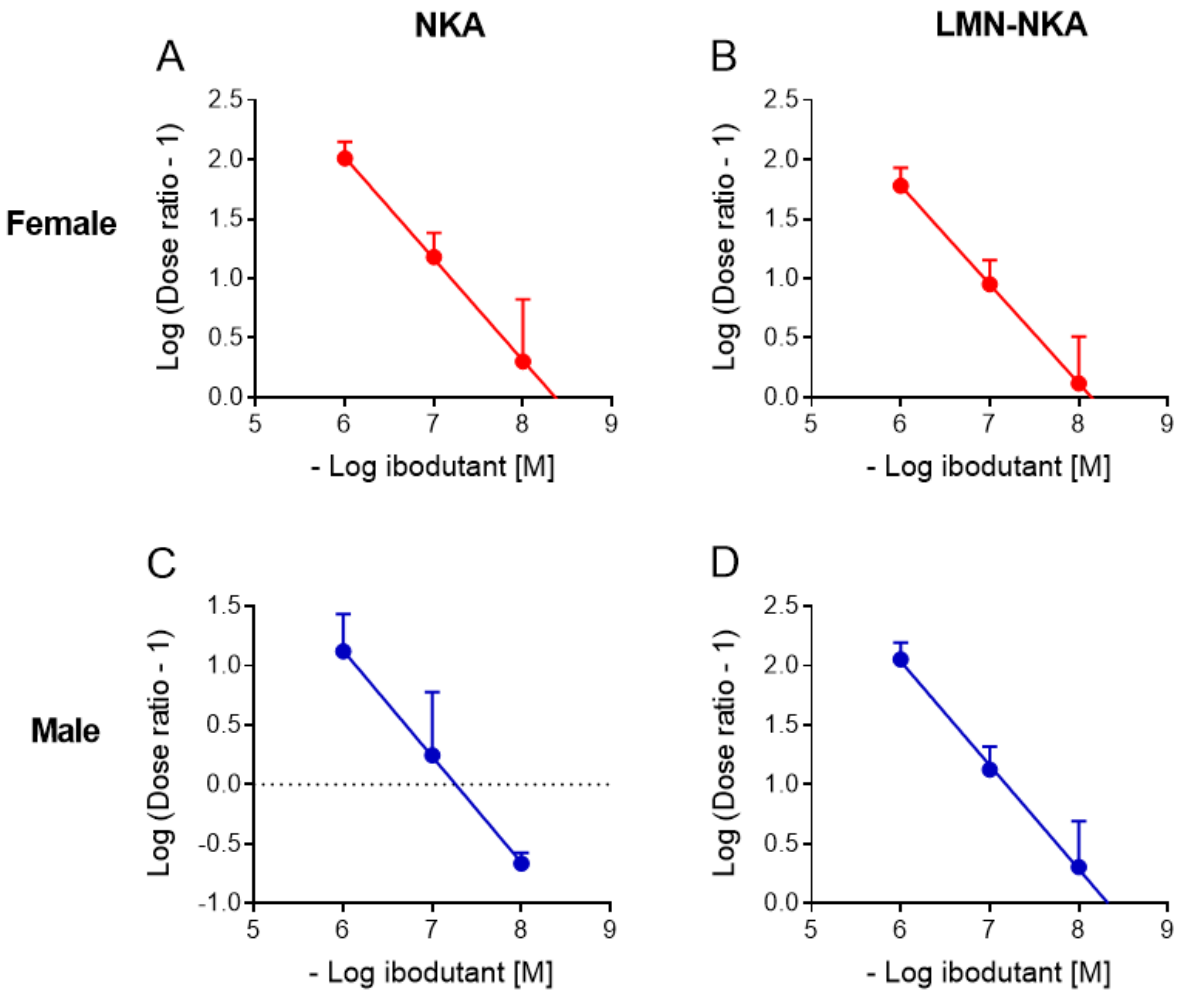


Figure 3

JPET Fast Forward. Published on August 6, 2020 as DOI: 10.1124/jpet.120.265967
This article has not been copyedited and formatted. The final version may differ from this version.

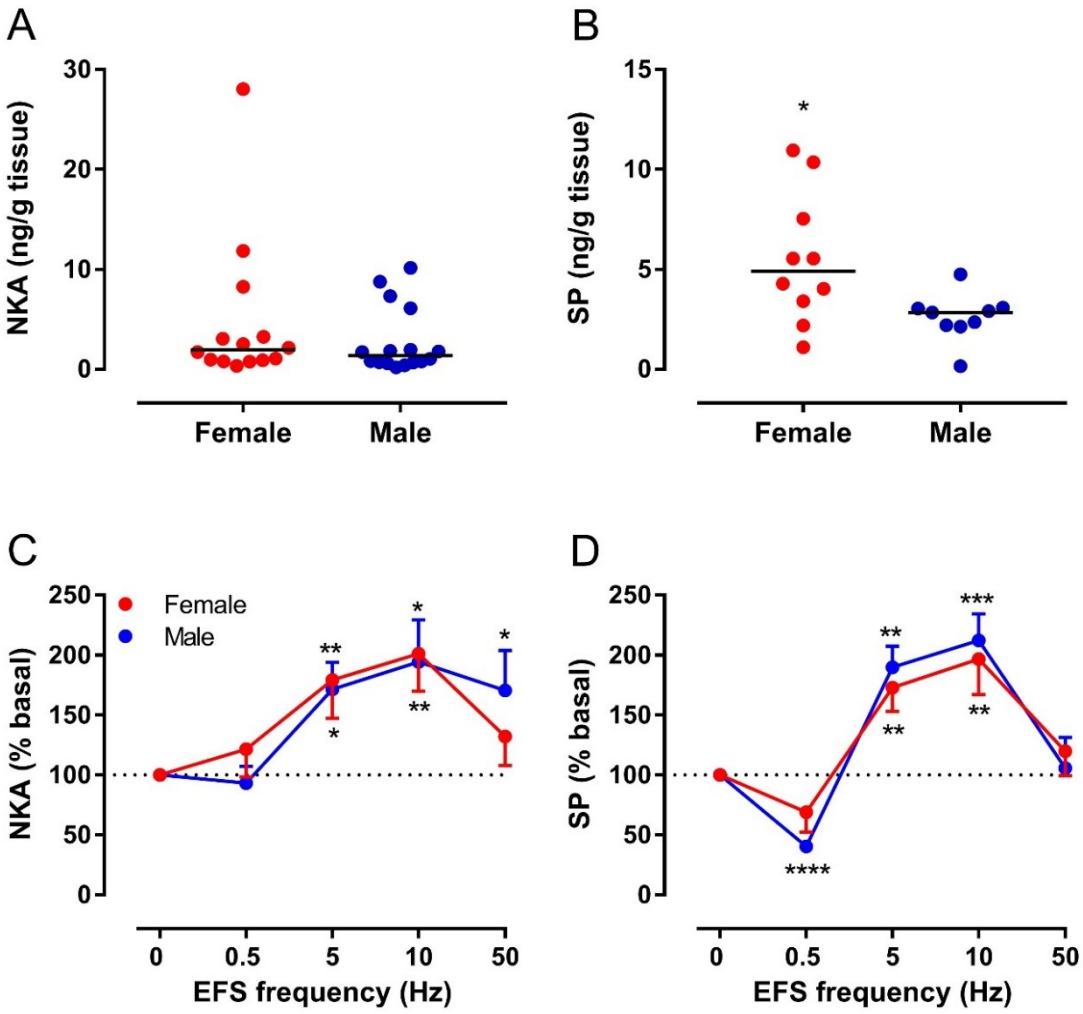


Figure 4

.

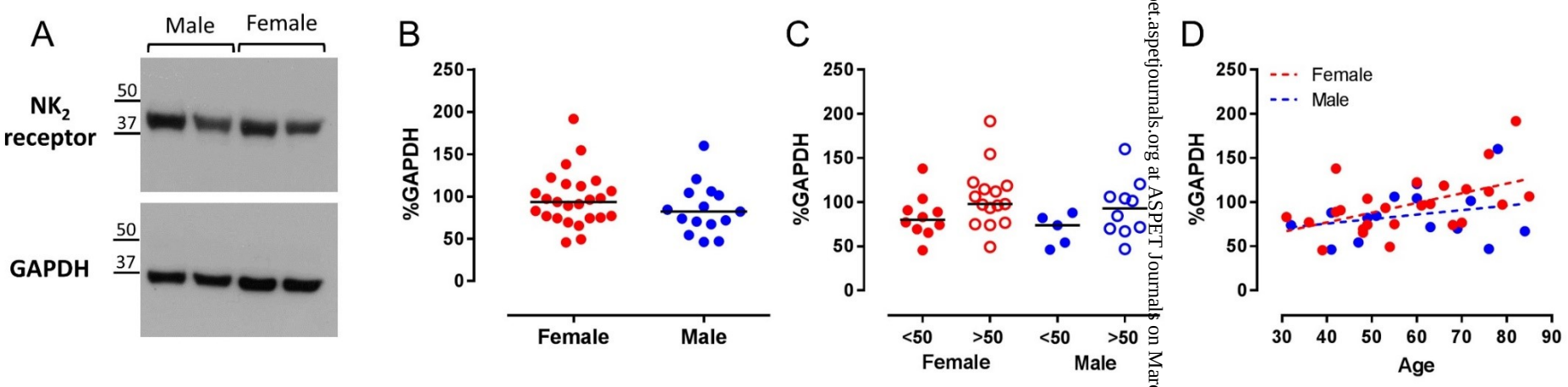


Figure 5

JPET Fast Forward. Published on August 6, 2020 as DOI: 10.1124/jpet.120.265967
This article has not been copyedited and formatted. The final version may differ from this version.

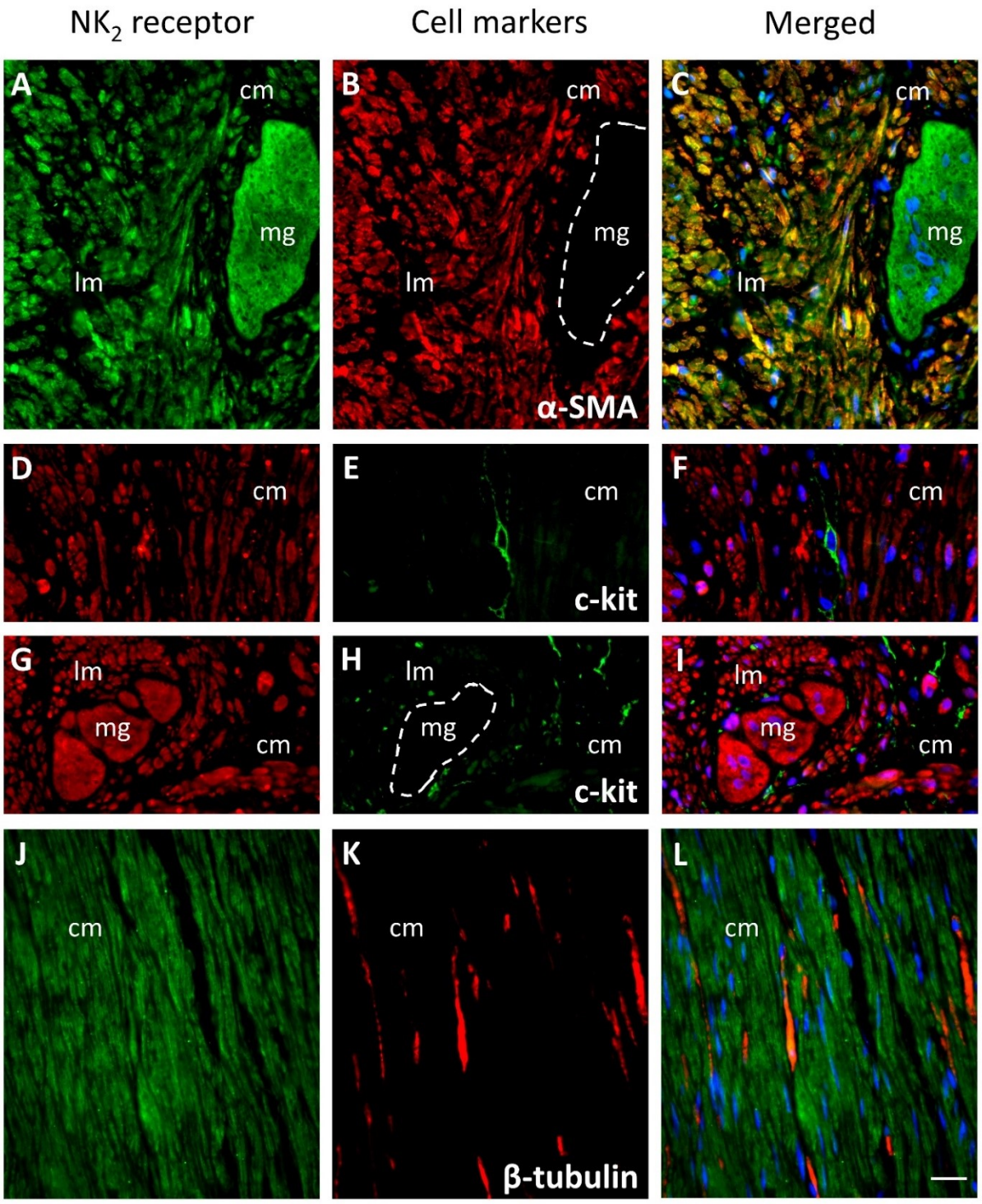


Figure 6

JPET Fast Forward. Published on August 6, 2020 as DOI: 10.1124/jpet.120.265967
This article has not been copyedited and formatted. The final version may differ from this version.

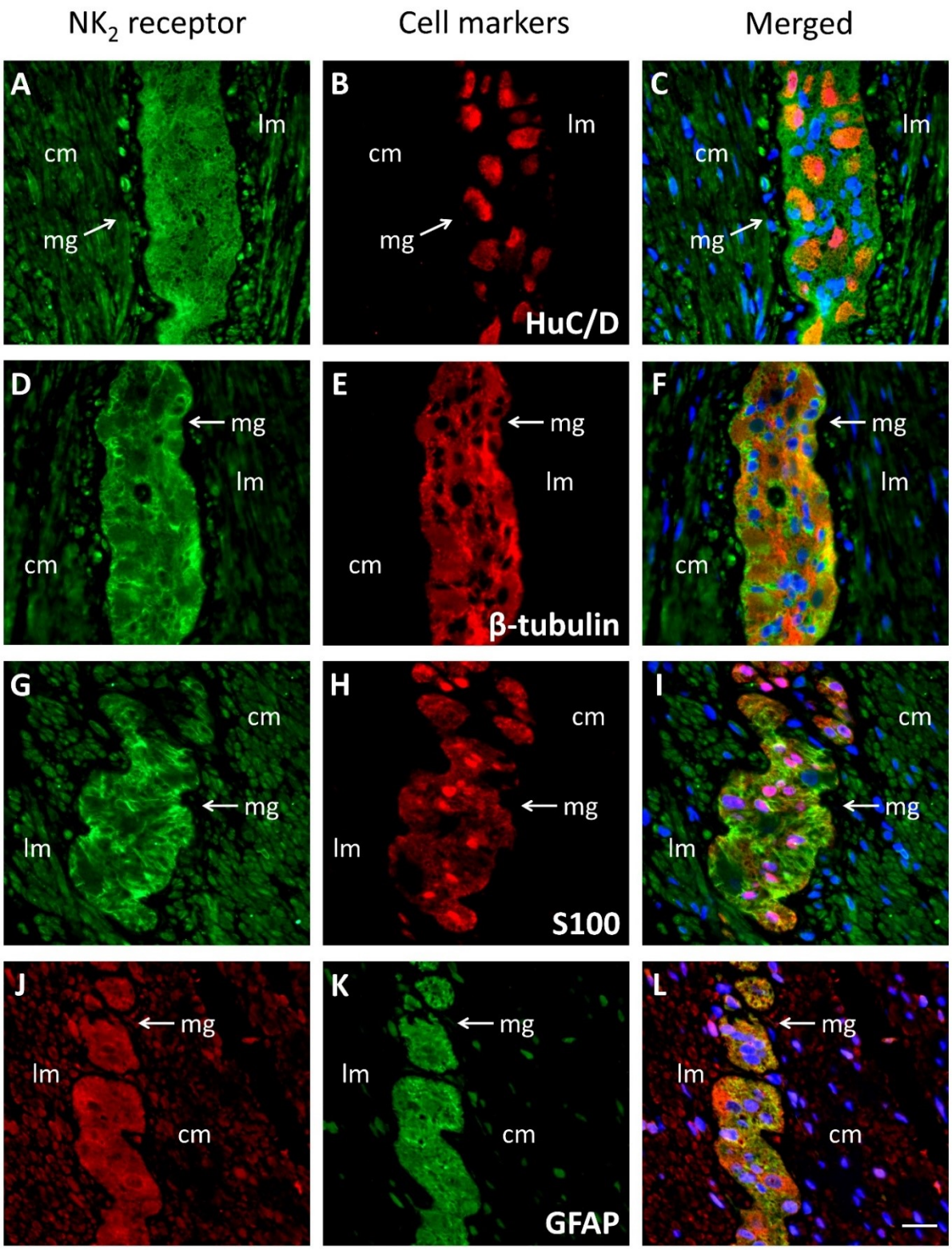


Figure 7

JPET Fast Forward. Published on August 6, 2020 as DOI: 10.1124/jpet.120.265967
This article has not been copyedited and formatted. The final version may differ from this version.

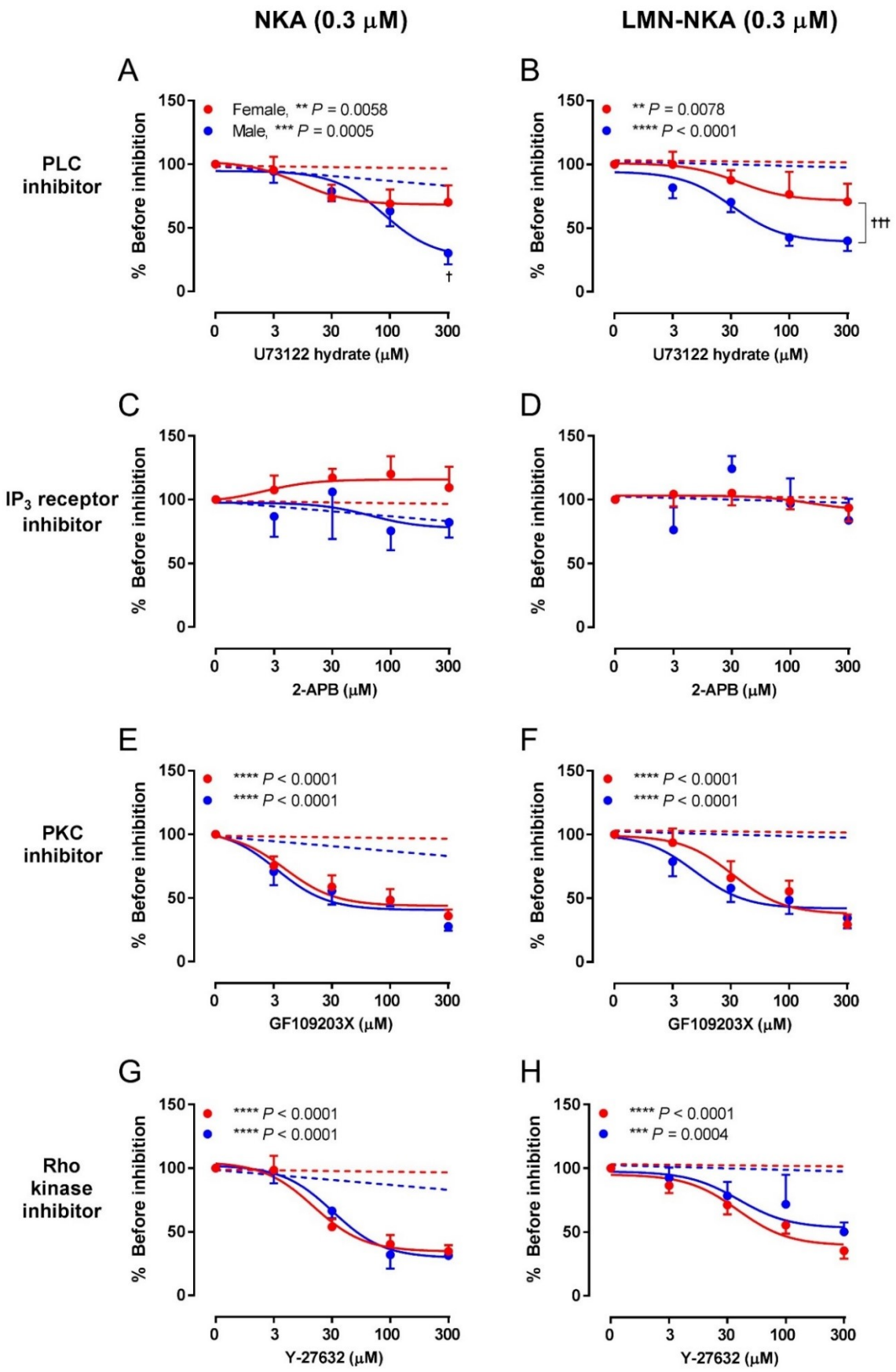


Figure 8

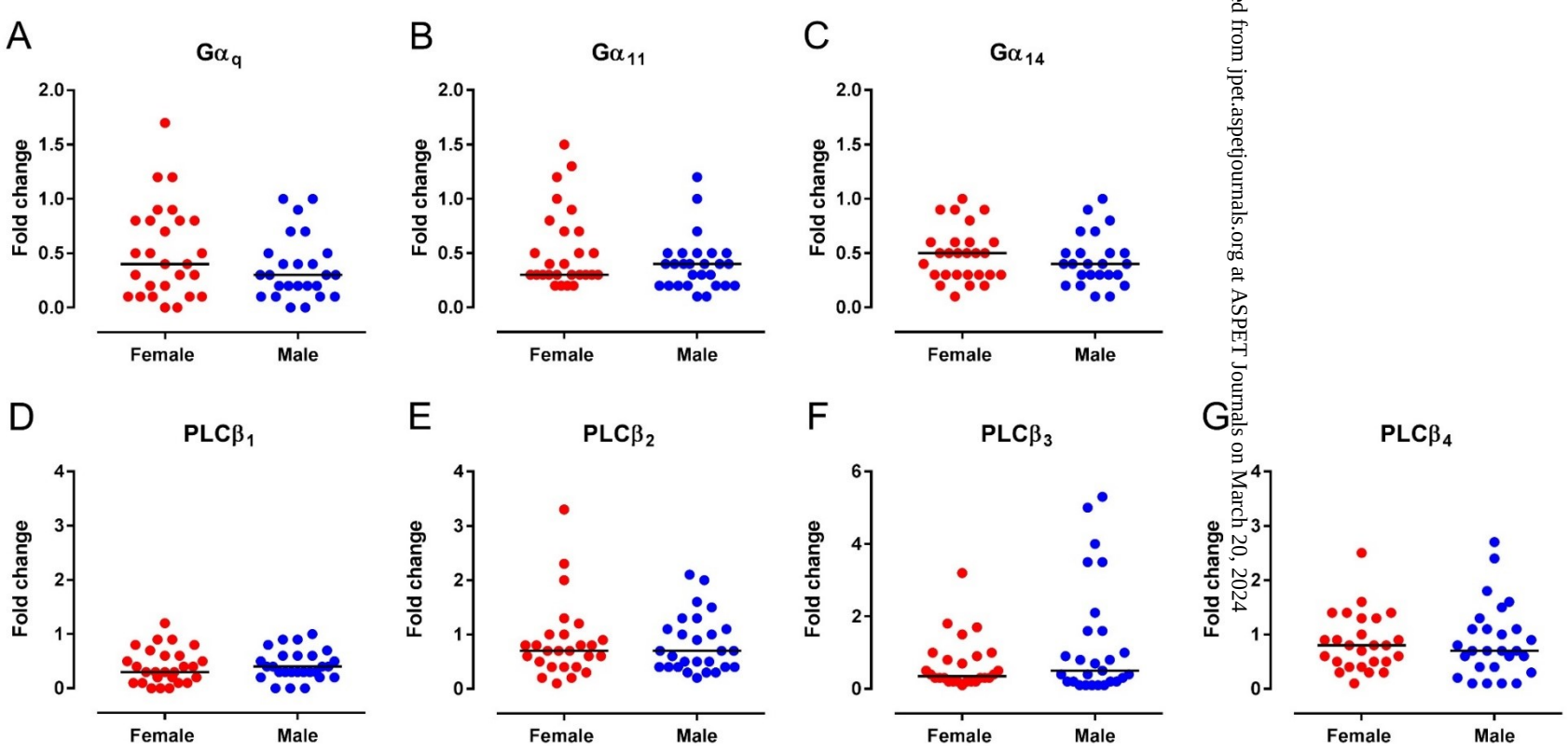


Figure 9

Supplemental Data JPET # 265967

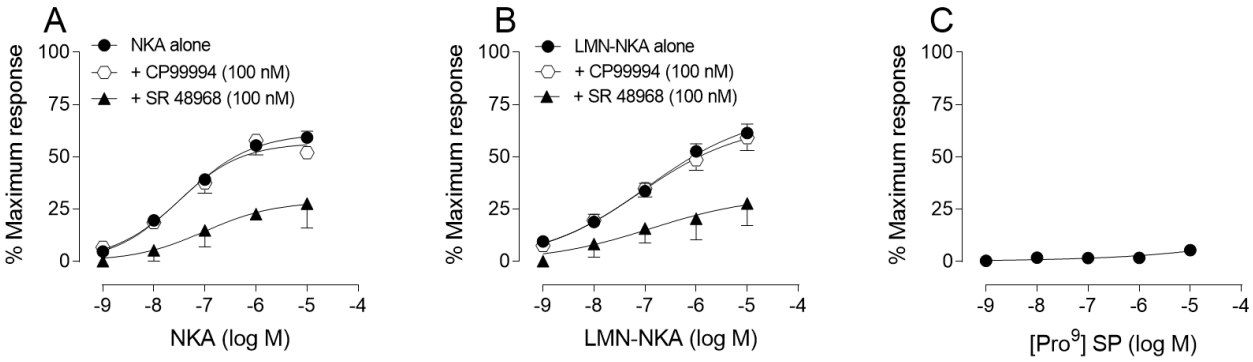
Gender-related differences of tachykinin NK_2 receptor expression and activity in human colonic smooth muscle

Stelina Drimousis, Irit Markus, Tim V Murphy, D Shevy Perera, Kim-Chi Phan-Thien, Li Zhang, Lu Liu

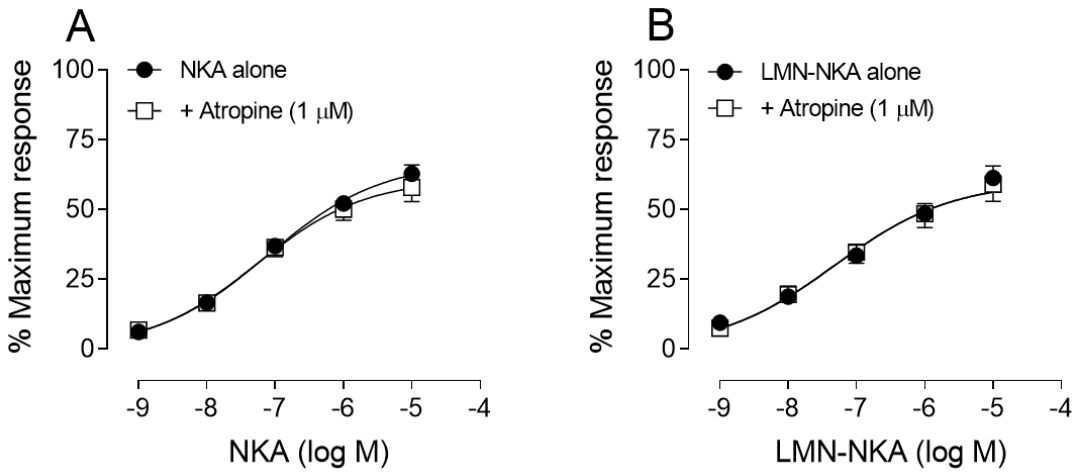
The Journal of Pharmacology and Experimental Therapeutics

Supplemental Table 1. Forward (FP) and reverse (RP) primers of human target and housekeeping genes used in real-time PCR studies. Primer melting temperatures and amplification efficiency scores are shown. Size of products are indicated as base pairs (bp).

Gene	NCBI No.	Primer	Sequence (5'-3')	Melting temp. (ºC)	Efficiency (%)	Size (bp)
Phospholipase C, β1	NM_182734	hPLC β1 FP	CCT CGT GAA CAT CTC CCA TT	81.3	89	178
		hPLC β1 RP	AAT ACG CCC TTC TGG AGT GA	81.3		
Phospholipase C, β2	NM_001284297	hPLC β2 FP	GTC TCT GCT CAA CCC TGT CC	82.7	110	213
		hPLC β2 RP	AAC TTC CCA AAG CGA GTA TCC	82.3		
Phospholipase C, β3	NM_000932	hPLC β3 FP	GGA GGA GGT AGG GCT TGA GA	84.2	124	152
		hPLC β3 RP	GCT TTT TGG GGT CTG TCT GT	84.0		
Phospholipase C, β4	NM_001172646	hPLC β4 FP	CTG GAA GGG CGG ATA GTT TG	80.1	84	156
		hPLC β4 RP	CAT TGG ACT GAC GTT GTT GG	80.2		
Guanine nucleotide-binding protein G(q) subunit α	NM_002072	hGNAQ FP	GGA CAG GAG AGA GTG GCA AG	80.8	85	195
		hGNAQ RP	GTG CAT GAG CCT TAT TGT GC	81.0		
Guanine nucleotide-binding protein G subunit α 11	NM_002067	hGNA11 FP	CCA CTG CTT TGA GAA CGT GA	83.2	90	185
		hGNA11 RP	GCA GGT CCT TCT TGT TGA GG	83.0		
Guanine nucleotide-binding protein G subunit α 14	NM_004297	hGNA14 FP	ATT CGT GCC TAC CCA ACA AG	82.0	85	220
		hGNA14 RP	GTT GTG ACA CTC AGC CAG GA	81.7		
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_001289745	hGAPDH FP	GCC AAA AGG GTC ATC ATC TC	82.4	83	176
		hGAPDH RP	AGT CCT TCC ACG ATA CCA AAG T	82.6		



Supplemental Fig. 1. Contractile responses of human colonic circular muscle to A) the endogenous NK_2 receptor ligand neurokinin A (NKA), B) the selective NK_2 receptor agonist [Lys^5,$MeLeu^9$, Nle^{10}]NKA(4-10) (LMN-NKA) and C) the selective NK_1 receptor agonist [Pro^9] substance P ([Pro^9] SP). Data are expressed as % ACh maximum response (n = 5 - 7). The responses to NKA and LMN-NKA were also determined in the presence of the selective NK_1 antagonist CP99994 and NK_2 antagonist SR 48968 (data from 4 males and 3 females with no observation of gender differences for both CP99994 and SR 48968).



Supplemental Fig. 2. The effect of atropine on the contractile responses of human colonic circular muscle to A) the endogenous NK_2 receptor ligand neurokinin A (NKA) and B) the selective NK_2 receptor agonist [Lys^5,$MeLeu^9$, Nle^{10}]NKA(4-10) (LMN-NKA). Data are expressed as % ACh maximum response (n = 5 - 7).