Anatomical and functional characterization of a duodeno-pancreatic neural reflex that can induce acute pancreatitis

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Running title: duodeno-pancreatic neural reflex and acute pancreatitis
Abstract

Objective: Neural cross-talk between visceral organs may play a role in mediating inflammation and pain remote from the site of the insult. We hypothesized such a cross-talk exists between the duodenum and pancreas and further it induces pancreatitis in response to intraduodenal toxins.

Design: A dichotomous spinal innervation serving both the duodenum and pancreas was examined and splanchnic nerve responses to mechanical stimulation of these organs were detected. This pathway was then excited on the duodenal side by exposure to ethanol followed by luminal mustard oil to activate TRPA1. Ninety minutes later, pancreatic inflammation was examined. Ablation of duodenal afferents by resiniferatoxin (RTX) or blocking TRPA1 by CHEM5861528 was used to further investigate the duodeno-pancreatic neural reflex via TRPA1.

Results: About 40% of dorsal root ganglia (DRG) from the spinal cord originated from both duodenum and pancreas via dichotomous peripheral branches; about 50% splanchnic nerve single units responded to mechanical stimulation of both organs. Ethanol sensitized TRPA1 currents in cultured DRG neurons. Pancreatic edema and myeloperoxidase (MPO) activity significantly increased after intraduodenal ethanol followed by mustard oil (but not capsaicin), but significantly decreased after ablation of duodenal afferents by using RTX or blocking TRPA1 by CHEM5861528.

Conclusion: We found the existence of a neural cross-talk between the duodenum and pancreas that can promote acute pancreatitis in response to intraduodenal chemicals. It also proves a previously unexamined mechanism by which alcohol can induce pancreatitis, which is novel both in terms of the site (duodenum), process (neurogenic) and receptor (TRPA1).

Key words: TRPA1, acute pancreatitis, alcohol, duodenum, neural cross-talk
**Introduction**

Visceral sensory nerves, either vagal or spinal in origin, may significantly modulate inflammation in various organs including the pancreas, a process called “neurogenic inflammation”. Thus functional denervation of the pancreas by neonatal capsaicin treatment, celiac ganglionectomy or resiniferatoxin (an ultrapotent capsaicin analog that produces desensitization of nociceptors) results in significant attenuation of acute experimental pancreatitis (33, 36). Two members of the transient receptor potential (TRP) cation channel family, TRPV1 (TRP subfamily V, member 1) and TRPA1 (subfamily A, member 1) are expressed by sensory nerves and play a key role in these processes including both pancreatic pain (12, 22-23) and inflammation (16, 19, 28-30, 35, 40, 55, 59). Activation of these receptors results in the local release of neuropeptides such as substance P and calcitonin gene related peptide (CGRP) amongst others, which produce arteriolar vasodilatation, increased vascular permeability, edema, and neutrophil infiltration (13, 17, 26, 38, 43, 62).

Spinal afferents in a given organ may be recruited in the inflammatory cascade either directly by noxious stimuli within the target organ or via neural reflexes originating in neighboring organs. Examples of the latter represent a form of viscero-visceral convergence and have been reported in several organs such as the urinary bladder and rectum, amongst others (27, 37, 39, 54). In this regard, enteropancreatic neural connections of a variety of types have also been described in the literature, although they have mainly been studied with respect to their physiological role (53). It can be hypothesized, however, that noxious triggers in the duodenum could induce inflammation in the pancreas utilizing these neural connections. Such a phenomenon could provide a potential explanation for conditions such as endoscopic retrograde cholangiopancreatography (ERCP)-induced pancreatitis, biliary pancreatitis as well as a link between ingested toxins and pancreatic injury.

In this study, we therefore hypothesized that pancreatic inflammation can be induced by duodenopancreatic nerve reflexes, specifically by extrinsic (spinal) afferents. Our aims were therefore to first establish anatomical evidence that dorsal root ganglia (DRG)
neurons receive convergent input from the duodenum and pancreas. A second aim was to examine whether stimulation of nerves in the duodenum or stomach could elicit acute pancreatitis. Finally, we wished to assess the effect of ethanol, a known pancreatic toxin, if any, in augmenting these processes.

MATERIALS AND METHODS

Animals

The animal protocols were approved by the institutional animal care and use committee at Stanford University. Experiments were performed on male Sprague Dawley rats (250-350 g) housed in a temperature-controlled environment with a 12-h light-dark cycle and free access to food and water.

Retrograde neural labeling

Under anesthesia with a cocktail of intra-peritoneal ketamine (80mg/kg)/xylazine (5mg/kg), two distinct retrogradely transported neuronal dyes (consisting of a cholera B toxin conjugated with an Alexa fluorescent dye) were injected into the rat’s exposed pancreas and the duodenal wall (“red” CTB-594 for pancreas and “green” CTB-488 for duodenum, both from Invitrogen, Carlsbad, CA) in 12 sites/each organ, using 2 µl per site for a total of 24 µl/organ for each dye. Seven days post retrograde labeling, the animals (n=3) were euthanized with ketamine/xylazine and transcardially perfused with 150 mL phosphate-buffered saline (PBS) followed by 400 mL ice-cold 4% paraformaldehyde (PFA) in PBS. Then the DRGs from segments T6-T13 of spinal cord were harvested and embedded in optimal cutting temperature (OCT). The 10µm frozen sections (5 sections per DRG/per rat) were cut and the sections were viewed using a Nikon Eclipse E600 microscope equipped with wavelength 450–500 nm for CTB-488 dye and 532–683 nm for CTB-594 and were counted from 5 sections per individual DRG per animal. Results were expressed as percentages of double-labeled neurons/segment/organ.

Immunofluorescent staining with TRPV1 and/or TRPA1 antibody

CTB-labeled DRG sections were obtained as described above and then respectively stained with rabbit anti-TRPV1 or goat anti-TRPA1 (both from Santa Cruz, Santa Cruz,
CA) or neither (as negative control) 1:200 in PBS containing 5% normal goat serum, 0.05% triton X-100 at 4°C for overnight. After washing in PBS, sections were incubated with anti-rabbit or anti-goat DyLight 649 secondary antibody (both from Jackson ImmunoResearch, West Grove, PA) following standard procedures, then the sections were viewed using a Zeiss LSM510 Meta inverted confocal microscope equipped with excitation filter wavelength 450–500 nm for CTB-488 dye, 532–683 nm for CTB-594 and 646-674 nm for DyLight 649, the percentages of triple-labeled neurons that were double labeled with CTB and also positively stained for TRPV1 or TRPA1 were counted (5 sections/DRG/ rat).

The immunofluorescent double-staining was performed on DRGs (T6-T13) sections from normal rats (n=3) after transcardial perfusion with 150 mL phosphate-buffered saline (PBS) followed by 400 mL ice-cold 4% paraformaldehyde (PFA) in PBS. Then the DRGs from segments T6-T13 of spinal cord were harvested and embedded in optimal cutting temperature (OCT). The 10µm frozen sections (5 sections /DRG/ rat) were cut and then incubated with mixtures of rabbit anti-TRPV1 and goat anti-TRPA1 primary antibodies or neither (as negative control) 1:200 each in PBS containing 5% normal goat serum, 0.05% triton X-100 at 4°C for overnight. After washing, sequential incubation with anti-rabbit Alexa 488 and anti-goat Alexa 594 secondary antibodies (both from Invitrogen, Carlsbad, CA). The sections were viewed using a Nikon Eclipse E600 microscope equipped with excitation filter wavelength 450–500 nm for Alexa 488 and 532–683 nm for Alexa 594. Double-labeled neurons which indicate coexpression of TRPV1 and TRPA1 were counted.

**Splanchnic nerve single unit response to gentle traction of pancreatic duct and duodenum**

We studied the response of splanchnic nerve single units to mechanical stimulation elicited by gentle traction of the pancreatic duct or duodenum. Rats (n=9) were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and maintained with a constant infusion of 50 mg sodium pentobarbital in 9 ml, 0.9% NaCl at 1.0 ml/h through the jugular vein. Tracheotomy was performed for artificial ventilation and rats were paralyzed with gallamine triethiodide (20 mg/kg. i.v; from Sigma, St. Louis, MO).
The duct and duodenum were exposed and a 4-0 suture was loosely placed in the pancreatic duct and the middle of the duodenum, with the other end left outside of the abdomen for applying traction. The left splanchnic nerve was exposed and as previously described (30), the electrical activity of single nerve units was measured in response to gentle traction (maintained for 10 sec) of the pancreatic duct or duodenum with an interval of 1 minute in between consecutive stimulations. A response ≥30% from baseline was considered as positive.

**Patch-clamp recording**

Patch-clamp recording was conducted in whole-cell configuration v-clamp. Data were acquired with Digidata interface 1200 series and pClamp software version 9.1 (Molecular Devices, Toronto, Canada). Sensory neurons in dorsal root ganglia (DRG) were cultured as previously described (63). The recording pipettes were pulled from borosilicate glass to give final resistances of 2–6 MΩ. The electrode solution contained (in mM): CsF (110), MgCl₂ (5), EGTA (11), NaCl (10), Hepes (10), pH was adjusted to 7.3–7.4. The cells were bathed in Tyrode saline contained (in mM): NaCl (135), KCl (5.4), MgCl₂ (1), CaCl₂ (2), NaH₂PO₄ (0.1), HEPES (10), glucose (10), pH was adjusted to 7.2–7.3. All agents were delivered through a valve control system.

**Activation of duodenal TRPV1 and TRPA1 receptors and administration of intraduodenal ethanol**

Under anesthesia with ketamin/xylazine, laparotomy was performed in rats (n=12) and loose ligatures were applied at the pyloric and distal duodenal ends. Various agents, as indicated, were then instilled into the closed duodenal lumen for 30 minutes. To activate TRPV1, 0.5mg/kg capsaicin in 0.5 ml saline; controls received saline only (n=3 for each group); to activate TRPA1, 2.5% mustard oil in 0.5 ml corn oil (from Sigma, St. Louis, MO); controls received corn oil only for control (n=3 for each group). Animals were sacrificed 90 minutes after instillation of these agents and blood and pancreatic tissue were collected for analysis of serum amylase and lipase, edema and MPO activity.

To test the effects of ethanol on TRPV1 or TRPA1, rats were given 0.5 ml of 10%, 20% and 40% ethanol by intraduodenal instillation (as described above). Control rats
were instilled with saline. After thirty minutes, the intra-duodenal contents were withdrawn and capsaicin or mustard oil with their respective controls (n=3 for saline+corn oil, n=4 for 40% ETOH+corn oil; n=9 for saline+mustard oil; n=9 each for 10%, 20% and 40% ETOH+mustard oil; n=6 for saline+capsaicin; n=6 for 40% ETOH+capsaicin) was instilled for another 90 minutes. Then the rats were sacrificed and the blood and pancreas were collected for serum amylase and lipase activity, pancreatic edema, pancreatic histology severity score and MPO activity.

Chemical ablation of duodenal nerves with resiniferatoxin

For these experiments, resiniferatoxin (RTX, from Sigma, St. Louis, MO) was instilled (2µg/kg in 0.5 ml saline) in the duodenum at laparotomy with pyloric and distal duodenal ligation for 1 hour to ensure exposure of luminal afferents to the drug. Subsequently, the intraduodenal contents were aspirated. Control rats received saline only.

TRPA1 antagonism

Chembridge-5861528 (C_{19}H_{23}N_{5}O_{3}; CHEM; a TRPA1 channel antagonist, a derivative of HC-030031) was synthesized by ChemBridge Corporation (San Diego, CA) and used as a selective TRPA1 channel antagonist that shows no TRPA1 channel agonism and no TRPV1 channel antagonism at doses up to 100uM (56-58). For these experiments, CHEM 50 µg in 10% DMSO in 0.5 ml saline, or vehicle (10% DMSO in 0.5 ml saline) was instilled into the duodenum along with 2.5% mustard oil for 90 minutes, as described above (n=6 for each group). Then the rats were sacrificed and the blood and pancreas were collected for serum amylase and lipase activity, pancreatic edema, pancreatic histology severity score and MPO activity.

Serum amylase and lipase activity: The blood was collected by decollation and centrifuged (10,000 g, 20 min), and serum was obtained for measurement of amylase and lipase activity using the Siemens Dimension Xpand Plus Integrated Chemistry System (Siemens Healthcare Diagnostics Ltd, Camberley, UK).

Pancreatic edema: The pancreas were dissected and separated into three parts (head,
middle and tail). For measurement of edema, the head region was weighed before and after dehydrating at 60°C for 24 hours. The pancreatic edema was expressed as water content (wet weight-dry weight) per dry weight.

**Pancreatic histology severity score (HSS):** The middle part of the pancreas used for histological analysis was immediately fixed in 10% formalin at 4°C for overnight. Then tissue was transformed to 75% ethanol and embedded in paraffin and processed for H&E staining. Evaluation of the pathologic changes was performed by an experienced GI pathologist who was blinded to the treatment groups. The severity of acute pancreatitis was graded by a semiquantitative assessment of edema, acinar necrosis and inflammatory cell infiltrate according to a scoring system described previously (16): Edema was scored as 0, absent; 1, focal increase between lobules; 2, diffuse increase between lobules; and 3, acini disrupted and separated. Inflammatory cell infiltrate was scored as 0, absent; 1, in ducts (around ductal margins); 2, in the parenchyma (in <50% of the lobules); and 3, in the parenchyma (in >50% of the lobules). Acinar necrosis was scored as 0, absent; 1, periductal necrosis (≤5%); 2, focal necrosis (5%-20%); and 3, diffuse parenchymal necrosis (20%-50%). The HSS was determined by the total score of edema, inflammation and necrosis.

**Pancreatic MPO activity:** A piece of tail part of the pancreas was immediately frozen and stored at -80°C for MPO (myeloperoxidase activity) measurement described previously (16). Tissue samples were thawed, homogenized in 20 mmol/L phosphate buffer (pH 7.4), and centrifuged (10,000g, for 10 minutes at 4°C), and the resulting pellet was resuspended in 50 mmol/L phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. The suspension was subjected to 4 cycles of freezing and thawing and was further disrupted by sonication (40 seconds). The sample was then centrifuged at 10,000g for 5 minutes at 4°C. The supernatant was used for the MPO assay. The reaction mixture consisted of the supernatant, 1.6 mmol/L tetramethylbenzidine, 80 mmol/L sodium phosphate buffer (pH 5.4), and 0.3 mmol/L hydrogen peroxide. This mixture was incubated at 37°C for 110 seconds and the changes of absorbance at 650 nm were used to calculate pancreatic MPO activity which was
expressed as units per mg of tissue.

**Statistical Analysis**

The results were expressed as means ± SE. T-test and One-Way ANOVA Post Hoc Tests was performed with SPSS statistical analysis software (IBM SPSS Inc., Chicago, USA). Values of $p<0.05$ were considered statistically significant.

**Results**

A subset of afferent neurons innervates both the duodenum and pancreas via dichotomous peripheral branches and expresses TRPA1 and TRPV1

Figure 1 shows the number of double-labeled cells expressed as a percentage of the total number of duodenal- and pancreatic-labeled cells in thoracic (T6-T13) DRG. 74.16% of all the neurons innervating both the duodenum and pancreas also expressed TRPA1 (Figure 2B), and 79.41% of all the neurons innervating both the duodenum and pancreas also expressed TRPV1 (Figure 2C). Co-expression of TRPV1 and TRPA1 widely existed in thoracic DRG segments (Figure 2A).

A total of 51 splanchnic nerve single units were recorded. Of these, 4 (7.84%) responded to traction of the pancreatic duct and 8 (15.69%) responded to traction of the duodenum; 26 (50.98%) responded to stimulation of both organs while 13 units (25.49%) had no response to either one (Figure 3).

**Ethanol sensitizes TRPA1 currents in sensory neurons**

Figure 4 illustrates the effect of 2.5% mustard oil (MO) or corn oil (CO) in combination with 3% ethanol on cultured DRG neurons. In whole-cell configuration, mustard oil and ethanol together resulted in an inward current of $-1677.1 \pm 520.86$ pA as compared with $-420 \pm 96.95$ pA after corn oil and ethanol ($p=0.027$) (Fig4A-C). We also analyzed single channel currents to examine the effects of mustard oil and ethanol on TRPA1 using excised patches of membrane (outside-out). Figure 4D-J shows a typical example. When membrane potential was held at $-60$mV, ethanol (3%) alone induced small currents, while corn oil alone did not invoke any significant current and the combination of corn oil and ethanol had no noticeable effect as compared to ethanol.
alone (data not shown). Exposure to mustard oil resulted in prominent activity combination of corn oil and the combination of mustard oil and 3% ethanol evoked multiple and persistent channel activity. As shown in Figure 4G-I, this was reversibly inhibited by CHEM (10uM), a specific antagonist of TRPA1. The channel open probability induced by ethanol and mustard oil was significantly reduced by CHEM from 0.75 ± 0.06 to 0.34 ± 0.09 (P=0.006).

**Intraduodenal ethanol enhances pancreatic inflammation induced by intraduodenal TRPA1 activation, an effect that is prevented by intraduodenal RTX or CHEM 5861528**

When mustard oil was used only in the duodenum, there was no significant difference compared with control corn oil. However, when animals received intraduodenal 40% ethanol, followed by mustard oil, there was a significant increase in pancreatic edema (F=11.018, p<0.001), histology severity score (F=3.959, p=0.022, Figure 9A-D) and MPO activity (F=4.493, p=0.014) as compared with the group receiving either 40% ETOH and corn oil or saline and mustard oil only (Figure 5). Lower concentrations of ETOH (10 and 20%) when followed by either saline or mustard oil had no effects on pancreatic inflammation (data not provided).

Local application of RTX significantly attenuated pancreatic inflammation induced by intraduodenal ethanol and mustard oil, with reductions in pancreatic edema, histology severity scores (Figure 9E-F) and MPO activity but not in serum amylase and lipase activity (Figure 6).

Figure 7 shows that the TRPA1 channel antagonist CHEM 5861528 had a similar effect as RTX and significantly reduced pancreatic edema, histology severity scores (Figure 9G-H) and MPO activity induced by intraduodenal ethanol and mustard oil, without affecting serum amylase and lipase activity.

**Intraduodenal capsaicin, with or without prior intraduodenal ethanol has no effect on pancreatic inflammation.**

With or without prior intraduodenal 40% ethanol instillation, the capsaicin used in the duodenum was no significant difference compared with control saline group on pancreatic edema (F=1.529, p=0.236), MPO activity (F=1.951, p=0.168), serum amylase
(F=1.595, \( p=0.235 \)) and lipase levels (F=3.185, \( p=0.057 \)). Pancreatic histology severity score of all these groups was zero (Figure 8, Figure 9I-L).

**Discussion**

Communication amongst sensory nerve serving anatomically close but separate organs is a common physiological and clinical phenomenon. It is best known in the context of referred pain, in which nerve fibers from two organs (e.g. a visceral organ and the overlying skin) are believed to converge on a common second order neuron in the spinal cord. However, neural communications between organs can take place in several other ways with consequences that are not only important for pain perception but also for inflammation, a phenomenon known as cross-sensitization (8). Central convergence can occur at spinal or even higher levels, subserved by both spinal and vagal nerves (14, 18, 20, 41-42). Peripheral mechanisms also exist and may take the form of cross-talk amongst fibers within the peripheral nerve bundle or amongst neurons within the dorsal root ganglion (3). Another theory, first espoused several decades ago (45), but one that has gained prominence recently is that of axon dichotomy in which a single sensory nerve gives rise to terminally divergent fibers that then innervate separate organs.

Using both anatomical and physiological techniques, axon dichotomy has been found to be a property of DRG neurons in many species, ranging from 0.5 to 15% of all afferents (32). This has been particularly of interest in pelvic and lower visceral organs (21); thus, up to 17% of rat and 21% of mice urinary bladder and colon afferents are presumed to be dichotomized based on dual labeling (9). Definitive proof of this phenomenon has come from single unit recordings of visceral afferents which show responses to stimulation of both the lower urinary tract and the colon (6). Neural convergence has physiological as well as anatomical implications and may be important in coordinating functions such as urination, defecation and coitus which should normally inhibit each other. The existence of such pathways could also provide a mechanism by which disease in one visceral organ induces pathology in another (37). Thus, acute cystitis in an otherwise healthy bladder can be observed in response to colitis, prostatitis or endometriosis in rats (60). Chronic colonic inflammation has been shown to produce neurogenic inflammation in the bladder accompanied by disturbed micturition,
recruitment and activation of bladder mast cells, and upregulation of neurotrophic growth factor (NGF) and stem cell factor (SCF) in the bladder (27, 53-54). The increased vascular permeability in the bladder in response to inflammation in the colon or uterine horn can be reduced by hypogastric nerve ablation implying the existence of hardwired neural pathways (60). Further, acute colitis can lead to upregulation of sodium currents in primary sensory neurons emanating from the bladder (31).

The duodenum communicates intimately with the pancreas via both hormonal and neural connections. The latter can be of several types (50). There is an indirect vagal pathway which is common to most upper gastrointestinal organs. Nerve bundles have also been shown to run directly between the duodenum (as well as the stomach) and the pancreas, connecting the intrinsic ganglia in both organs via cholinergic neurons (4, 22-23). In this study we first established the dichotomous nature of the sensory innervation of the pancreas and duodenum, using well-established retrograde labeling techniques. Our findings suggest a remarkable level of “neural intimacy” between these two neighboring visceral organs. In DRG segment T8 for instance, about half of all pancreatic segments also received innervation from the duodenum; similarly, in T9 about half of all duodenal neurons also innervated the pancreas. Functional correlation of these observations were provided by electrophysiological studies with 50% splanchnic nerve single units responding to mechanical stimulation of both the pancreatic duct and duodenum. Our results are in keeping with studies on neural convergence in other organs, as discussed above. We therefore hypothesized that activation of duodenopancreatic nerves could have pathological consequences, as has been demonstrated in other organs.

Neurogenic pancreatitis is now well described, generally in relationship to sensory nerves that directly innervate the pancreas, and act via pathways that involve TRPV1 and substance P signaling (28, 33, 35-36, 40). However, in our study simple activation of intraduodenal nerves with ethanol or agonists to either TRPV1 or TRPA1 did not lead to any significant inflammation in the pancreas. We then showed that pre-exposure to ethanol followed by TRPA1 activation (but not TRPV1) resulted in acute pancreatitis, manifested by edema, histology and myeloperoxidase activity. Amylase and lipase levels were not significantly elevated, which is in keeping with other forms of neurogenic pancreatitis (28). The neurogenic nature of this effect was proven by the fact
that it could be prevented by the application of resiniferatoxin, a potent TRPV1 agonist that can reliably produce long-lasting chemical sensory denervation (1-2, 5, 11, 24, 34, 61). The discrepancy in the effects of TRPV1 versus TRPA1 activation are somewhat surprising since all TRPA1 expressing fibers also express TRPV1 in rat DRG neurons (25), TRPV1 is a major player in other forms of neurogenic pancreatitis (28), and TRPV1 and TRPA1 have been shown to participate synergistically in the pathogenesis of acute pancreatitis, the downstream effects of TRPA1 may involve mechanisms that include TRPV1 currents, it is possible that ethanol may modulate TRPA1 indirectly by acting on TRPV1 (44). The results of our study also confirm co-expression of these receptors by neurons innervating both the duodenum and the pancreas. Our results therefore suggest that although these receptors may share some downstream signaling elements, their activation may produce distinct physiological effects.

What are the clinical implications of our study? Cross-modulation between the pancreas, duodenum and esophagus has been suggested by experimental studies in humans (12). Duodenopancreatic neural reflexes have also been speculated to play a role in biliary pancreatitis, although rigorous experimental proof does not exist (48). In a closed duodenal loop rodent model of acute pancreatitis, autonomous arc reflexes initiated in the peri-Vaterian duodenum have been implicated in the pathogenesis based on the ability of local lidocaine to attenuate pathological changes (10). Such a mechanism could also provide a satisfactory answer to the vexing and serious problem of ERCP induced pancreatitis which can occur even without canulation or injection of contrast into the pancreatic duct. Interestingly, one of the highest risk factors for post-ERCP pancreatitis, sphincter of Oddi dysfunction (49), may plausibly be associated with altered neural responses that may predispose them to activation of a pro-inflammatory reflex from manipulation of the papillary mucosa. Theoretically, this mechanism may also be invoked by passage of gallstones and irritation of the papilla, providing an alternative explanation for biliary pancreatitis as well.

Another novel finding in this study was the interaction between ethanol and TRPA1. Previous reports have suggested that ethanol can directly stimulate the TRPV1 receptor as measured by a capsazepine-sensitive calcium response in trigeminal and dorsal root ganglia neurons; more importantly perhaps, it can also sensitize DRG neurons...
to the effects of capsaicin (52). Finally, it can also cause the release of SP from various tissues in a capsazepine sensitive manner, again suggesting the involvement of TRPV1 (51). Our results suggest that ethanol may have similar effects on TRPA1 via an unknown mechanism. In support of our findings, it has been shown that high doses of intraluminal ethanol in the upper gastrointestinal tract modulates pancreatic secretion, possibly via a neural mechanism (49). The physiological significance of our findings is worthy of discussion. We used a very high concentration of ethanol (40% by volume or about 32 g/dL) and the final concentration to which the duodenal mucosa would be exposed to under physiological conditions may be considerably less, given the dilution by intestinal fluid. However, in alcoholic volunteers, concentrations of alcohol as high as 5 g/dL have been reported after ingestion of drinks containing 20-25% ethanol (15). Thus conceivably, in some patients, particularly those with rapid gastric emptying, the duodenal concentration of ethanol can get close to the range required to cause sensitization of TRPA1 receptors. It is also possible that congeners in alcoholic beverages such as acetaldehyde, which can activate TRPA1, may provide the “double hit” that seems to be required to trigger this pathway (7).

In conclusion, we have shown the existence of a previously unexamined mechanism by which noxious stimulation of the duodenum can induce pancreatitis, via a neurogenic process mediated by TRPA1 and enhanced by high concentrations of ethanol. Further studies will be required to understand the clinical significance of these findings but they can theoretically provide an explanation for some forms of pancreatitis such as that seen after ERCP or in response to heavy drinking.

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Contributions of authors:

Cuiping Li: Performed experiments, analyzed the results, wrote and revised the manuscript
Yaohui Zhu: Performed experiments, analyzed results and revised the manuscript
Mohan Shenoy: Performed experiments and revised the manuscript
Reetesh Pai: Evaluation of the pancreatic histology severity score
Liansheng Liu: Performed experiments, analyzed results and revised the manuscript
Pankaj Jay Pasricha: The principal investigator of this NIH grant, designed the studies, analyzed the results, wrote and revised the manuscript.
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**Figure Legends**

**Figure 1.** Dichotomizing duodenalpancreatic nerves at thoracic T6-13 segments. A, DRG neurons originated from pancreas (pancreatic-specific neurons shown in red); B, DRG neurons originated from duodenum (duodenal-specific neurons shown in green); C, overlay of A and B (double labeled neurons shown in yellow). Magnification of these pictures is 200X (object lens 20X, eyepiece 10X); D, percentage of double labeled DRG of total number of duodenal- and pancreatic labeled DRG neurons at various segments.

**Figure 2.** Immunofluorescent staining with TRPV1 and/or TRPA1 antibody. A, one DRG section from normal rats double staining with TRPV1 and TRPA1 (TRPV1-positive neurons shown in green, TRPA1-positive neurons shown in red, double stained neurons shown in yellow, magnification is 400X); B, CTB-labeled DRG neurons staining with TRPA1 (pancreatic-specific neurons shown in red, duodenal-specific neurons shown in green, TRPA1-positive neurons shown in pseudo-colored blue, triple stained neurons shown in purple, magnification is 200X); C, CTB-labeled DRG neurons staining with TRPV1 (pancreatic-specific neurons shown in red, duodenal-specific neurons shown in green, TRPV1-positive neurons shown in pseudo-colored blue, triple stained neurons shown in purple, magnification is 200X).

**Figure 3.** Splanchnic nerve single unit response to gentle traction of the pancreatic duct and duodenum. A, representative recording of GSN fiber response to gentle traction of organs; B, splanchnic nerve single unit response to gentle traction of pancreatic duct and duodenum. A total of 51 splanchnic nerve single units were recorded. Of these, 4 (7.84%) responded to traction of the pancreatic duct and 8 (15.69%) responded to traction of the duodenum; 26 (50.98%) responded to traction of both organs while 13 units (25.49%) had response to neither organ.

**Figure 4.** The TRPA channel inhibitor CHEM inhibits ethanol and mustard oil
sensitive inward currents. A, Whole cell patch recording of current from corn oil in combination with 3% ETOH (ethanol); B, Whole cell patch recording of current from mustard oil in combination with 3% ETOH; C, whole cell amplitudes activated by ETOH + corn oil and ETOH+ mustard oil (n=5 each group); D-I: Single channel currents from a representative cell patch (c in panels represents closed state). D, Single channel currents from 3% ETOH; E, current from corn oil; F, Single channels current from mustard oil; G, Single channel currents activated by mustard oil in combination with 3% ETOH; H, CHEM inhibition of single channel currents invoked by mustard oil in combination with 3% ETOH; I, Single channel currents re-activated by mustard oil in combination with 3% ETOH after CHEM washout; J, Single channel open probabilities in the presence of ETOH and mustard oil and the effects of CHEM (n=5 each group). *p <0.05, **p <0.01 and ***p <0.001. Arrows denote time of application of agents (CHEM was perfused for 30 seconds before exposure to ETOH+MO).

Figure 5. Effects of ethanol on pancreatic inflammation induced by intraduodenal TRPA1 activation. A, pancreatic edema; B, pancreatic histology severity score; C, serum amylase level; D, serum lipase level; E, MPO activity. Results were expressed as means ± SE. (n=3 for saline+corn oil, n=4 for 40%ETOH+corn oil, n=9 each for saline+mustard oil and 40%ETOH+mustard oil). *p <0.05, **p <0.01 and ***p <0.001.

Figure 6. Effects of intraduodenal RTX on pancreatic inflammation induced by intraduodenal ethanol and mustard oil. A, pancreatic edema; B, pancreatic histology severity score; C, serum amylase level; D, serum lipase level; E, MPO activity. Results were expressed as means ± SE. (n=13 each for RTX+40%ETOH+mustard oil and saline+40%ETOH+mustard oil). *p <0.05, **p <0.01 and ***p <0.001.

Figure 7. The role of the TRPA1 channel antagonist CHEM on pancreatic inflammation induced by intraduodenal ethanol and mustard oil. A, pancreatic edema; B, pancreatic histology severity score; C, serum amylase level; D, serum lipase
level; E, MPO activity. Results were expressed as means ± SE. (n=6 for 40%ETOH+mustard oil+vehicle and n=5 for 40%ETOH+mustard oil+CHEM). *p <0.05, **p <0.01 and ***p <0.001.

Figure 8. The effects of intraduodenal capsaicin, with or without prior intraduodenal ethanol on pancreatic inflammation. A, pancreatic edema; B, MPO activity; C, serum amylase level; D, serum lipase level; pancreatic histology severity score of all these groups was zero. Results were expressed as means ± SE. (n=3 each for capsaicin and saline group; n=6 each for saline+capcaicin and 40%ETOH+capsaicin).

Figure 9: Representative images of pancreatic histology severity score. saline+corn oil (A), 40%ETOH+corn oil (B), saline+mustard oil (C), 40%ETOH+mustard oil (D), RTX+40%ETOH+mustard oil (E), saline+40%ETOH+ mustard oil (F), CHEM+40%ETOH+mustard oil (G), vehicle+40%ETOH+mustard oil (H), saline (I), capsaicin (J), saline+ capsaicin (K), 40%ETOH+ capsaicin (L).
Percentage of double labeled DRG of total number of duodenal- and pancreatic-labeled DRGs

![Bar chart showing percentage of double labeled DRGs for T6 to T13, with separate bars for duodenum and pancreas.](image)
A  GSN fiber response to gentle traction of organs

Response to pancreatic duct

Response to duodenum

Response to both

Response to neither

B  Splanchnic nerve single unit response to gentle traction of pancreatic duct or duodenum

% Total fibers respond

0  10  20  30  40  50  60

pancreatic duct  duodenum  both  neither

7.84  15.69  50.98  25.49
Whole-cell amplitudes activated by ETOH+corn oil and ETOH+mustard oil

Single channel open probability (ETOH+mustard oil) inhibited by CHEM