Received: 12 October 2016 | Accepted: 23 February 2017

DOI: 10.1111/nmo.13070

ORIGINAL ARTICLE

WILEY Neurogastroenterology & Motility NGM

Tryptase potentiates enteric nerve activation by histamine and serotonin: Relevance for the effects of mucosal biopsy supernatants from irritable bowel syndrome patients

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Funding Information
Funded by DFG Graduate School RTG-1373/2 and DFG Sche 267/7-2.

Abstract

Background: We previously showed that mucosal biopsy supernatants from irritable bowel syndrome patients activated neurons despite low concentrations of tryptase, histamine, and serotonin which individually would not cause spike discharge. We studied the potentiating responses between these mediators on excitability of enteric neurons.

Methods: Calcium-imaging was performed using the calcium-sensitive dye Fluo-4 AM in human submucous plexus preparations from 45 individuals. Histamine, serotonin, and tryptase were applied alone and in combinations to evaluate nerve activation which was assessed by analyzing increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$), the proportion of responding neurons and the product of both defined as Ca-neuroindex (NI). Protease activated receptor (PAR) 2 activating peptide, PAR2 antagonist and the serine protease-inhibitor FUT-175 were used to particularly investigate the role of proteases.

Key Results: Histamine or serotonin (1 μmol/L each) evoked only few small responses (median NI [25%/75%]: 0 [0/148]; 85 [0/705] respectively). Their combined application evoked statistically similar responses (216 [21/651]). Addition of the PAR2 activator tryptase induced a significantly higher Ca-NI (1401 [867/4075]) compared to individual application of tryptase or to coapplied histamine and serotonin. This synergistic potentiation was neither mimicked by PAR2 activating peptide nor reversed by the PAR2 antagonist GB83, but abolished by FUT-175.

Conclusions & Inferences: We observed synergistic potentiation between histamine, serotonin, and tryptase in enteric neurons, which is mediated by proteolytic activity rather than PAR2 activation. This explained neuronal activation by a cocktail of these mediators despite their low concentrations and despite a relatively small PAR2-mediated response in human submucous neurons.

KEYWORDS
enteric neurons, histamine, irritable bowel syndrome, neuronal excitability, serotonin, tryptase

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1 | INTRODUCTION

The enteric nervous system (ENS) consists of two ganglionated plexuses: the myenteric and submucous plexus.[1] These neuronal networks can regulate gut function independently from the central nervous system. There is, however, some modulation of intestinal functions by central input[2], and mucosal and immune cells[3]. Abberant signaling between these elements may play a role in gastrointestinal disorders. In particular, immune activation causing enhanced signaling between immune cells and neurons has been suggested to play a role in irritable bowel syndrome (IBS).[4] Not only mediators released by immune cells, but also the release of serotonin by enterochromaffin cells may play a role in symptom generation.[5,6] Moreover, serine proteases produced either by the pancreas or cells in the gut wall possibly contribute, as proteases generate hypersensitivity symptoms.[7]

In IBS-D patients the proteolytic activity was dramatically enhanced in fecal samples in comparison to healthy controls, and this was mainly of host and probably of pancreatic origin.[8–10] Also trypsin and tryptase mRNA expressions were significantly increased in biopsy samples from IBS patients.[7] Furthermore, elevated concentrations of soluble mediators like histamine and proteases have been measured in mucosal biopsy supernatants of IBS patients.[6] All mentioned mediators might influence gut functions and contribute to symptom generation since they have excitatory effects on neurons. Proteases can activate enteric neurons via three protease activated receptors (PARs). Mainly PAR1 and to a much lesser degree PAR2 and PAR4 have been shown to activate human submucous or myenteric neurons.[11,12] This is in contrast with findings in guinea pig and mouse where PAR2 activation caused strong activation of enteric neurons.[7,11] The action of histamine is mediated via four receptor subtypes[13] while serotonin mainly activates human enteric neurons via the 5-HT_3 receptor.[14] Proteases, histamine, and serotonin are among the most prominent nerve activating mediators contained in mucosal biopsy supernatants of IBS patients.[15] A combination of these substances plus TNF-α, the level of which is systemically enhanced in IBS, was used to mimic the action of mucosal biopsy supernatants and activate human submucous neurons.[16] The concentration of each substance in human samples, however, was about 10- to 1000-fold lower compared to the concentrations that are necessary to evoke spike discharge in enteric neurons (compare Refs. 11,13,14). This raises the question, if cross-potentiation might explain the nerve activating properties of IBS supernatants. We therefore investigated the so far unknown synergistic effects between histamine, serotonin, and proteases on enteric neurons.

Key points

- Biopsy supernatants of irritable bowel syndrome patients activate enteric neurons despite the low concentrations of tryptase, histamine, and serotonin which individually would not cause spike discharge.
- We found that tryptase synergistically potentiated the response to individual and combined application of histamine and serotonin. This potentiation was mediated by proteolytic activity of tryptase rather than protease activated receptor 2 activation.
- Our findings identified synergism between neuroactive substances as a plausible explanation for their pronounced effects as a cocktail.

2 | MATERIAL AND METHODS

2.1 | Human tissue samples

For the neuroimaging experiments human tissue samples of 45 individuals (62±12 years; 19 female) from small (20) and large (25) intestine were obtained from clinics in Munich and Freising. The patients had to undergo surgery for various reasons, mostly cancer (31) or diverticulosis/diverticulitis (7). Other indications were tubulovillous adenoma (1), stenosis (1), chronic fistula (1), perforation of the small intestine (1), and ileostomy reversal (3). For the Ussing chamber experiments tissue samples of five different individuals (62±19 years; 3 female) from small (4) and large intestine (1) were used, who underwent surgery due to cancer (4) or diverticulitis (1). Immediately after removal of the tissue a macroscopically unaffected area was transferred to a cooled sterile bottle filled with Hepes/Krebs solution (composition in mmol/L: 135 NaCl, 3 HEPES, 12.2 Glucose, 5.4 KCl, 1 $MgCl_2 \cdot 6H_2O$, 1.25 $CaCl_2 \cdot 2H_2O$, 1.2 NaH_2PO_4; pH=7.4; 293 mosmol/L) and further on continuously cooled at 4°C during transportation. The time from removal of the tissue until arrival at the laboratory was about 1-2 hours.

All procedures were approved by the ethical committee of the Technical University of Munich (1746/07) and written informed patient's consent was obtained from every participant. All procedures were in accordance with the declaration of Helsinki.

2.2 | Tissue preparation

Immediately after arrival, the tissue was removed from the transportation bottle and washed three times in fresh, ice-cold Krebs solution for preparation (composition in mmol/L: 117 NaCl, 25 $NaHCO_3$, 11 Glucose, 4.7 KCl, 1.2 $MgCl_2 \cdot 6H_2O$, 2.5 $CaCl_2 \cdot 2H_2O$, 1.2 NaH_2PO_4; pH=7.4, 293 mosmol/L). The tissue was dissected by removing both the muscle and the mucosa and continuously perfused with ice-cold Krebs solution. Small pieces of submucous tissue were stretched and pinned flat on platelets made of Sylgard®184 (Dow Corning, Wiesbaden, Germany).

2.3 | Imaging technique

The imaging technique has been described previously.[16] Briefly, calcium imaging was performed to measure intracellular calcium increase ([Ca2+]i) as a reflection of neuronal activation. The calcium sensitive dye Fluo-4 AM (10 μmol/L) was used. Preparations were incubated for 45 minutes and subsequently washed for 20 minutes.

The sylgard-platelet carrying the submucous plexus preparation was transferred to a recording chamber suitable for an upright microscope and perfused with Krebs solution for experiments (mmol/L: 117 NaCl, 20 $NaHCO_3$, 11 Glucose, 4.7 KCl, 1.2 $MgCl_2 \cdot 6H_2O$, 2.5 $CaCl_2 \cdot 2H_2O$, 1.2 NaH_2PO_4; pH=7.4; 293 mosmol/L; 37°C). All solutions from incubation until perfusion during the experiment contained 500 μmol/L Probenecid to prevent the dye from leaking out of the cell through unspecific anion transporters. Calcium signals were recorded with a CCD camera and a sampling frequency of 2 Hz (time-lapse-recording). All recordings were performed as single trial recordings. Data from camera pixels belonging to individual neurons (cells responding to nicotine, see below) was averaged and displayed as percentage change of resting fluorescence (ΔF/F). For [Ca2+]i measurements we identified the maximal value during the measurement period. It was always possible to determine a peak [Ca2+]i response as the transients started to return to baseline level during the recording period. To avoid bleaching of the dye we limited the recording periods to 30-60 seconds. Therefore, in many experiments we were not able to determine the duration of the responses directly because the signals did not return to baseline during the recording period. We used curve fitting to determine the duration of the response (see Results). Signals were only included if their amplitude was at least twice the background noise signal.

2.4 | Drug administration

If not stated otherwise, all substances have been purchased from Sigma-Aldrich (Steinheim, Germany). The following substances have been used as single applications or in combinations: histamine (1 μmol/L); serotonin (1 μmol/L); tryptase (10 nmol/L; Merck KGaA, Darmstadt, Germany); human specific PAR2-activating peptide (AP) (SLIGKV- NH_2; 10 μmol/L; Peptide Synthesis Core Facility, University of Calgary, Calgary, Alberta, Canada).

Each substance was tested individually and in combination with other substances. The protocol was the same for all substances. On every ganglion only one substance was tested to avoid cross-sensitization or desensitization effects. The first application was always 100 μmol/L nicotine to test the viability and to identify neurons. Afterward either a single substance or the mixtures were locally administered for 600 milliseconds with 0.7 bar pressure ejection onto single ganglia. According to our previous measurements, substances applied with pressure ejection for 600 milliseconds are diluted to approximately 1:10 once it reaches the ganglion.[13]

Experiments were performed to block tryptase activity with PAR2 antagonist GB83[17] (Axon Medchem, Groningen, The Netherlands; 2 μmol/L), or with the serine-protease inhibitor FUT-175 (50 μg/mL; Calbiochem, Darmstadt, Germany). Experiments with blockers were not performed in a paired fashion on the same ganglion to avoid desensitizing effects. Per tissue, 2-3 ganglia were exposed with the substance before perfusion of the PAR blockers and 2-3 different ganglia were exposed after 20 minutes perfusion of the antagonist. In case of FUT-175, the inhibitor was added to the mixture of histamine, serotonin, and tryptase, 10 minutes before pressure ejection on the neurons.

2.5 | Ussing chamber

GB83 has been shown to specifically inhibit PAR2 mediated relaxations in the guinea pig internal anal sphincter,[18] but it was previously not tested in human intestinal tissue. Therefore, we have tested the potency of GB83 to block PAR2 activation in Ussing chamber experiments with human intestinal tissue and the human specific PAR2-AP, SLIGKV-NH_2. We tested the specificity of GB83 on secretory activity rather than enteric neuron excitability for the following reason. We previously showed in human intestinal preparations that PAR2 activation reliably evoked a secretory response but the neural response was rather marginal.[11,12] The Ussing chamber voltage clamp technique was used to measure the secretory activity of the mucosa/submucosa preparations (Easy Mount chambers; Physiologic Instruments, San Diego, CA, USA), as previously described in detail.[19] Mucosal and serosal sides were bathed in 5 mL Krebs solution, maintained at 37°C and continuously bubbled with carbogen (5% CO_2, 95% O_2). The setup allowed measurements of electrogenic transport (short circuit current) in μA/cm^2 (I_{SC}); positive I_{SC} indicated a net anion current from basolateral side to the lumen. Sliders fixing the tissue between chambers were equipped with platinum electrodes, connected to a constant voltage stimulator (Grass SD-9; Astro-Med Inc., West Warwick, RI, USA), allowing electrical field stimulation (EFS) of nerves. The tissues were allowed to equilibrate for at least 45 minutes before measuring resistance. The effect of serosal PAR2-AP (10 μM) application on I_{SC} was measured in one preparation while another preparation from the same patient was first preincubated with 2 μmol/L GB83. The effect of GB83 on EFS was also tested in three human tissue samples, by performing EFS 20 minutes before and 20 minutes after the application of GB83.

2.6 | Data analysis

The application of nicotine was used to identify and distinguish neurons from glia cells.[11,16] The nicotine responsive cells matched PGP 9.5 immunoreactive neurons.[11,16] Based on staining with an antibody recognizing α1, α3, and α5 subunits 98% of human submucous neurons express nicotinic receptors (own unpublished results). Nevertheless, we cannot claim that the nicotine responsive neurons represent all neurons in a given ganglion. In our hands, nicotine is the most reliable tool to selectively activate enteric neurons. The number of nicotine responsive cells was taken as 100% for the calculation of the percentage of neurons responding to individual substances or a mixture of substances. The parameter to determine neuronal activation was the peak $[Ca^{2+}]_i$ response for each neuron expressed as ΔF/F. To determine a value for the overall activity in each ganglion, we multiplied the mean peak $[Ca^{2+}]_i$ response with the percentage of responding neurons per ganglion to obtain a Ca-neuroindex (NI).[20] The unit of the Ca-NI is % of maximal ΔF/F×% responding neurons/ganglion, but for simplicity it will be further used in the text as an absolute value without unit.

Analysis of the neuroimaging data was performed with Neuroplex 10.1.2. (RedShirtImaging, Decatur, GA, USA) and Igor Pro 7 (Wavemetrics, Inc., Lake Oswego, OR, USA).

2.7 | Statistical analysis

Since the majority of data were not normally distributed statistical analysis was performed with non-parametric tests (Mann–Whitney Rank-Sum test and ANOVA on ranks). $P<.05$ was considered significant. For multiple comparisons Bonferroni corrections were used to adjust α. To test whether an increased activation induced by a mixture was greater than the addition of the effects of the single substances, an expected median was calculated by summing up the medians evoked by the single components. This expected median was tested with a One Sample Signed Rank Test against the measured values for this combination of substances.

Potentiation was defined as follows: the mixture of mediators induces a significantly higher neuronal activation compared to the single applications for at least one of the three analyzed parameters (NI, peak $[Ca^{2+}]_i$ response or percentage responding neurons per ganglion). Since we were mostly interested in potentiating effects of proteases, we also compared the effects of combined application of histamine and serotonin with that after addition of proteases.

Statistical analysis was performed with Sigma Plot 12.5 (Systat Software Inc., Erkrath, Germany). Values in the text will be given as median with 25% and 75% quartiles in brackets. For graphs dot plots together with the median are used.

3 | RESULTS

In preliminary experiments, the concentrations for histamine and serotonin to evoke a detectable increase in $[Ca^{2+}]_i$ when applied in combination was 1 μmol/L each. This value is 1/100 or 1/1000 of what had been used in our previous experiments to evoke action potential discharge in human submucous plexus to histamine[13] or serotonin[14] respectively. For tryptase or PAR2-AP 1/10 or 1/100 of the previously used concentrations to evoke spike discharge[11] were applied respectively. In these studies, we never observed inhibition of neuronal activity; if effective the mediators always increased spike discharge without inhibiting ongoing "spontaneous" spike discharge. We therefore interpret increase in $[Ca^{2+}]_i$ transients as neuronal activation. We acknowledge the remote possibility that the observed Ca^{2+} transients may be associated with slow after spike hyperpolarization due to activation of Ca^{2+} activated K^+ currents as shown in guinea-pig myenteric neurons,[21] although we have not recorded slow after spike hyperpolarizations or spikes with a Ca^{++} hump in human submucous plexus.[22]

According to our previously published study the median concentrations in the IBS mucosal biopsy supernatants were 48 nmol/L (range: 0-123 nmol/L) for serotonin, 98 nmol/L (range: 18-858 nmol/L) for histamine and 0.23 nmol/L (range: 0.03-4 nmol/L) for tryptase.[15] Taking into account the 1:10 dilution the concentrations of serotonin, histamine and tryptase at the ganglion level were around 100 nmol/L, 100 nmol/L, and 1 nmol/L respectively. Thus, the concentrations are all within the range measured in IBS biopsy supernatants.

3.1 | Histamine and serotonin do not potentiate each other

Application of histamine or serotonin evoked only marginal neuronal activation, which was not different from baseline $[Ca^{2+}]_i$ transients (Figure 1A,B). The combination of both substances induced a slightly stronger activation, which was not different from the effect of histamine or serotonin alone (Figure 1B), but significantly larger than baseline $[Ca^{2+}]_i$ transients ($P=.004$, Figure 2B). For further comparisons, effects of the combined application of histamine and serotonin were tested against the effects after adding tryptase or PAR2-AP. For comparison, nicotine evoked an average peak Ca^{2+} response of 32.7%±14.6% ΔF/F.

3.2 | GB83 blocks the pro-secretory effect of PAR2-AP in Ussing chamber

The PAR2-AP induced an increase in I_{SC}, which was completely abolished by the PAR2 antagonist GB83 ($P=.016$; 4.86±1.85 μA/cm^2 vs −0.91±0.93 μA/cm^2). GB83 had no effect on the response to EFS ($P=.762$). These findings confirmed the PAR2 antagonistic effect of GB83 in human intestine.

3.3 | Tryptase potentiates the effect of histamine and serotonin via a protease-dependent, but PAR2 independent pathway

Tryptase activates neurons via PAR2. These receptors do not play a functionally important role in protease evoked neuronal activation in human submucous ganglia.[11] Therefore, we observed a rather small $[Ca^{2+}]_i$ response of submucous neurons after tryptase application (Figure 2A,C). The duration of the response was determined by fitting an exponential function in Igor Pro 7.0 to the falling phase of the $[Ca^{2+}]_i$ transient (Figure 2A). A value of ΔF/F=0.5% marked the end of the response and the fitted function determined the duration. The combination of histamine, serotonin, and tryptase induced a significantly larger $[Ca^{2+}]_i$ transient with a longer duration compared to both the application of tryptase or the combination of histamine and serotonin (Figure 2). The Ca-NI evoked by combined application of histamine, serotonin, and tryptase (1401 [867/4075]) was significantly higher compared to histamine plus serotonin (216 [21/651]) and also compared to application of tryptase alone (450 [74/783]). Therefore, the sum of the neuroindeces for histamine plus serotonin and for tryptase was 666 and hence, significantly smaller than the actually measured Ca-NI of the combined application of histamine, serotonin, and tryptase ($P=.012$ one sample signed rank test). The PAR2 antagonist GB83 had no significant effect on the neuronal activation after histamine, serotonin, and tryptase and hence did not prevent the potentiation by tryptase (Figure 3A).

We investigated whether the potentiation could be mimicked by the PAR2-AP SLIGKV-NH_2. PAR2-AP evoked only a very small response. Strikingly, addition of PAR2-AP to histamine and serotonin did not cause enhanced responses (Figure 3B).

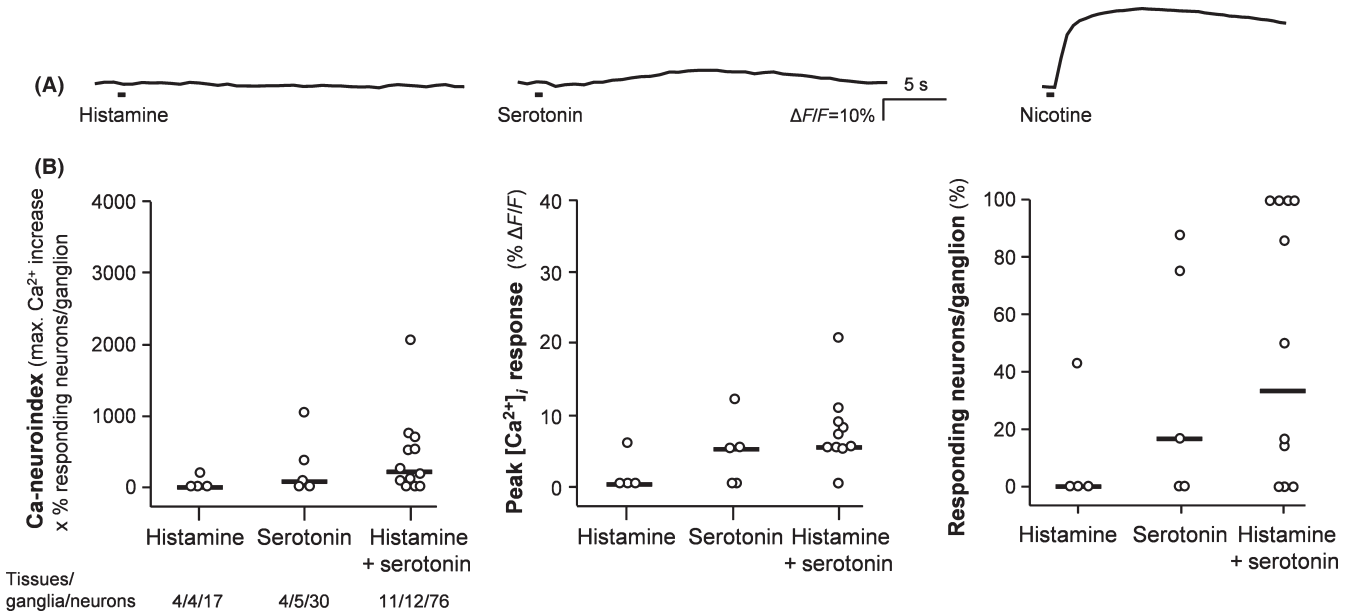

FIGURE 1 Effects of histamine and serotonin as single application and in combination on human submucous neurons. (A) It shows two representative traces. The bars below the curves indicate the beginning and end of application. The third trace shows an example of the response of another neuron to nicotine. (B) It is a graphical representation of the results. The effects of serotonin and histamine were not significantly different from baseline $[Ca^{2+}]_i$ transients (P=1, P=.25 respectively). There is no difference in peak $[Ca^{2+}]_i$ responses (P=.253), percentage of responding neurons per ganglion (P=.208) or the Ca-neuroindex (P=.235) between co-application of histamine and serotonin and their individual application

So far the results suggested that PAR2 may not be involved in the potentiating effect of tryptase. However, the proteolytic activity must be important because pre-incubation of the mixture of histamine, serotonin, and tryptase with the serine protease inhibitor FUT-175 completely abolished the potentiation (Figure 3C). The remaining neuronal activation was not different from that of the combination of histamine and serotonin in the same tissues (NI: 289 [58/481], P>.05).

4 | DISCUSSION

To the best of our knowledge, this is the first description of a potentiation of neuronal activity in response to histamine and serotonin by a serine protease in the human submucous plexus. The findings demonstrate the potency of tryptase to enhance and even synergistically potentiate the effects of other neuromodulators, in our study specifically serotonin and histamine. Histamine and serotonin did not potentiate the effect of each other. The potentiation by tryptase was not mediated via the classical activation of PAR2 receptors, as it could not be reproduced by using the human specific PAR2-AP (SLIGKV-NH_2) and not be reversed by a PAR2 antagonist. However, the response to the mixture of histamine, serotonin, and tryptase was dramatically suppressed by the serine-protease inhibitor FUT-175.

In this study, we used lower concentrations of serotonin, histamine, and PAR activators than in previous experiments. It is important to realize that the reason why we have seen effects at low concentrations is due to the use of Ca^{2+} measurement which is more sensitive than voltage sensitive dye recordings which require that the membrane depolarization is strong enough to reach threshold for spike discharge.[23] Because of the higher signal to noise ratios, Ca^{2+} imaging is capable of detecting more subtle neuronal changes even in the absence of action potential firing.

Tryptase has been shown to potentiate histamine and serotonin induced contractions in dog airway smooth muscle cells via direct or indirect effects on Ca^{2+} channels.[24] In human sensitized bronchi, tryptase also potentiated the contractile response to histamine via a calcium-related mechanism.[25] The receptor of tryptase mediating this potentiating effect is not known.

As suggested by the present study, the synergistic potentiation of serotonin and histamine by tryptase was not blocked by the PAR2 antagonist. We nevertheless cannot fully exclude a PAR2 involvement because PAR2 antagonists may selectively target intracellular pathways. Thus, it has been demonstrated in human cell models that the PAR2 antagonist GB88 selectively inhibits PAR2/Gq/11/Ca^{2+}/PKC (protein kinase C) but not cyclic adenosine monophosphate (cAMP), extracellular signal-regulated kinases (ERK) or Rho, which were rather activated by GB88.[26]

Despite the above reservation, the finding that the PAR2-AP did not reproduce the potentiation caused by tryptase strongly suggests a proteolytic, but PAR2 independent mechanism. In the rat colonic myocyte model, the effects of tryptase could be blocked by a selective tryptase inhibitor or a serine protease inhibitor, suggesting that it is linked to its enzymatic activity.[27] In our experiments, the synergistic effect of tryptase, histamine and serotonin was blocked by FUT-175, a serine protease inhibitor, which clearly indicates that it is the enzymatic property of tryptase which is responsible for the potentiation.

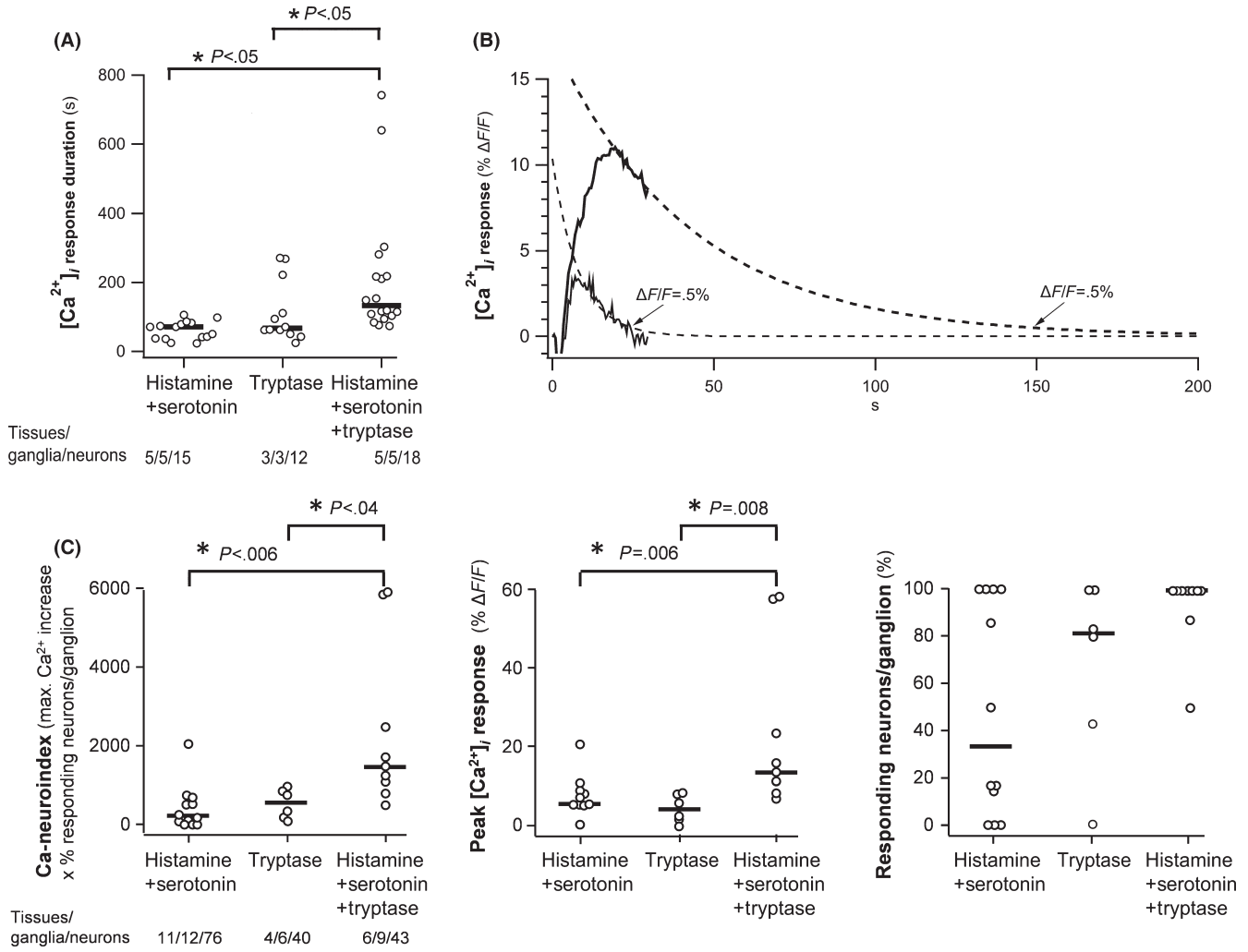

FIGURE 2 Application of tryptase as single application and in combination with 1 μmol/L histamine and 1 μmol/L serotonin on human submucous neurons. (A) The response to histamine, serotonin, and tryptase has a significantly longer duration than the response to tryptase alone or the combination of histamine and serotonin. (B) It shows two traces illustrating the difference between co-applied histamine and serotonin with and without tryptase. The bars below the curves indicate the beginning and end of application. In addition, shown are the exponential functions that are fit to the falling phase of the responses to determine the duration $[Ca^{2+}]_i$ transients. The end of the responses is defined when ΔF/F reached again 0.5%. (C) There is a significant difference between the neuronal activity reflected by the peak $[Ca^{2+}]_i$ response and the Ca-neuroindex after histamine, serotonin, and tryptase in comparison to the single application of tryptase and combined application of histamine plus serotonin

Similar to our observations, the tryptase-induced histamine release from human lung cells was blocked by a tryptase inhibitor, but was not mimicked by the PAR2-AP.[28] Furthermore, in airway smooth muscle cells, tryptase stimulated IL-8 production, which was blocked by protease inhibitors including FUT-175, but could not be reproduced by the PAR2-AP.[29] In these studies the authors concluded that the effect of tryptase is PAR2-independent. Tryptase can cleave a variety of other substrates, but none of these other substrates has receptor activating properties.[30] In dog airway smooth muscle cells, tryptase induced Akt phosphorylation and presumably PI 3 kinase stimulation by a proteolytic action independent of PAR2.[31] However, the responsible mechanisms are unknown.

We did not observe desensitization to the response of IBS biopsy supernatants[6] despite the strong desensitization to PAR activators.[11] This may be related to the lower concentrations of PAR activators in biopsy supernatants. Although we used concentrations of proteases in the range measured in IBS biopsy supernatants, we decided to not test different combinations of substances on the same ganglion to avoid the smallest risk of desensitization in particular to PAR activators,[11,32] and yet to a lesser extent also to serotonin[14] and histamine.[13] While desensitization can be excluded by an unpaired experimental protocol, it theoretically may increase the variability of responses between ganglia in one tissue and between tissues from different patients. The nerve activation induced by mediators applied alone or in combination were performed on different ganglia in the same tissue. The experiments where we tested combined applications before and after perfusion of PAR2 antagonist confirmed the findings as the effects of the mediators were comparable. This suggested that

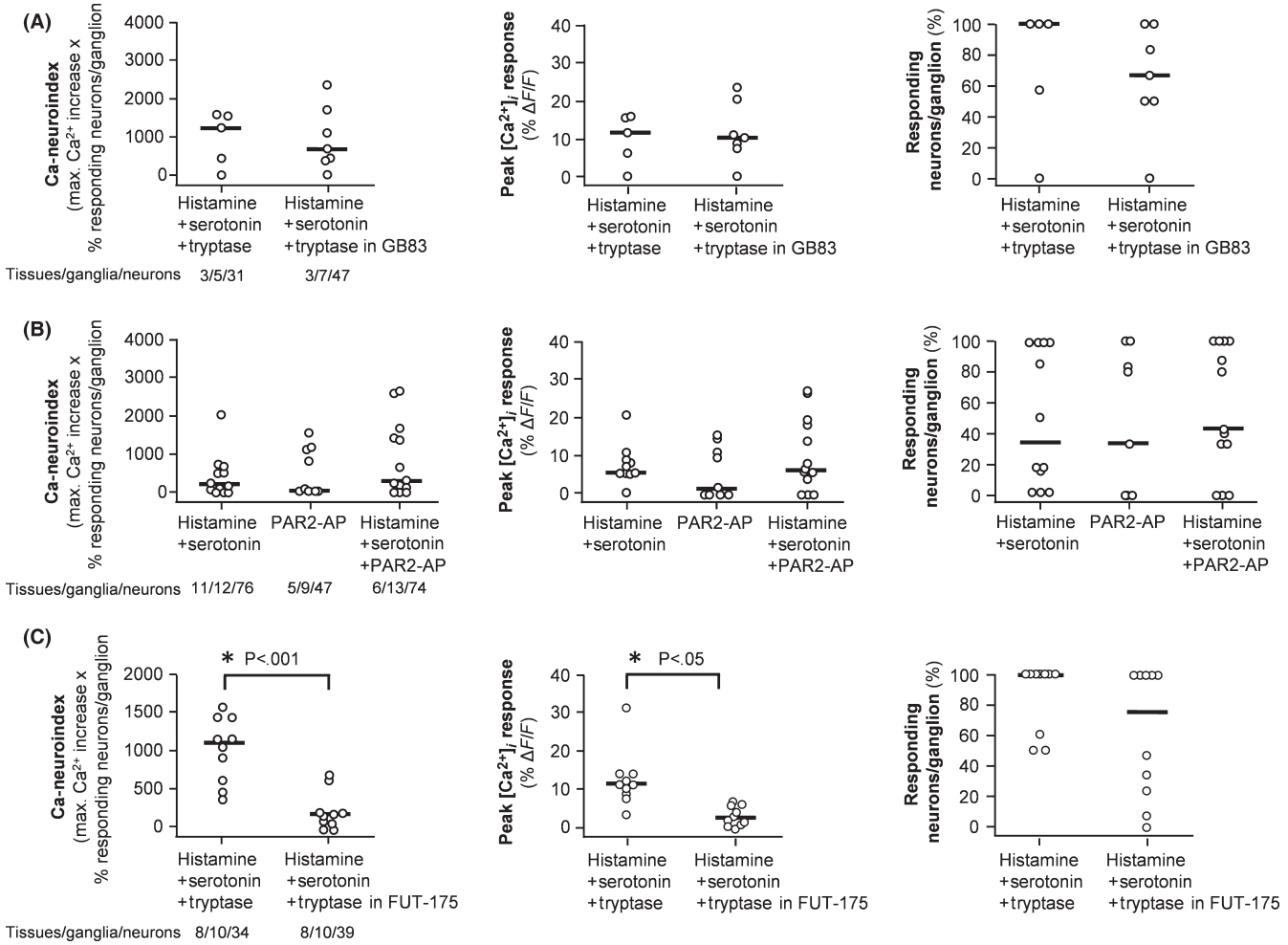

FIGURE 3 The potentiation of the serotonin and histamine response by tryptase is mediated by its protease activity rather than by PAR2 mechanisms. (A) The nerve response to the mixture of histamine, serotonin, and tryptase on human submucous ganglia is not reduced by the PAR2 antagonist GB83 (NI: P=.876; peak $[Ca^{2+}]_i$ response: P=.876; percentage of responding neurons: P=.53). (B) The potentiating effect of tryptase (see Figure 2) could not be mimicked with the PAR2-AP. (C) In contrast with GB83, the serine protease inhibitor FUT-175 prevented the tryptase mediated potentiation of co-applied histamine and serotonin. Both the Ca-neuroindex and the peak $[Ca^{2+}]_i$ response were significantly reduced

the effects of mediators were even comparable between tissues from different patients.

Our data demonstrated that a cocktail containing mediators at concentrations which individually did not cause spike discharge in human submucous neurons[11,13,14] evoked nerve activation by synergistic actions. This finding is particularly relevant for mucosal biopsy supernatants of IBS patients. In the present study we focused on those mediators that we previously identified in these supernatants as most relevant for activating enteric nerves.[6,15] It seems plausible to suggest a similar potentiation of other neurotransmitters or modulators by proteases although this requires confirmation. We acknowledge that the supernatants contain an even richer variety of nerve activating substances. Our findings show that already the combination of three different substances at low concentrations can lead to robust activation of human submucous neurons through synergistic potentiation. This further demonstrates the impact of small changes in the immediate milieu of enteric ganglia, for example as a result of immune or enteroendocrine cell activation. Sustained low grade inflammation has often been associated with IBS and an increased number of mast cells and enhanced protease levels have been demonstrated.[7,33] Protease inhibition has been shown to abolish the nerve activation of IBS mucosal biopsy supernatants.[6,15] Results of the current study explain the potency of tryptase and probably other serine proteases as they are required for the other mediators to evoke meaningful response, at least when applied at concentration present in the supernatants. Our findings further stress the importance of serine proteases and their potential to even synergistically increase the activity of other substances. As the proteolytic activity in IBS patients was found to be increased compared to controls,[10] this synergistic effect should be even more pronounced in IBS patients than in controls. The link between potentiation of nerve activation by proteases and IBS symptoms remains to be studied. The degree of nerve activation is certainly not related to stool irregularities as it is comparable between biopsy supernatants from IBS patients with diarrhea or constipation.[6,33] Furthermore, enteric neurons

in biopsies from IBS patients with diarrhea or constipation show similar responses to nicotine, synaptic activation, or a cocktail of inflammatory mediators.[16] However, the degree of nerve activation correlated with the pain threshold assessed by rectal barostat distension.[15] It is therefore conceivable that the potentiation of intracellular calcium increase varies with visceral pain perception. We previously reported that PAR2 is functionally rather irrelevant in human ENS because tryptase or PAR2 activating peptides evoked marginal spike discharge in a small population of neurons.[11,12] Findings of the present study revealed that tryptase potentiates nerve excitation by other mediators; however, this effect seemed independent of classical PAR2 activation.

ACKNOWLEDGMENTS

This work was supported by DFG Graduate School RTG-1373/2 and DFG Sche 267/7-2.

DISCLOSURE

The authors have nothing to disclose.

AUTHOR CONTRIBUTION

DO study design, performed imaging experiments, data analysis, and interpretation; manuscript draft, approved the final version and is accountable for all aspects of the work; AA performed imaging experiments, data analysis and interpretation; manuscript draft, approved the final version and is accountable for all aspects of the work; DK performed Ussing chamber experiments, data analysis and interpretation. She drafted the manuscript, approved the final version and is accountable for all aspects of the work; KM data analysis and statistics; ID provided human samples and medical council; critically revised and approved manuscript. He is accountable for correct patient characterization and material handling; GC provided human samples and medical council; critically revised and approved manuscript. He is accountable for correct patient characterization and material handling; FZ provided human samples and medical council; critically revised and approved manuscript. He is accountable for correct patient characterization and material handling; MS formed the concept and study design, obtained funding, reviewed the manuscript critically for important intellectual content, gave final approval for publication and is accountable for all aspects of the work.

REFERENCES

1. Furness JB, Costa M. Types of nerves in the enteric nervous system. *Neuroscience*. 1980;5:1-20.
2. Blackshaw LA, Brookes SJH, Grundy D, Schemann M. Sensory transmission in the gastrointestinal tract. *Neurogastroenterol Motil*. 2007;19(1 Suppl):1-19. doi:10.1111/j.1365-2982.2006.00871.x.
3. Buhner S, Schemann M. Mast cell-nerve axis with a focus on the human gut. *Biochim Biophys Acta*. 2012;1822:85-92. doi:10.1016/j.bbadis.2011.06.004.
4. Barbara G, Stanghellini V, de Giorgio R, et al. Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. *Gastroenterology*. 2004;126:693-702. doi:10.1053/j.gastro.2003.11.055.
5. Gershon MD. Nerves, reflexes, and the enteric nervous system: pathogenesis of the irritable bowel syndrome. *J Clin Gastroenterol*. 2005;39:S184-S193.
6. Buhner S, Li Q, Vignali S, et al. Activation of human enteric neurons by supernatants of colonic biopsy specimens from patients with irritable bowel syndrome. *Gastroenterology*. 2009;137:1425-1434. doi:10.1053/j.gastro.2009.07.005.
7. Cenac N, Andrews CN, Holzhausen M, et al. Role for protease activity in visceral pain in irritable bowel syndrome. *J Clin Invest*. 2007;117:636-647. doi:10.1172/JCI29255.
8. Roka R, Rosztoczy A, Leveque M, et al. A pilot study of fecal serine-protease activity: a pathophysiologic factor in diarrhea-predominant irritable bowel syndrome. *Clin Gastroenterol Hepatol*. 2007;5:550-555. doi:10.1016/j.cgh.2006.12.004.
9. Gecse K, Roka R, Ferrier L, et al. Increased faecal serine protease activity in diarrhoeic IBS patients: a colonic lumenal factor impairing colonic permeability and sensitivity. *Gut*. 2008;57:591-599. doi:10.1136/gut.2007.140210.
10. Tooth D, Garsed K, Singh G, et al. Characterisation of faecal protease activity in irritable bowel syndrome with diarrhoea: origin and effect of gut transit. *Gut*. 2014;63:753-760. doi:10.1136/gutjnl-2012-304042.
11. Mueller K, Michel K, Krueger D, et al. Activity of protease-activated receptors in the human submucous plexus. *Gastroenterology*. 2011;141:2088-2097.e1. doi:10.1053/j.gastro.2011.08.034.
12. Kugler EM, Mazzuoli G, Demir IE, Ceyhan GO, Zeller F, Schemann M. Activity of protease-activated receptors in primary cultured human myenteric neurons. *Front Neurosci*. 2012;6:133. doi:10.3389/fnins.2012.00133.
13. Breunig E, Michel K, Zeller F, Seidl S, Weyhern C, Schemann M. Histamine excites neurones in the human submucous plexus through activation of H 1, H 2, H 3 and H 4 receptors. *J Physiol*. 2007;583:731-742. doi:10.1113/jphysiol.2007.139352.
14. Michel K, Zeller F, Langer R, et al. Serotonin excites neurons in the human submucous plexus via 5-HT3 receptors. *Gastroenterology*. 2005;128:1317-1326. doi:10.1053/j.gastro.2005.02.005.
15. Buhner S, Li Q, Berger T, et al. Submucous rather than myenteric neurons are activated by mucosal biopsy supernatants from irritable bowel syndrome patients. *Neurogastroenterol Motil*. 2012;24:1134-e572. doi:10.1111/nmo.12011.
16. Ostertag D, Buhner S, Michel K, et al. Reduced responses of submucous neurons from irritable bowel syndrome patients to a cocktail containing histamine, serotonin, TNFα, and tryptase (IBS-Cocktail). *Front Neurosci*. 2015;9:465. doi:10.3389/fnins.2015.00465.
17. Barry GD, Suen JY, Le GT, Cotterell A, Reid RC, Fairlie DP. Novel agonists and antagonists for human protease activated receptor 2. *J Med Chem*. 2010;53:7428-7440. doi:10.1021/jm100984y.
18. Huang S-C. Proteinase-activated receptor-1 (PAR1) and PAR2 mediate relaxation of guinea pig internal anal sphincter. *Regul Pept*. 2014;189:46-50. doi:10.1016/j.regpep.2014.03.001.
19. Krueger D, Michel K, Zeller F, et al. Neural influences on human intestinal epithelium in vitro. *J Physiol*. 2015;357-372. doi:10.1113/JP271493.
20. Buhner S, Braak B, Li Q, et al. Neuronal activation by mucosal biopsy supernatants from irritable bowel syndrome patients is linked to visceral sensitivity. *Exp Physiol*. 2014;99:1299-1311. doi:10.1113/expphysiol.2014.080036.
21. Hillsley K, Kenyon JL, Smith TK. Ryanodine-sensitive stores regulate the excitability of AH neurons in the myenteric plexus of guinea-pig ileum. *J Neurophysiol*. 2000;84:2777-2785.

22. Schemann M, Neunlist M. The human enteric nervous system. *Neurogastroenterol Motil*. 2004;16(Suppl 1):55-59. doi:10.1111/j.1743-3150.2004.00476.x.
23. Michel K, Michaelis M, Mazzuoli G, Mueller K, Vanden Berghe P, Schemann M. Fast calcium and voltage-sensitive dye imaging in enteric neurones reveal calcium peaks associated with single action potential discharge. *J Physiol*. 2011;589(Pt 24):5941-5947. doi:10.1113/jphysiol.2011.219550.
24. Sekizawa K, Caughey GH, Lazarus SC, Gold WM, Nadel JA. Mast cell tryptase causes airway smooth muscle hyperresponsiveness in dogs. *J Clin Invest*. 1989;83:175-179. doi:10.1172/JCI113855.
25. Johnson PR, Ammit AJ, Carlin SM, Armour CL, Caughey GH, Black JL. Mast cell tryptase potentiates histamine-induced contraction in human sensitized bronchus. *Eur Respir J*. 1997;10:38-43.
26. Suen JY, Cotterell A, Lohman RJ, et al. Pathway-selective antagonism of proteinase activated receptor 2. *Br J Pharmacol*. 2014;171:4112-4124. doi:10.1111/bph.12757.
27. Corvera CU, Dery O, McConalogue K, et al. Mast cell tryptase regulates rat colonic myocytes through proteinase-activated receptor 2. *J Clin Invest*. 1997;100:1383-1393. doi:10.1172/JCI119658.
28. He S, Aslam A, Gaca MD, et al. Inhibitors of tryptase as mast cell-stabilizing agents in the human airways: effects of tryptase and other agonists of proteinase-activated receptor 2 on histamine release. *J Pharmacol Exp Ther*. 2004;309:119-126. doi:10.1124/jpet.103.061291.
29. Mullan CS, Riley M, Clarke D, et al. Beta-tryptase regulates IL-8 expression in airway smooth muscle cells by a PAR-2-independent mechanism. *Am J Respir Cell Mol Biol*. 2008;38:600-608. doi:10.1165/rcmb.2007-0082OC.
30. Hallgren J, Pejler G. Biology of mast cell tryptase. An inflammatory mediator. *FEBS J*. 2006;273:1871-1895. doi:10.1111/j.1742-4658.2006.05211.x.
31. Brown JK, Hollenberg MD, Jones CA. Tryptase activates phosphatidylinositol 3-kinases proteolytically independently from proteinase-activated receptor-2 in cultured dog airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol*. 2006;290:L259-L269. doi:10.1152/ajplung.00215.2005.
32. Bohm SK, Khitin LM, Grady EF, Aponte G, Payan DG, Bunnett NW. Mechanisms of desensitization and resensitization of proteinase-activated receptor-2. *J Biol Chem*. 1996;271:22003-22016.
33. Barbara G, de Giorgio R, Stanghellini V, Cremon C, Salvioli B, Corinaldesi R. New pathophysiological mechanisms in irritable bowel syndrome. *Aliment Pharmacol Ther*. 2004;20(Suppl 2):1-9. doi:10.1111/j.1365-2036.2004.02036.x.

How to cite this article: Ostertag D, Annahazi A, Krueger D, et al. Tryptase potentiates enteric nerve activation by histamine and serotonin: Relevance for the effects of mucosal biopsy supernatants from irritable bowel syndrome patients. *Neurogastroenterol Motil*. 2017;29:e13070. https://doi.org/10.1111/nmo.13070