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Vagal gut-brain signaling mediates amygdaloid plasticity, affect and pain in a functional dyspepsia model

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Abbreviations:
FD = functional dyspepsia; CeA = central nucleus of amygdala; CRH = corticotrophin releasing hormone; BDNF = Brain derived neurotrophic factor
Abstract

Functional dyspepsia (FD) is associated with both chronic gastrointestinal distress and anxiety and depression. Here, we hypothesized that aberrant gastric signals, transmitted by the vagus nerve, may alter key brain regions modulating affective and pain behavior. Using a previously validated rat model of FD characterized by gastric hypersensitivity, depression- and anxiety-like behavior, we found that vagal activity in response to gastric distention was increased in FD rats. The FD phenotype was associated with gastric mast cell hyperplasia and increased expression of corticotrophin-releasing factor (Crh) and decreased brain-derived neurotrophic factor genes in the central amygdala. Subdiaphragmatic vagotomy reversed these changes and restored affective behavior to that of controls. Vagotomy partially attenuated pain responses to gastric distention, which may be mediated by central reflexes in the periaqueductal gray, as determined by local injection of lidocaine. Ketotifen, a mast cell stabilizer, reduced vagal hypersensitivity, normalized affective behavior and attenuated gastric hyperalgesia. In conclusion, vagal activity, partially driven by gastric mast cells, induces long-lasting changes in Crh signaling in the amygdala that may be responsible for enhanced pain and anxiety- and depression-like behaviors. Together, these results support a “bottom-up” pathway involving the gut-brain axis in the pathogenesis of both gastric pain and psychiatric co-morbidity in FD.

Key words: Gut-brain axis, vagus, mast cells, functional dyspepsia, amygdala, CRH, BDNF.
Introduction

Functional dyspepsia (FD), a highly prevalent clinical gastrointestinal syndrome, has been variously defined but generally includes some form of upper abdominal discomfort or pain. The Rome Committee criteria subclassify FD into two types, one of which is also defined predominantly by pain (epigastric pain syndrome or FD/EPS) and the other (postprandial distress syndrome or FD/PDS) although there are varying degrees of overlap (1, 2). Regardless of the symptomatic phenotype, patients with FD commonly have psychiatric comorbidities, such as anxiety and depression (3, 4). It has been widely accepted that these can drive gastrointestinal symptom perception (“top-down” model) (5, 6). However, recent evidence from animal models (7) as well as human subjects suggests that gastrointestinal pathology may actually induce anxiety and depression (“bottom-up” model) (8). Thus the gut-brain axis may actually be involved in a bidirectional role in the genesis of both abdominal symptoms and associated mood disorder (9). However, the mechanisms by which primary gastric insults can initiate changes in affect and mood have not been investigated and is the subject of the present study.

A leading candidate mechanism in mediating these changes is the vagus nerve, a major route of communication between visceral organs and the brain (10). Sensory (afferent) vagal neurons, whose cell bodies are located in the nodose ganglia, project to a variety of visceral organs including the gastrointestinal tract. In the gut, they respond to physiological and pathological stimuli and relay this information to second-order neurons in the nucleus tractus solitarius (NTS). The NTS neurons further project to almost every other brain region, including centers involved in mood regulation such as the amygdala. Indeed, the vagus nerve is known to induce or modulate behavioral changes in response to peripheral inflammatory insults (10), and has been implicated in the pathogenesis of anxiety and depression in irritable bowel syndrome (IBS), contributing directly to affective behavior and indirectly, to enhanced nociception in rodent
We therefore hypothesized that increased gastric vagal activity may contribute to the changes in affective behavior and nociception associated with FD, and that gastric mast cells may have a mechanistic role given their well-established functional relationship with neurons (14). Several studies have reported an increase in mucosal mast cells in the upper gastrointestinal tract in both children and adults with FD, similar to what has been described in patients with irritable bowel syndrome (IBS) (15-17). In the current study, we tested these hypotheses using a previously validated rat model of FD in which mild gastric irritation in neonatal rats induces a state of persistent gastric hyperalgesia, impaired gastric motility, and anxiety-like and depression-like behavior in adulthood which was associated with brain alterations (7, 18).

Results

Vagal activity drives depression- and anxiety-like behaviors in FD rats.

We first examined the vagal afferent responses to gastric distention (GD) in the FD model using single nerve unit recordings. While baseline activity was similar in both groups, vagal nerve activity in response to GD was significantly increased in FD rats as compared with controls (Figure 1A, Two-ANOVA: main effect of model $F_{(1,280)}=23.54$, $P<0.001$, main effect of pressure $F_{(3,280)}=23.99$, $P<0.001$, interaction of model and pressure $F_{(3,280)}=2.3$ $P=0.078$). These data indicate that the FD phenotype is associated with gastric vagal hypersensitivity to distention. We next examined the effects of vagotomy on affective behaviors in FD rats.

FD rats that underwent sham surgery showed increased time spent immobile and reduced latency to immobility in the forced swim test (FST), indicative of depression-like behavior. These alterations were reversed by vagotomy (Figure 1B; Two-Way ANOVA, Immobile time: main effect of model $F_{(1,28)} = 43.7$ $P<0.001$, main effect of vagotomy $F_{(1,28)}= 1.54$ $P = 0.224$, interaction of model and vagotomy $F_{(1,28)} = 8.27$ $P<0.05$; Latency: main effect of
Further, relative to control, FD rats showed increased immobile time and decreased rearing time in the open field test, suggesting an increase in anxiety-like behavior (19, 20) (Figure 1C). These behavioral changes were also normalized by vagotomy (Figure 1C; Two-Way ANOVA, Immobile: main effect of model $F_{(1,28)} = 25.9$, $P < 0.001$, main effect of vagotomy $F_{(1,28)} = 25.4$ $P < 0.001$, interaction of model and vagotomy $F_{(1,28)} = 15.59$, $P < 0.001$; Rearing: main effect of model $F_{(1,28)} = 3.91$ $P < 0.001$, main effect of vagotomy $F_{(1,28)} = 2.98$ $P = 0.096$, interaction of model and vagotomy $F_{(1,28)} = 11.15$ $P < 0.05$). Vagotomy had no effect on these behaviors in control rats.

**FD is associated with gene expression changes in the amygdala that are partially normalized by vagotomy.**

Given that vagotomy robustly normalized the depressive- and anxiety-like phenotype of FD rats, we further examined the effects of vagotomy on the expression of relevant genes in the amygdala, a key brain nucleus in regulating affective behavior. In the central amygdala (CeA), corticotrophin releasing hormone ($Crh$) gene expression was significantly increased in FD rats, an effect that was reversed by vagotomy (Figure 2A, Two-way ANOVA: main effect of model $F_{(1,19)} = 2.35$, $P = 0.142$, main effect of vagotomy $F_{(1,19)} = 0.0727$, $P = 0.79$, interaction of model and vagotomy $F_{(1,19)} = 8.882$. $P < 0.05$). Furthermore, post-hoc tests showed significant differences between control/sham and FD/sham and between FD/sham and FD/vagotomy (Figure 2A). The expression of the $Crh$ receptor 1 gene ($Crhr1$; Figure 2B), but not $Crhr2$ (Figure 2C), was increased in the CeA of FD rats. However, $Crhr11$ expression was not reversed with vagotomy (Figure 2B, Two-way ANOVA: main effect of model $F_{(1,20)} = 7.089$ $P < 0.05$, main effect of vagotomy $F_{(1,20)} = 0.092$ $P = 0.765$, interaction of model and vagotomy $F_{(1,20)} = 1.039$ $P = 0.32$). The expression of brain derived neurotrophic factor ($Bdnf$) gene in the CeA was significantly lower in FD rats, but normalized by vagotomy (Figure 2D, Two-way ANOVA: main
effect of model $F_{(1,20)} = 0.515, P = 0.483$, main effect of vagotomy $F_{(1,20)} = 1.946, P = 0.181$, interaction of model and vagotomy $F_{(1,20)} = 10.043, P < 0.05$). Post-hoc tests showed significant differences between control/sham and FD/sham and between FD/sham and FD/vagotomy.

**Vagal activity contributes to nociceptive sensitization via central descending modulatory pathways**

Because the vagus does not directly convey sensory information to the spinal cord, we hypothesized that indirect central nervous system reflexes, generated in response to vagal signals, may participate in nociception. Both facilitatory and inhibitory pathways from supraspinal centers such as the ventrolateral periaqueductal gray (PAG) and the rostral ventromedial medulla can significantly modulate spinal responses to nociceptive signaling from the periphery. We therefore first examined the role of descending pathways in pain behavior in this model by blocking PAG activity with local infusion of lidocaine. As Figure 3A showed, FD rats show increased nociceptive response to gastric distention (GD) with both 40 mmHg and 80 mmHg while PAG infusion with lidocaine attenuated the VMR response induced by 80 mmHg GD, but not 40 mmHg (Figure 3A). Of note, GD with 40 mmHg is not considered noxious in rats (21). These effects began 10 minutes after the infusion (Figure 3A) and began to resolve by 30 minutes post-infusion (data not shown). These results therefore indicate that descending pathways from the PAG are enhancing the spinal responses to noxious stimulation in this model.

We then tested whether increased vagal activity in FD rats contributes to hyperalgesia in these rats. We assessed the effects of vagotomy on expression of FOS (an immediate early gene product that indicates neuronal activation) in layer 1-2 of the dorsal horn in thoracic spinal levels 8-10 (T8-10) in response to gastric distention (GD). We found that GD-induced FOS expression is increased in FD rats, indicating nociceptive sensitization (Figure 3B). Further, vagotomy significantly attenuated, but did not eliminate, this sensitization and had no effect in
control rats. Together, these results suggest that vagal hypersensitivity contributes to gastric hyperalgesia in this model, possibly via activating descending facilitatory reflexes from the brain to the spinal cord.

**Increased numbers of mast cells in the stomach may drive vagal hypersensitivity, changes in affect and hyperalgesia in FD rats**

Having shown that vagal hypersensitivity may maintain the FD phenotype in this model, we next examined the potential mechanistic role of mast cells, based on our original hypothesis and supported by our previously published report (22). We found that tryptase-positive mast cells were significantly increased in the mucosa and submucosa of the stomach of FD rats (Figure 4A, Student’s t-test: P<0.001 for both mucosa and submucosa). By co-staining with PGP9.5, a pan-neuronal marker, it was observed that the mast cells were in close proximity to nerve terminals in the mucosa (Figure 4A). When treated with ketotifen, a histamine H1 receptor antagonist and mast cell stabilizer, we found that vagal hypersensitivity to gastric distention in FD rats was partially reversed (Figure 4B. Three-way ANOVA, main effect of model $F_{(1,146)} = 66.2$, $P<0.001$, main effect of treatment: $F_{(1,146)} = 11.06$, $P<0.001$, main effect of pressure: $F_{(3,146)} = 51.14$, $P<0.001$).

We then tested the effects of ketotifen on affective behavior in this model. As demonstrated previously, FD rats spent a significantly more time immobile in the open field test. This effect was reversed by treatment with ketotifen (Figure 4C, Two-Way ANOVA showed significant difference in main effects of model, $F_{(1,17)} = 19.92$, $P < 0.001$, main effect of ketotifen treatment $F_{(1,17)} = 4.45$, $P = 0.05$, Interaction of model and treatment, $F_{(1,17)} = 11.67$, $P < 0.05$). Likewise, FD rats spent significantly more time immobile in the forced swim test, and the effect was reversed by treatment with ketotifen (Figure 4C, Two-Way ANOVA showed main effects of model, $F_{(1,17)} = 7.34$, $P < 0.05$, main effect of ketotifen treatment $F_{(1,17)} = 18.415$, $P < 0.001$, Interaction of model and treatment, $F_{(1,17)} = 4.76$, $P < 0.05$).
Unlike vagotomy, however, ketotifen administration did not reverse the enhanced $Crh$ expression in the CeA in FD rats. $Crh$ mRNA was significantly increased in the CeA of FD rats (the $\log_2{}^{\Delta\Delta Ct}$ in Control/Water 0 ± 0.079, Control/ketotifen -0.019 ± 0.072, FD/Water 0.22 ± 0.104, FD/ketotifen 0.21 ± 0.123). Two-way ANOVA revealed a significant difference between control and FD rats, but no effect on ketotifen (main effect of Model: $F(1,15) = 5.623$, $P < 0.05$, main effect of ketotifen treatment $F(1,15) = 0.028$, $P = 0.868$, interaction of model and treatment, $F(1,15) = 0.00129$, $P = 0.972$).

We also examined the effects of ketotifen on the pain behavior response to gastric distention (GD) using the visceral-motor reflex (VMR) response, measured by electromyography (EMG). As compared with control rats, FD rats showed a significant increase in the VMR response to GD, an effect that was attenuated by ketotifen at lower distention pressures, but not at the highest (80 mmHg) (Figure 4D, Two-Way ANOVA main effects of groups, $F(3,60) = 17.1$, $P < 0.001$, main effect of pressure $F(3,60) = 71.5$, $P < 0.001$, Interaction of groups and pressure, $F(9,60) = 3.6$, $P < 0.001$).

Together, these results indicate that activated gastric mast cells play at least a partial role in maintaining hyperactivity of the gastric vagus nerve afferents, affective behavior and hyperalgesia in this model of functional dyspepsia.

Discussion
In this study, we hypothesized that the primary manifestations of functional dyspepsia (FD), including both sensory and psychological disturbances, are mediated by aberrant signaling via the abdominal vagus to key regions in the CNS responsible for pain and behavioral responses. Our results (Figure 1) show a robust sensitization of the vagal response to gastric distention, even at pressures previously shown to be in the physiological range as evaluated previously (18, 23). Sub-diaphragmatic vagotomy reversed behavioral changes suggestive of depression and anxiety in rats with FD (Figure 1). It is important to acknowledge that this method does have
limitations, as do all vagal ablation procedures. The vagus nerve contains approximately 90% afferent and 10% efferent fibers (13), and total vagotomy severs both connections to abdominal viscera, with effects on motility as well. Though there are other approaches to selectively disturb vagal afferents, they do not seem to inactive all vagal afferents, which presents its own limitations(62). Regardless of the exact mechanisms, however, our results do establish the role of an intact vagus in mediating brain plasticity in the FD model.

We then examined the possible CNS mechanisms responsible for the effect. Ascending fibers in the vagus nerve relay signals to the nucleus tracts solitarius (NTS), which in turn connects to multiple regions of the brain, both directly and indirectly (24-26). Intragastric acid in high concentrations activates neurons in the NTS, lateral parabrachial nucleus, thalamic and hypothalamic paraventricular nucleus, supraoptic nucleus, central amygdala and medial/lateral habenula, an effect that is nearly abolished by bilateral vagotomy (27). Among these connections, the amygdala stands out as a key component of the limbic system that been implicated in the pathogenesis of anxiety, depression and other mood disorders. Acute noxious stimulation of the gastrointestinal tract has previously been reported to result in activation and/or plasticity in the amygdala (28-30). Transcriptomic changes in the amygdala have been described in germ free mice with altered behavioral and nocifensive responses. Functional imaging studies also suggest hyperactivity of the amygdala in humans with IBS,(31, 32), indicating a potentially important role of this center in the pathogenesis of chronic gastrointestinal hypersensitivity syndromes that are also associated with altered affective behavior.

We therefore hypothesized that the amygdala may mediate the effects of vagal hypersensitivity on affective behaviors in FD rats, via the production of neuroactive factors. Since we had previously shown that stress-induced circulating ACTH and corticosterone levels are increased in this model and that antalarmin, a CRH receptor antagonist, attenuated
depression-like behavior (7), we first focused on the CRH system. Our results show increased Crh and Crhr1 (but not Crhr2) gene expression in the CeA in FD rats (Figure 2), supporting a potential role for augmented CRH signaling in the pathogenesis of depression and anxiety in this model. Such a role for amygdaloid CRH is consistent with the results of multiple studies in the literature (33-36). We further linked upregulation of the CRH/CRHR1 pathway to vagal activity, as Crh expression was normalized after vagotomy, corresponding with normalization of the FD behavioral phenotype. Crhr1 expression was not significantly changed after vagotomy, suggesting that other, yet unknown, factors may be responsible for this effect.

We also examined the role of BDNF as its expression is decreased in the amygdala in models of anxiety while enhanced by anxiolytic interventions (37-39). Most antidepressant drugs also cause an increase in brain BDNF expression (40, 41). Our results show that, Bdnf gene expression was decreased in rats with FD and normalized after a vagotomy (Figure 2), consistent with its putative role in mood disorders.

Our data therefore indicates that vagal hypersensitivity in a disease model can result in modulation of CRH and BDNF in the amygdala, which to the best of our knowledge has not previously been described. However, studies on the effects of vagal nerve stimulation (VNS) provide indirect evidence in support of these results. VNS has been shown to induce plasticity in the amygdala (42), and upregulate CRH in the hypothalamus as well as increase plasma ACTH and corticosterone levels(43). In healthy mice, VNS also induces BDNF expression and enhances ligand-induced activation of the cognitive receptor for BDNF, TrkB (44, 45). On the other hand, subdiaphragmatic vagotomy decreases Bdnf mRNA in the hippocampus (46). Our results indicate a decrease in amygdaloid Bdnf gene expression after vagotomy in control rats. In FD rats, however, the effects of vagotomy are the opposite and suggest that in disease models, increased vagal activity has a pathophysiologic effect on brain plasticity that is different than what the vagus does in health, whether at baseline or in response to VNS.
Together, these findings are in keeping with the results of several other studies. For example, vagotomy consistently blocks depression-like behaviors induced by peripheral injection of LPS and IL-1beta-induced behavioral depression (12, 47, 48). In a more recent study, sub-diaphragmatic deafferentation of the vagus reduced anxiety-like behavior as measured by the elevated plus maze test, open field test, and food neophobia test (49). On the other hand, data from colonic models demonstrate somewhat conflicting results- with some studies showing that vagotomy blocked the therapeutic effect of probiotics on anxiety- and depression-related behavior in mice under conditions of stress (11, 12), but not in the setting of infectious colitis (50). Thus, it appears that the vagus can both augment and attenuate emotional responses to noxious gastrointestinal stimulation, perhaps depending on the gut region and/or activation of model-specific (e.g., disease versus normal or acute versus chronic) pathways. Our results therefore have implications not only for the pathogenesis of affective behavior in FD but also for possibly predicting the effects of VNS in this condition, which may not be extrapolated from the results obtained in non-disease models or healthy volunteers.

A secondary aim of this study was to examine the role of vagal activity in gastric nociceptive sensitization in FD. Classically, pain is initiated via activation of spinal afferent pathways and we have previously shown hyperactivity of these nerves in this model of FD (18, 51). It is less certain as to how vagal activity modulates nociception, if at all. An intact vagus and NTS are required for the hyperalgesic effect of pro-inflammatory cytokines such as IL-1 or TNF (52). Pharmacological or electrical stimulation of the vagus has been reported to result in both facilitation and inhibition of the nociceptive response and appears to involve central circuits, originating in the NTS and then via several relay nuclei, activating descending pathways to the spinal cord region to modulate incoming noxious stimuli via spinal afferents (53, 54). In our study, vagotomy did not affect the FOS response to gastric distention in control rats, which is similar to what has been reported earlier by other another group (55). However, vagotomy
significantly attenuated, although did not eliminate, enhanced dorsal horn FOS expression in response to gastric distention. Thus, while nociceptive sensitization is still present and maintained by gastric spinal afferents, the subdiaphragmatic vagus is an important contributor. This effect may be mediated by a central pathway as demonstrated by the reduced pain behavior in response to injection of lidocaine into the ventrolateral PAG. Together, our results support the hypothesis that activated vagal nerves in FD rats attenuates the central inhibitory pathway, resulting in increased sensitivity of the spinal pathway. In this regard, our findings on amygdaloid plasticity suggest that this region may also mediate such a role. The amygdala can receive pro-algesic vagal input such as that induced by cholecystokinin (CCK) (13), and in turn transmit this to centers of descending pain control, such as the periaqueductal grey (PAG) (56, 57). CRH signaling within the CeA has been particularly implicated in the pathogenesis of pathological pain in both somatic and visceral pain states (36, 58-61).

We then examined the peripheral factors that may be responsible for persistent vagal activity with a focus on gastric mast cells. These cells contain numerous potent effector molecules, which they can release in response to a variety of stimuli, and can exert profound effects on secretion, gut barrier integrity, enteric neuronal function and sensory nerve activity. This includes both spinal and vagal nerves; indeed, evidence exists of a bidirectional mast cell-vagus axis (63, 64). We found that mast cell number was increased in the stomach of FD rats and that pharmacological doses of the mast cell stabilizer ketotifen were effective in suppressing not only the vagal hypersensitivity to gastric distention but also significantly attenuating pain and affective behavior indicating a potentially key role for these cells in the pathogenesis of FD. This is consistent with our previous report, showing a negative association between mast cell numbers and sucrose intake (22). However, unlike vagotomy, ketotifen did not reverse the increased expression of Crh in the amygdala, which may reflect its partial attenuation of vagal hypersensitivity or other mechanisms not related to mast cell activity.
Although the exact mechanism for mast cell proliferation and activation in this model are yet to be fully understood, a variety of experimental models of early life stress suggest that this may be due a disruption of the normal maturing process of the mucosal immune system in neonates (65). Gastric CRH may also play a role in recruiting mast cells in our model of neonatal gastric irritation, as previously reported by us (22). These experimental studies have corroborative evidence in humans. An increase in mast cell density, sometimes along with eosinophils, has also been noted in antral as well as duodenal biopsies from children with FD, and correlates with symptoms, gastric myoelectrical activity and altered gastric emptying (15, 16). Gastric mucosal mast cells are also increased in adults with Helicobacter pylori-negative functional dyspepsia, particularly in post-infectious cases, where an increased release of histamine and 5-hydroxytryptamine from gastric mucosa has also been noted (66, 67). In a recent study of children with FD, parent report of anxiety and depression correlated significantly with antral mast cell density, but not with any other inflammatory cell type (e.g., eosinophils or T-cells) or the presence of esophagitis, gastritis, or duodenitis (68).

In conclusion, our study reveals novel biological mechanisms responsible for pain and affective behavior in a model of chronic gastric hypersensitivity/functional dyspepsia. Vagal activity driven in part by gastric mast cells, induces long lasting changes in CRF signaling in the amygdala that may be responsible for both enhanced pain, anxiety and depression (Figure 5). Together, these results support a role for “bottom-up” pathogenesis of both gastric pain and associated psychological co-morbidity, which offers a new paradigm for functional dyspepsia as well as novel therapeutic targets based on these aspects of the gut-brain axis.
Methods

Animals
Sprague-Dawley rat dams with their litters of pups (10-12 male pups/dam) at postnatal day 6 were purchased from Harlan Laboratories (Indianapolis, IN). All rats were housed in a temperature-, humidity-, and light-controlled room (12 h light/dark cycles). Food and water were available *ad libitum*. After weaning, rats were housed 2-3 per cage. In all experiments, animals were randomly assigned into the experimental groups.

Functional Dyspepsia (FD) Model
The FD model was generated as previously described.(18) Briefly, a weak acid solution, 0.1% iodoacetamide (IA) in 2% sucrose (0.2 ml), was administered to rat pups (beginning on postnatal day 10-12) by oral gavage once a day for 6 days. Control pups received oral gavage of 2% sucrose (0.2 ml). The rats were then weaned at 3 weeks of age and housed under standard conditions until 8 –12 weeks of age when used for the studies.

Subdiaphragmatic vagotomy
Adult male control and FD rats (12 weeks old) underwent surgery for bilateral subdiaphragmatic vagotomy as previously described (69, 70). Briefly, after a midline abdominal incision, the esophagus with the associated sub- diaphragmatic vagal trunks and their major branches were exposed. The vagal trunks were cut proximal to the bifurcations of the hepatic and celiac branches. Sham surgery was performed in a similar procedure, but the vagus was left intact. Since vagotomy reduces stomach motility, rats were fed liquid food for three days before and for one week following the surgery. Behavior assessment was begun 2 weeks after recovery from vagotomy. Depression- and anxiety-like behaviors in FD rats were examined by open field test and forced swim test (see below). Each test was conducted in a day with at least one day
interval between tests. In a separate study, the brains of FD and control rats were collected 3 weeks after vagotomy and were immediately frozen until used for gene expression (See below).

**Spinal FOS response to gastric distention**

A balloon was inserted into stomach of FD rats during the vagotomy or sham surgery as described above. Two weeks after the surgery, rats were fasted overnight. On the next day, gastric distention with 80 mmHg pressure was conducted for 20 second. Twenty minutes later, the rats underwent cardiac perfusion with 0.1M PBS followed by 300 ml of 4% paraformaldehyde in 0.1M PBS. The T8-T10 spinal cord segments were collected and post-fixed with 4% paraformaldehyde overnight and incubated in 30% sucrose solution at 4°C until they sank to the bottom of the vials. Coronal sections (14 µm) of the spinal cord were then used for FOS staining.

**Vagal nerve single unit recording**

Vagal nerve single unit recording was conducted as previously reported by this laboratory and others with modifications.(18)(71, 72) After separating the vagal nerve from the carotid artery and sympathetic nerve and transecting the vagus below the nodose ganglion, the nerve was teased into fine bundles and spilt further to obtain a single-unit recording. Nerve activity was recorded by draping the fiber over one arm of the bipolar silver electrode while an equally thin connective tissue was placed on the other arm of the electrode to allow differential recordings. Single units that innervate to stomach were identified by consistent spick rate in response to gastric distention. Signals were amplified 1000X with Iso-DAM8A Bio-amplifier (WPI), filtered with 300Hz to 3 KHz, and monitored with TDS 2012 digital oscilloscope (Tektronix). Then the wave-mark template in SPIK 2 computer software program (Cambridge Electronic Design, UK) was used to record and analyze nerve activity. Gastric distention (GD) with graded pressure (20, 40, 60 and 80 mmHg) was applied in an ascending graded manner by
rapidly inflating a balloon implanted in the stomach for a duration of 30 s using a pressure transducer and sphygmanometer, with at least 2 min interval between stimuli.

**Stereotaxic injection of lidocaine into ventrolateral periaqueductual gray (PAG)**

A cannula was implanted into the periaqueductal gray (PAG) using stereotaxic coordinates Bregma AP -7.8, L 0.5 and DV -4.5 mm in 8-week-old FD and control rats (73), while a balloon was implanted for distention and subsequent testing for pain behavior using the abdominal withdrawal reflex (AWR). One week later, after a baseline test (0 min) of the AWR response to GD (40 and 80 mmHg), 0.5 µL of 2% lidocaine or normal saline was infused into PAG (Bregma AP -7.8, L 0.5 and DV -5.5 mm) in 80 sec and the needle was left for additional 40 sec. The AWR response to GD (40 or 80 mmHg, 20 sec) was measured at 10 min after the infusion by behavioral scores as previously described (18). Behavioral responses were graded as: 0, no behavioral response to GD; 1, brief head movement followed by immobility; 2, contraction of abdominal muscles; 3, lifting of abdomen; 4, body arching, lifting of pelvic structures, and stretching of body. The highest score of behaviors in the 20 sec of GD period was counted. Behaviors were videotaped and analyzed by a blinded observer.

**Effect of ketotifen on vagal nerve activity and behaviors of IA-treated rats**

Ketotifen, a mast cell stabilizer, was purchased from Santa Cruz Biotechnology Inc (sc-201094, Santa Cruz, CA). Adult rats that had received neonatal IA or vehicle were treated with ketotifen through drinking water (0.1 mg/ml) for 7 days. The average intake of drinking water by two groups of rats was 52-62 ml. There was no significant difference in the amount of drinking water consumed between control and ketotifen treated rats. On the day after the last treatment, all rats underwent vagal afferent recording in response to gastric distension as described above. In a separate study, rats were treated with ketotifen for 10 days prior to behavioral and gastric nociceptive sensitivity tests using VMR response to GD measured by EMG as we previously
reported (18) (see below). Treatment with ketotifen in drinking water was continued during testing.

**Behavioral tests**

All behavioral tests are conducted during the light cycle, between 1000 and 1700h. Rats were tested in a randomized order.

*Open Field*

The open field (OF) consists of an arena (60 cm x 60 cm x 40 cm) with a circular inner zone that has a 30 cm diameter. Each rat was gently placed into the center of the field and allowed to explore the arena for 10 minutes. Behavior was scored by a blinded observer for time spent in immobile and rearing.

*Forced Swim Test*

The forced swim test (FST) was conducted using a clear Plexiglas cylinder (65 cm tall X 25 cm diameter) that was filled to 48 cm with 23°C water. On the first day, each rat was placed in the swim chamber and allowed to habituate for 10 minutes. The water was changed, and the swim chamber cleaned after each rat. 24 hours later, each rat was placed in the swim chamber for 4 minutes. Behavior on the second day was scored by a blinded observer for latency to immobility and total immobility time.

*Visceromotor reflex*

Visceromotor reflex (VMR) response to gastric distention (GD) was used to assess hyperalgesia in FD rats as we previously described.(18) Briefly, a balloon was inserted into the stomach and a pair of electrodes were implanted into the acromiotrapezius muscle one week before the test. The VMR responses to GD were measured by electromyography (EMG). After basal EMG activity was recorded for 20 s, intragastric pressures (20, 40, 60 and 80 mmHg) were applied by
a gastric balloon for 20 s and EMG were recorded. EMG activity was calculated as the area under the curve by SPIKE2 program. The data were expressed as increase of GD response to baseline.

**Tissue collection and molecular studies**

**Brain dissection**

Animals were euthanized during the light cycle between 1000 and 1400h. After rapid decapitation, rat brains were flash frozen in dry ice-cold 2-methybutane and then placed in powdered dry-ice for at least 10 min. The brains were stored at -80°C until use. To dissect the brain regions, 300µm coronal brain slices were sectioned at the level of amygdala. Central amygdala (CeA, Bregma -1.92mm to -2.76 mm) were dissected (73). The tissue was stored at -80°C until use for qPCR.

**RT-qPCR**

Total RNA was extracted from the central nucleus of the amygdala (CeA) using RNeasy plus micro kits (Qiagen) and 100ng RNA was used to make cDNA using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA). Taqman assays for specific genes of interest were used to examine the expression of the genes for corticotrophin releasing factor (*Crh*, Taqman assay ID: Rn01462137_m1), CRH receptor 1 (*Crhr1*, Taqman assay ID: Rn00578611_m1), CRH receptor 2 (*Crhr2*, Taqman assay ID: Rn00575617_m1) and brain-derived neural factor (*Bdnf*, Taqman assay ID: Rn02531967_s1) (Thermo Fisher Scientific, Waltham, MA). The qPCR was normalized by house-keeping gene, Hprt1 (Taqman assay ID: Rn01527840_m1). qPCR was performed for each sample in triplicate using TaqPath™ qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA) following manufacturer's suggested protocol. To calculate the gene expression, a threshold was set in the linear range of amplification plot in the Rotor-gene Q software. The mean of threshold cycle number (CT) from
the triplicate of target gene was subtracted to that of Hprt1 (ΔCT). The mean of ΔCT from control/sham group, as reference, was subtracted by each ΔCT of target genes, resulting in ΔΔCT. The gene expression was presented as Log2^(-ΔΔCT) relative to control/sham group.

**Immunohistochemistry for Tryptase and FOS**

For tryptase staining in the stomach, paraffin-embedded tissue sections were used. After de-paraffinizing, sections were washed with PBS 5 min for 3 times followed by blocking with 10% normal goat serum (NGS), 5% BSA in PBS in 1 hour. The sections were then incubated with mouse-anti mast cell tryptase (1:300, #NBP2-26444, Novus Biologicals, Centennial CO) and Rabbit-anti-PGP9.5 (1:400 #Z5116 DAKO/Aligent, Santa Clara, CA) in PBS containing 5% normal goat serum, 1 % BSA and 0.1% Triton X-100, overnight. After 3 washes with PBS, sections were incubated with Goat-anti-mouse IgG-conjugated with Alexa 488 and Goat-anti-rabbit IgG conjugated with Alexa 594 (1:500, #A-11001 and #A-11012, Thermo Fisher Scientific Inc, Waltham, MA) for 2 hours. After wash, the slides were mounted with ProLong™ Gold Antifade Mountant solution (#P36931, Thermo Fisher Scientific Inc, Waltham, MA).

For FOS staining, the spinal cord (Thoracic 8-10) was collected and sectioned to 14µm slices that were mounted on slides. The immuno-staining procedure was similar as that for tryptase, except goat-anti-FOS (sc 271243, Santa Cruz Biotechnology Inc, Dallas, TX) at 1:100 dilution was used.

The images were captured by a fluorescent microscope with 20x lens. The images were analyzed using ImageJ software. Four to six sections from each animal and 4-5 rats in each group were analyzed.

**Statistical Analysis**

Data were analyzed by two-way analysis of variance (ANOVA) or Student’s t-test using SigmaPlot (Systat software Inc. San Jose, CA), unless otherwise noted. If a significant
difference was detected, a Student-Newman-Keuls post-hoc test was used to evaluate differences between individual groups. Data are expressed as mean ± standard error of the mean (SEM) of the group (n=6-8, unless otherwise noted). For all tests, p<0.05 was considered significant.

**Study Approval**

All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University.

**Author contributions (CRediT taxonomy):**

- Conceptualization: PJP, THM, AB
- Formal Analysis: QL
- Funding acquisition: PJP, AB
- Investigation: ZC, LL, KT, QL
- Methodology: TH, QL, KT, PJP
- Project administration: PJP, QL
- Resources: PJP, THM
- Supervision: PJP, THM
- Writing – original draft: PJP, QL
- Writing – review & editing: All

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Figure 1. Vagal nerve hypersensitivity to gastric distention and relationship to affect in FD. (A) Vagal nerve activity is increased in adult FD rats induced by neonatal intragastric iodoacetamide (IA) as compared with those exposed to vehicle alone (control). The left panel shows an example of single vagal nerve activity in response to gastric distention (GD) (20-40 sec.). The top trace represents the firing frequency (spikes/s) calculated from nerve action potentials as shown in bottom trace; the right panel shows summated data of vagal nerve activity in response to GD in control and IA rats. Data are expressed as mean ± SEM (n=30-42 fibers from 5-8 rats). Data were analyzed using two-way ANOVA followed by post-hoc testing (*significantly different from controls at same pressure, P < 0.05 by Student-Newman-Keuls post-hoc test). (B and C) Vagotomy reversed depression- and anxiety-like behaviors assessed by forced swim test (B) and open field test (C), respectively. The dot plots represent individual values (circles and triangles) and means (diamonds) ± SEM (bars) (n= 8 rats). Data were analyzed using two-way ANOVA followed by post-hoc testing (*significantly different between two groups; P < 0.05 by Student-Newman-Keuls post-hoc test).
Figure 2. Effects of vagotomy on amygdaloid gene expression in adult FD rats. Expressions of *Cshr* (A), *Crhr1* (B) and *Crhr2* (C) receptors and BDNF (D) in the central amygdala of control and FD rats were measured by RT-qPCR. The dot plots represent individual values (circles and triangles) with means (diamonds) ± SEM (bars) (n= 5-8 rats). Data were analyzed using two-way ANOVA followed by post-hoc testing (*significantly different between two groups; P < 0.05 by Student-Newman-Keuls post-hoc test).
Figure 3. Contribution of central pathways and vagal activity to nociceptive sensitization in adult FD rats induced by neonatal intragastric iodoacetamide (IA) as compared with those exposed to vehicle alone (control). (A) Pain behavior in response to GD (40 or 80 mmHg) was measured 10 minutes after lidocaine infusion into the periaqueductal gray (PAG). Increased pain behavioral responses to gastric distention (GD) in FD rats can be attenuated by lidocaine infusion at 80, but not 40 mmHg of pressure. Data were analyzed using two-way ANOVA followed by post-hoc testing. The dot plots represent individual values (circles and triangles) with means (diamonds) ± SEM (bars) (n= 5-8 rats) (*significantly different between two groups; P < 0.05 by Student-Newman-Keuls post-hoc test). (B) Vagotomy attenuates increased spinal responsiveness to gastric distention (GD) as measured by FOS expression in the dorsal horn of the spinal cord (T8-10). The top panel shows an example of FOS staining in the layer I and II of the dorsal horn of spinal cord (Scale bar = 50 µm). The bottom panel shows summated data of FOS positive cells/field from one side of the dorsal horn. Data are expressed as means ± SEM (n= 39-44 field from 4-5 rats / group). Data were analyzed using two-way ANOVA followed by post-hoc testing. The dot plots represent individual values (circles and triangles) with means (diamonds) ± SEM (bars) (n= 39-44 field from 4-5 rats / group) (*significantly different between two groups; P < 0.05 by Student-Newman-Keuls post-hoc test).
Figure 4: Role of mast cells in vagal hypersensitivity, affect and pain behavior. 
(A) Left: Gastric mucosa and submucosa stained for mast cell tryptase (green, arrow) and nerves (PGP 9.5, red). Scale bar = 15 µm. Right: Summated data; individual values (circles and triangles) with means (diamonds) ± SEM (bars) (n= 40-42 field from 4-5 rats/ group).
(*significantly different between two groups; P < 0.05 by Student t-test). (B) Left: An example of vagal nerve activity in response of gastric distention (GD) (20-40 sec). Right: Summated data on vagal nerve activity expressed as a percentage of the baseline (10-20 sec) (means ± SEM; n= 24-30 nerves from 5-6 rats /group) and analyzed by two-way ANOVA and post-hoc testing (*significantly different from control/vehicle group at the indicated GD pressure; $significantly different from IA/vehicle group; #significantly different between IA/vehicle and IA/ketotifen; &: significantly different between IA/vehicle and control/vehicle; P < 0.05 by Student-Newman-Keuls post-hoc test). (C) Effects of ketotifen on FD-induced changes on affective behavior, as assessed by open field test (left) and forced swim test (right). Individual values (circles and triangles) with means (diamonds) ± SEM (bars) (n= 7-8 rats/group). Data were analyzed using two-way ANOVA followed by post-hoc testing (*significantly different between two groups; P < 0.05 by Student-Newman-Keuls post-hoc test). (D) Effect of ketotifen treatment on hyperalgesia in FD rats in response to gastric distention (GD). Left: An example of visceromotor responses (VMR) to GD. Right: Summated data (percentage of baseline, means ± SEM; n= 7-8 rats /group), analyzed by two-way ANOVA and post-hoc testing (*significantly different from control/vehicle group at the indicated GD pressure; #significantly different from IA/vehicle group; $significantly different between IA/vehicle and IA/ketotifen; &significantly different between IA/vehicle and control/vehicle; P < 0.05 by Student-Newman-Keuls post-hoc test).
Figure 5. A proposed paradigm for pain and affective behavior in functional dyspepsia. Based on our results, we propose that mast cell hyperplasia and activation results in sensitization of vagal sensory nerves. The latter then causes changes in the amygdaloid region of the brain with associated changes in key neurotransmitters such as CRH (increased) and BDNF (decreased). Further, this is associated with suppression of descending inhibitory pathways that augment nociceptive signals conveyed by gastric spinal sensory nerves. Together, these mechanisms can contribute to pain and discomfort as well as the psychological morbidities. (CRH = corticotrophin releasing hormone; BDNF = Brain derived neurotrophic factor; NG = nodose ganglion; PAG = periaqueductal gray)