Extrinsic Afferent Nerve Sensitivity and Enteric Neurotransmission in Murine Jejunum in vitro


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Abstract (maximum 250 words)

Introduction Enteric and extrinsic sensory neurons respond to similar stimuli. Thus, they may be activated in series or in parallel. As signal transmission via synapses or mediator release would depend on calcium, we investigated its role for extrinsic afferent sensitivity to chemical and mechanical stimulation.

Methods Extracellular multiunit afferent recordings were made in vitro from paravascular nerve bundles supplying the mouse jejunum. Intraluminal pressure and afferent nerve responses were recorded under control conditions and under 4 conditions designed to interfere with enteric neurotransmission.

Results Phasic intestinal contractions ceased after switching perfusion to Ca\(^{2+}\)-free buffer with or without PPADS (purinergic P2-receptor antagonist) or cadmium (blocking all Ca\(^{2+}\)-channels) but not following \(\omega\)-conotoxin GVIA (N-type Ca\(^{2+}\)-channel blocker). Luminal HCl (pH 2) and 5-HT (500 µM) evoked peak firing of 17±4 imp/sec\(^{-1}\) (n=10) and 21±4 imp/sec\(^{-1}\) (n=13) under control conditions. These responses were reduced to 4±2 imp/sec\(^{-1}\) and 5±2 imp/sec\(^{-1}\) by cadmium (n=7, p<0.05), to 7±2 imp/sec\(^{-1}\) and 6±1 imp/sec\(^{-1}\) by Ca\(^{2+}\)-free perfusion (n=6; p<0.05) and to 3±1 imp/sec\(^{-1}\) and 4±1 imp/sec\(^{-1}\) by Ca\(^{2+}\)-free perfusion with PPADS (n=6; p<0.05). Responses were unchanged by \(\omega\)-conotoxin GVIA. Mechanical ramp distension of the intestinal segment to 60 cmH\(_2\)O was not altered by any of the experimental conditions.

Conclusions HCl and 5-HT activate extrinsic afferents via a calcium dependent mechanism which is unlikely to involve enteric neurons carrying N-type calcium channels. Extrinsic mechanosensitivity is independent of enteric neurotransmission. It appears that cross-talk from the enteric to the extrinsic nervous system does not mediate extrinsic afferent sensitivity.

Keywords HCl, intestinal neurophysiology, 5-HT, mouse, visceral sensitivity
Introduction

The gastrointestinal tract is endowed with an elaborate network of surveillance systems that comprise both intrinsic and extrinsic sensory neurons as well as an array of immune cells and specialized epithelial cells (9). Sensory neurons can be classified according to the location of their cell bodies and the pattern or projection of their axons. Intrinsic primary afferent neurons (IPANS) are estimated to make up about 30% of the enteric nervous system (11). These have cell bodies either in the submucosal or the myenteric plexus and afferent axons that innervate both the mucosa and the muscle layers of the gut wall. IPANS synapse with each other and in this way form a self-reinforcing network, providing sensory input from the mucosa and muscle that in turn modulates the enteric reflex circuitry that controls gastro-intestinal function. The sensory nerve endings have a number of transduction channels that transform stimulus energy into a coded sequence of action potentials that are then transmitted synaptically through the enteric network (3, 5, 37).

However, in addition, these sensory endings have ligand gated receptors and channels that transmit sensory information from other structures within the gut wall. One important source of such input arises from the proximity of sensory terminals in the lamina propria to enterochromaffin cells (EC cells) in the mucosal epithelium. 5-HT released from these cells has been shown to contribute to the mechanical and chemical sensitivity of IPANS supplying the intestinal mucosal (2). In addition to these intrinsic sensory neurons there are 2 groups of extrinsic sensory neurons that project information out of the gut wall to the central nervous system. Vagal afferents have their cell bodies in the jugular and nodose ganglia, while spinal afferents have their cell bodies in the dorsal root ganglia and project
into the spinal cord. The visceral endings of the vagal and spinal afferents are located in different sites within the gut wall; mucosa, sub-mucosa, muscle, myenteric plexus, serosal and mesenteric connections (1, 21). These terminals are positioned to respond to changes in the mechanical and chemical environment both within the lumen and within the gut wall and its mesenteric connections (49). Intrinsic and extrinsic afferents share a number of characteristics i.e. both groups have similar innervation territories and are responsive to similar modalities of mechanical and chemical stimulation (11, 14, 21).

Both spinal and vagal afferents can be seen coursing through the enteric nervous system, where they are potentially exposed to chemical mediators released during enteric reflex activation (5, 14, 15). Vagal afferents in particular form elaborate structures around enteric ganglia. These so-called intraganglionic lamina endings form basket-like structures around ganglia and have been shown by Brookes and colleagues to be the site of mechanosensitive hotspot (49). This proximity between enteric and extrinsic sensory neurons raises the intriguing possibility that they may share a common mechanism of activation with enteric neural activity being transmitted to the terminals of extrinsic afferents following release of chemical mediators into the enteric ganglionic neuropil. This would give rise to “cross talk” between the enteric and extrinsic nervous system and as such sensory input to enteric reflex circuits would also be available to the central nervous system. Such cross talk would likely depend upon calcium dependent mechanisms responsible for vesicular release of mediators into the synaptic neuropil. Calcium entry through mainly N-type calcium channels is required for transmitter release (29) and, therefore, removal of extra cellular calcium or calcium channel blockers should attenuate this process. However, extra-cellular calcium
can interact with some ion channels to increase membrane conductances (20). In contrast, increases in intra-cellular calcium may lead to the modulation of various ion channels including calcium-gated potassium channels that decrease the excitability of neurons (8). Therefore, calcium can have both positive and negative effects on sensory neuron excitability and the relative contribution of these counteractive influences can only be determined empirically.

In the present study we aimed to manipulate calcium channels by different techniques to examine the effect on GI afferent sensitivity. By recording from mesenteric paravascular bundles in-vitro, we simultaneously sampled the activity of both vagal and spinal afferents supplying the jejunum in order to determine the sensitivity in both mechanical and chemical stimulation. We hypothesized that mesenteric afferent sensitivity depends on functional calcium channels in the intestinal wall, i.e. on intact synaptic transmission in the enteric nervous system.
Methods

Tissue Preparation

All experiments were performed with female mice (C57BL6, 20-30 g), which were fed a standard laboratory diet. Animals were anesthetized with pentobarbitone (60 mg kg⁻¹ i.p.). Following laparotomy, a 2 cm segment of jejunum with mesenteric attachment was excised and placed into a custom-made organ bath. In one chamber of the organ bath (perfusion chamber), the jejunal segment was superfused with Krebs buffer equilibrated with 95 % O₂ and 5 % CO₂ (composition (mM) Na⁺ 143.5, K⁺ 5.9, Cl⁻ 126, Ca²⁺ 2.5, Mg²⁺ 1.2, H₂PO₄ 1.2, SO₄ 1.2, HCO₃⁻ 25, glucose 10 and sodium butyrate 1, pH 7.4, rate 10 ml min⁻¹, temperature 32 °C, a maximum of 2 segments was used per animal). The mesenteric arcade was pulled through an aperture into a separate chamber (recording chamber). The aperture was sealed with Vaseline and the recording chamber filled with colourless heavy liquid paraffin, pre-warmed to 32°C to insulate the recording electrodes. The gut lumen was cannulated at both ends, and perfused with isotonic saline from the proximal end (perfusion rate 10 ml per hour), while the distal cannula remained open to the atmosphere for drainage except during mechanical ramp distension (see below). The intraluminal pressure was recorded continuously at the proximal end of the intestinal segment via a Y-piece which was connected to a pressure transducer (Ohmeda, DTX Plus Transducer, Ohmeda, NJ, USA). The pressure was typically 1-3 cmH₂O at baseline on top of which phasic contractile events could be observed. A separate segment of jejunum was used for each experiment. The committee for animal experiments at the University of Tuebingen approved of the
protocol prior to these procedures. N-numbers vary for technical reasons as it was not always possible to run the whole protocol in a single preparation.

Nerve recording
A single paravascular nerve bundle was dissected out from the mesenteric arcade and attached to one of a pair of platinum recording electrodes. A strip of connective tissue was wrapped around the other electrode to act as a reference. The electrodes were connected to a CED single channel 1902 preamplifier/filter (Cambridge Electronic Design (CED), Cambridge, UK), and the signal was differentially amplified 10000 times and filtered with a bandwidth of 100 Hz to 1 kHz. The output from the 1902 amplifier was passed into a power Micro 1401 interface system (CED), captured, and viewed online by a PC running Spike 2 software (version 4.01; CED), together with the output from the pressure transducer showing intraluminal pressure.

Protocol
HCL (pH 2 for 4 min) and 5-HT (500 µM for 2 min) were perfused through the gut lumen at a rate of 10 ml h⁻¹ separated by an interval of 10 min which served for washout with normal saline at the same perfusion rate. Subsequently, the outlet cannula was closed and the jejunal segment distended with normal saline which was infused at a rate of 1.5 ml min⁻¹ by a perfusion pump (IVAC 711, IVAC Corp., San Diego, USA) until the maximum intraluminal pressure of 60 cmH₂O was reached.
In control experiments this protocol with HCl, 5-HT, and mechanical ramp distension was run 20 min after the preparation was allowed to stabilize during Krebs buffer perfusion. In separate experimental subgroups the protocol was run 10 to 20 min after perfusion was switched to one of the following solutions:

1. Ca\(^{2+}\)-free Krebs (containing 2.5 mM Mg\(^{2+}\) + 0.1 mM EGTA, a Ca\(^{2+}\) chelator) – to inhibit synaptic transmission by removal of extracellular Ca\(^{2+}\)

2. Ca\(^{2+}\)-free Krebs (containing 2.5 mM Mg\(^{2+}\) + 0.1 mM EGTA) + Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) (PPADS) (50 μM) – since ATP is released by removal of extracellular Ca\(^{2+}\) and this may influence afferent firing indirectly PPADS was added as a purinergic P2-receptor antagonist to block any activation of afferents by ATP

3. Krebs plus ω-conotoxin GVIA (500 nM) – to block N-type Ca\(^{2+}\) channels

4. Cadmium (250 µM) in HEPES buffer (50 mM) - to block all Ca\(^{2+}\) channels unselectively

Use of HEPES buffer in the 4th experimental group was necessary to maintain cadmium in solution. In pilot experiments afferent nerve responses to HCL, 5-HT and distension were unchanged in HEPES compared to Krebs buffer. This allowed comparison of the 4 experimental subgroups with each other. Ca\(^{2+}\) free solutions (1. and 2.) were perfused for at least 20 minutes prior to subsequent stimuli (HCL, 5-HT, ramp distension) to ensure complete Ca\(^{2+}\) washout in the preparation.
Drugs

Pentobarbitone was obtained from Rhône Mérieux, Lyon, France. 5-HT, cadmium, heavy liquid paraffin, PPADS (Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) hydrate tetrasodium salt), EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid) and all buffer salts were from Sigma Chemicals (St. Louis, MO, USA). ω-conotoxin GVIA was obtained from Alomone Laboratories (Jerusalem, Israel).

Data analysis

Baseline discharge frequency (imp sec⁻¹) during perfusion with Krebs solution was calculated by averaging afferent nerve firing for a 60 second period following a 20 minute period for signal stabilisation. After perfusion was changed to a modified solution according to different experimental subgroups (see above 1.-4.), baseline discharge was again quantified in this manner.

Following HCL, 5-HT and mechanical ramp distension, peak increase in afferent nerve discharge frequency above baseline was determined. These peak responses were calculated as mean discharge per second from a 3 second bin analyzed during peak response. Intestinal motility was quantified as mean of peak amplitudes from phasic contractile events observed during 200 seconds following stabilization of afferent firing in each of the solutions in the perfusion chamber. Data are presented as mean ± SEM and compared by one way ANOVA followed by Dunnet’s corrections for multiple comparisons. P < 0.05 was considered statistically significant.


**Results**

**Intestinal motility**

Under control conditions with Krebs buffer perfusion, phasic increases in intraluminal pressure were recorded with a peak pressure of $11.2 \pm 0.9$ cmH$_2$O ($n=23$). When Ca$^{2+}$ free Krebs buffer was perfused, peak pressures were reduced to $1.2 \pm 0.5$ cmH$_2$O and to $1.9 \pm 0.3$ cmH$_2$O during perfusion with Ca$^{2+}$ free Krebs and PPADS (50 µM; $n=6$; $P<0.05$).

Contrary to Ca$^{2+}$ free conditions, contractile activity continued with peak pressures of $9.1 \pm 3.4$ cmH$_2$O when the perfusion was switched to a solution containing the N-type calcium channel antagonist ω-conotoxin GVIA (500 nM; $n=6$). A reduction of phasic pressure events to $2.4 \pm 0.2$ cmH$_2$O was also observed during perfusion with cadmium containing HEPES buffer ($n=6$; $P<0.05$) which was similar to Ca$^{2+}$ free conditions (Fig. 1A-C).

**Baseline afferent discharge**

Under control conditions with Krebs buffer perfusion, spontaneous multiunit afferent nerve discharge was observed at baseline. This afferent discharge was maintained over the time course of the experiments and consisted of spikes with different amplitudes and waveforms indicative of multi-unit recordings. Baseline afferent nerve discharge was $14 \pm 4$ imp sec$^{-1}$, when afferent discharge was evaluated during the initial control conditions with Krebs buffer perfusion from all experimental subgroups ($n=23$).
Afferent firing was increased from $14 \pm 3$ imp sec$^{-1}$ during the initial recording period under control conditions to $24 \pm 3$ imp sec$^{-1}$ when perfusion was switched to Ca$^{2+}$ free Krebs solution ($p<0.05$; $n=6$). This increase in afferent firing was from $12 \pm 2$ imp sec$^{-1}$ to $26 \pm 4$ imp sec$^{-1}$ when perfusion was switched to Ca$^{2+}$ free Krebs buffer containing PPADS (50 µM; $p<0.05$; $n=6$). Baseline afferent firing was unchanged compared to control conditions when perfusion was continued with a solution containing $\omega$-conotoxin GVIA ($14 \pm 2$ imp sec$^{-1}$; $n=6$) or cadmium ($12 \pm 3$ imp sec$^{-1}$; $n=6$; Figure 1A-C).

Afferent sensitivity to HCl, 5-HT and mechanical ramp distension

Intraluminal acid at pH 2 evoked a robust increase in afferent nerve discharge which peaked at $17 \pm 4$ imp sec$^{-1}$ above baseline ($n=10$). This response to HCl was attenuated following perfusion with Ca$^{2+}$ free buffer to $7 \pm 2$ imp sec$^{-1}$ ($n=6$; $P<0.05$), and to $3 \pm 1$ imp sec$^{-1}$ ($n=6$, $P<0.05$) following Ca$^{2+}$ free buffer containing PPADS. It was unchanged following $\omega$-conotoxin GVIA with a peak firing of $20 \pm 4$ imp sec$^{-1}$ ($n=6$), but also attenuated following cadmium ($4 \pm 2$ imp sec$^{-1}$; $n=7$, $P<0.05$, Figure 2A). Intestinal motility was unchanged during intraluminal perfusion with HCl (Figure 3).

Similar to HCl, intraluminal 5-HT (500 µM) was followed by a robust increase in afferent discharge to $21 \pm 4$ imp sec$^{-1}$ above baseline ($n=13$). The response to 5-HT was reduced to $6 \pm 1$ imp sec$^{-1}$ in Ca$^{2+}$ free conditions ($n=6$, $P<0.05$) and to $4 \pm 1$ imp sec$^{-1}$ in Ca$^{2+}$ free buffer with PPADS ($n=6$, $P<0.01$), while $\omega$-conotoxin GVIA had no effect ($27 \pm 6$ imp sec$^{-1}$; $n=6$). Afferent discharge to 5-HT was reduced to $5 \pm 2$ imp sec$^{-1}$ during perfusion
with a cadmium containing solution (n=7, p<0.05; Figure 2B). Intraluminal perfusion with 5-HT did not alter intestinal motility (Figure 3).

Mechanical ramp distension of the intestinal segment to 60 cmH₂O stimulated a pressure-dependent increase in afferent discharge (n=12). Afferent responses to distension were similar in all experimental subgroups as regards response profile and peak firing, independent of the solution perfused in the organ bath (Figure 4).
Discussion

The present study investigated the role of Ca\textsuperscript{2+} dependent mechanisms for intestinal motility and mesenteric afferent nerve discharge at baseline and following mechanical and chemical stimuli.

Phasic intestinal contractions were abolished under all Ca\textsuperscript{2+} free conditions. Calcium is needed to maintain the smooth muscles’ calcium balance and for contractile processes (36). It is therefore, not surprising that intestinal motor events were attenuated in the absence of extracellular calcium since L-type calcium channels play an important role in excitation/contraction coupling in intestinal smooth muscle (22). Similarly, cadmium which will block all voltage-gated calcium channels, including L-type channels, causes a pronounced inhibition of intestinal motility. In addition, Ca\textsuperscript{2+} dependent mechanisms are necessary for synaptic transmission which will be attenuated in the absence of extracellular calcium or in the presence of cadmium (27). Synaptic transmission relies more on N-type calcium channels and these can be selectively blocked by \textit{ω}-conotoxin GVIA. However, in contrast to Ca\textsuperscript{2+}-free and cadmium, \textit{ω}-conotoxin had no effect on intestinal motility indicating that under the current experimental conditions the motor activity was driven largely by myogenic mechanisms.

Spontaneous mesenteric afferent nerve discharge at baseline was unchanged when cadmium was added to the buffer solution despite the marked reduction in phasic intestinal motor activity. Under baseline conditions, therefore, it appears that contractile activity does
not drive afferent discharge. Similarly, blocking N-type Ca\(^{2+}\) channels with \(\omega\)-conotoxin GVIA failed to alter spontaneous afferent firing. Since both cadmium and \(\omega\)-conotoxin will attenuate synaptic transmission within the enteric nervous system (41, 44, 45), their lack of effect on baseline afferent discharge would indicate that spontaneous afferent firing does not require mediator release or synaptic transmission in the enteric nervous system and rather suggests that it is a property of the afferent ending itself. Strikingly, baseline afferent firing was increased under Ca\(^{2+}\)-free conditions at a time when intestinal motility was attenuated. The most likely explanation is that excitability of the afferent endings is increased in the absence of external Ca\(^{2+}\) potentially subsequent to disinhibition of certain ion channels in the afferent membrane. Indeed, there is considerable evidence from sensory neurons maintained in cell culture that extracellular Ca\(^{2+}\) modulates the gating properties of a number of ion channels that regulate neuronal excitability (40). Moreover, Ca\(^{2+}\) is required by ecto-nucleotidases for the breakdown of ATP to adenosine (33, 34 50, 51). In the absence of extracellular calcium there is an increase in ATP content (33) and since intestinal afferents express P2X receptors (26) there may be a secondary increase in afferent excitability via ligand-gated activation. This possibility, however, was ruled out since adding the purinergic P2-receptor antagonist PPADS to the Ca\(^{2+}\) free perfusate did not alter increased afferent discharge at baseline under Ca\(^{2+}\) free conditions.

Mechanical ramp distension of the jejunal segment used in the current study was designed to assess the contribution of low threshold and high threshold afferents travelling via vagal and spinal afferent pathways (4, 12, 31). The afferent response during ramp distension was
unchanged following the various conditions designed to manipulate calcium metabolism. This may not be surprising as regards spinal afferents since many have mechanosensitive endings and mesenteric connections at some distance from enteric ganglia (1). However, the response to distension was also unchanged at lower distending pressure that might be considered more physiological. Vagal afferents that terminate as intraganglionic laminar endings (49) are considered the site of low threshold mechanosensitivity. In this location these endings may be exposed to the chemical environment in the synaptic neuropil and therefore are a likely candidate for cross talk between extrinsic and enteric sensory endings. The absence of any attenuation of mechanosensitivity following calcium manipulation suggests that this too is independent of synaptic activity which is consistent with previous observations (48). Nevertheless, the multi-unit signal does not completely rule out the possibility that a minor subpopulation of extrinsic afferents depends on synaptic activity. A third population of afferents in the mesenteric nerve bundles arise from intestinofugal fibres that project to the prevertebral ganglia. These receive cholinergic synaptic input following mechanical stimulation (39) which would be eliminated under \( \text{Ca}^{2+} \) free conditions and during Cd superfusion. That the response to distension is independent of calcium implies that these intestinofugal fibres do not contribute greatly to the sensory signals conveyed in the mesenteric nerve bundles at the level of the mouse jejunum which is in keeping with their anatomical distribution in which is predominantly in the ileum and colon (10). In summary, mechanosensitivity in the mouse jejunum seems to be independent of calcium regardless of whether it is stimulated by a physiological or noxious trigger.
While mechanosensitivity appears to be clearly mediated by direct activation of afferent endings there may be a good rationale to suggest that chemosensitivity may be indirect. In particular the evidence that EC cells are responsible for luminal “tasting” and influence both enteric and extrinsic afferent following the release of 5-HT (2, 13). We therefore examined luminal sensitivity to acid and 5-HT in order to assess potential involvement at the level of the EC cell – sensory terminal interface - or following activation of enteric sensory neurons and transmission within enteric ganglia.

We found that the afferent nerve response to acid and 5-HT was attenuated by removal of calcium from the perfusate and by adding the non-selective calcium channel blocker cadmium, while the N-type calcium channel blocker ω-conotoxin GVIA had no effect. Thus, irrespective of changes in excitability at the level of the sensory terminal, the absence of any influence of ω−conotoxin (17) suggests that cross-talk from intrinsic primary afferent neurons to extrinsic afferents does not contribute to the serotonin sensitivity of extrinsic afferents.

But how is the attenuated response to 5-HT explained during Ca^{2+}-free conditions? 5-HT may activate enteric neurons in the myenteric plexus via 5-HT₃, 5-HT₄ and 5-HT₁P receptor (2, 36, 43). As cross-talk from the enteric nervous to the extrinsic nervous system does not seem to occur, extrinsic afferent nerve fibers were probably directly stimulated by luminal 5-HT. Indeed, it was shown previously that extrinsic afferents respond to 5-HT directly via the 5-HT₃ receptor subtype (18) which is located on vagal afferents (17). Considering that the 5-HT₃ receptor is a ligand gated ion channels (6) that - when activated by 5-HT - permits Ca^{2+} influx (16), it is likely that this activation is attenuated in the absence of
extracellular calcium. A potential secondary effect by accumulated ATP during Ca^{2+}-free conditions (33) was again ruled out by adding the purinergic P2 receptor antagonist PPADS in a different series of experiments.

This mechanism of activation of the 5-HT_{3} receptor does not explain inhibition of the 5-HT response in the presence of cadmium which blocks calcium channels unselectively. It was shown, however, that desensitisation of the 5-HT_{3} receptor is regulated by other voltage gated Ca^{2+}-channels (24). In other words, Ca^{2+}-channels in the cell wall of extrinsic afferents may have been blocked by cadmium with a subsequently enhanced desensitisation of the 5-HT_{3} receptor. Alternatively, cadmium may have bound directly to the 5-HT_{3} receptor (23) with subsequent potential inhibition or it may change overall excitability of intestinal afferents which depends on external calcium (40). This mechanism may involve TTX-R Na^{+} channels which have been described in sensory neurons (NaV1.8 and 1.9) and potentially contribute to the mechanisms that regulate sensory neuronal function, especially in the context of hypersensitivity (19). That NaV1.8 is sensitive to cadmium has also been demonstrated (28) and indeed the current that these channels mediate in isolated DRGs is more sensitive to cadmium than that mediated by Nav1.9. Therefore, cadmium at concentrations below those used in the current study markedly attenuate both NaV1.8 and NaV1.9 currents and potentially result in attenuated excitability. What is more difficult to explain with this concept is why the effects of cadmium are seen only with responses to luminal chemical stimulation, while baseline firing and mechanosensitivity were unaffected. One possibility is that the latter drives the afferents beyond the level at which
subtle changes in excitability might become manifest. However, this remains speculative pending further investigation.

Several possible explanations exist that may account for the observation that the afferent nerve response to acid was reduced during Ca\(^{2+}\)-free perfusion and in the presence of cadmium. Although minor subpopulations of afferents may behave differently, cross-talk from the enteric to the extrinsic nervous system seems again unlikely as blocking neuronal N-type calcium channels in the enteric nervous system was without effect. One possible mechanism to explain the effect observed during acid exposure is that acid has the potential to activate transient receptor potential vanilloid 1 receptors (TRPV1) or acid sensitive ion channels (ASIC) on afferent nerve terminals directly (35, 38, 42). Both mechanisms would be attenuated under Ca\(^{2+}\)-free conditions as calcium as a substrate for these ion channels would be missing (46). However, this does not explain, why the response was attenuated during Cd exposure rendering this suggested explanation unlikely. A second possible explanation is that acid triggers the release of 5-HT from enteroendocrine cells (EC-cells) which was shown in previous investigations (25, 47). The mechanism involves the transient receptor potential ankyrin 1 receptor (TRPA 1; 32) which seems to be is sensitive to alterations in pH (7). This mechanism involves calcium influx in the EC-cell (32) which explains the attenuation of the acid response during Ca-free conditions and Cd by reduced 5-HT release. 5-HT would then be subject to the mechanisms illustrated above for 5-HT sensitivity which would elegantly explain why a similar modulation of the afferent response to acid and 5-HT occurred during different types of calcium manipulation.
In conclusion, extrinsic afferent nerve discharge to acid and 5-HT is stimulated by a calcium dependent mechanism suggesting mediator release with subsequent activation of afferents. This mediator release does not involve N-type calcium channels and is, therefore, unlikely to stem from neurons in the enteric nervous system rendering EC cells as likely candidates. Extrinsic afferent nerve discharge to mechanical stimulation was independent of calcium indicating that mechanosensitive afferents were directly activated. These observations suggest that cross-talk from the enteric to the extrinsic nervous system does not occur. It is of note, however, that mechanisms of afferent activation may be different for other stimuli and that in contrast to naive animals cross-talk may occur under pathological conditions such as intestinal inflammation.
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Figure 1: Effects on baseline afferent discharge and contractility. The vertical arrows indicate the point of time at which the different solutions (A-C) were added to the preparation.

A: Ca\(^{2+}\) free Krebs’ buffer

The upper trace shows the raw nerve recording. The lower trace gives the recording of the decreasing pressure in the intestinal segment after Ca\(^{2+}\) free Krebs’ buffer perfusion. Note the increase in baseline afferent discharge although the jejunal segments of the small intestine were flaccid and showed no phasic increases in intraluminal pressure.

B: ω-conotoxin GVIA Krebs’ buffer

Representative recording of mesenteric afferent nerve discharge after switching to ω-conotoxin GVIA Krebs’ buffer in jejunal segments. The upper trace shows a rate histogram of mesenteric afferent nerve discharge frequency in imp sec\(^{-1}\). The lower trace gives the recording of intraluminal pressure. Note that there is no change in baseline afferent nerve activity and motility.

C: Cadmium

The upper trace shows the quantified afferent discharge frequency in imp sec\(^{-1}\). The lower trace gives the recording of the reduced contractility after cadmium perfusion. Note the unchanged afferent firing frequency in the flaccid jejunal segments.

Figure 2: Chemical stimulation with acid and 5-HT.

A: Histogram showing the peak afferent firing in response to HCl (pH2) under the following conditions. Mean ± SEM, (*P<0.05).
B: Histogram showing the peak afferent firing response to 5-HT under the following conditions. Mean ± SEM, (*P<0.05).

Figure 3.
Effects of intraluminal acid pH 2 and 5-HT on intraluminal pressure in control experiments. The arrows indicate the beginning of the intraluminal perfusion of acid pH 2 and 5-HT.

Figure 4: Distension profiles (cmH₂O ± SEM), (n.s.).
Note baseline afferent discharge was set at 0. The increase in afferent discharge was quantified as the peak discharge frequency above baseline.
Figure 1

A

Ca\textsuperscript{2+}-free

Impulse frequency (imp s\textsuperscript{-1})

Intraluminal pressure (cmH\textsubscript{2}O)

B

Omega-conotoxin GVIA

Impulse frequency (imp s\textsuperscript{-1})

Intraluminal pressure (cmH\textsubscript{2}O)

C

Cadmium

Impulse frequency (imp s\textsuperscript{-1})

Intraluminal pressure (cmH\textsubscript{2}O)
Figure 3

A

5-HT (500 μM)

Impulse frequency (imp s⁻¹)

Intraluminal pressure (cmH₂O)

B

Intraluminal acid pH2

Impulse frequency (imp s⁻¹)

Intraluminal pressure (cmH₂O)