Enhanced sympathetic nerve activity induced by neonatal colon inflammation induces gastric hypersensitivity and anxiety-like behavior in adult rats

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ABSTRACT

Gastric hypersensitivity (GHS) and anxiety are prevalent in Functional Dyspepsia patients; their underlying mechanisms remain unknown largely because of lack of availability of live visceral tissues from human subjects. Recently, we demonstrated in a preclinical model that rats subjected to neonatal colon inflammation show increased basal plasma norepinephrine, which contributes to GHS through the up-regulation of nerve growth factor (NGF) expression in the gastric fundus. We tested the hypothesis that neonatal colon inflammation increases anxiety-like behavior and sympathetic nervous system (SNS) activity, which upregulates the expression of NGF to induce GHS in adult-life. Chemical sympathectomy, but not adrenalectomy, suppressed the elevated NGF expression in the fundus muscularis externa and gastric hypersensitivity. The measurement of heart rate variability showed a significant increase in the LF/HF ratio in GHS vs. the control rats. Stimulus-evoked release of NE from the fundus muscularis externa strips was significantly greater in GHS than in the control rats. Tyrosine hydroxylase expression was increased in the celiac ganglia of the GHS vs. the control rats. We found an increase in trait but not stress-induced anxiety-like behavior in GHS-rats in elevated plus maze. We concluded that neonatal programming triggered by colon inflammation upregulates tyrosine hydroxylase in the celiac ganglia, which upregulates the release of NE in the gastric fundus muscularis externa. The increase of NE release from the sympathetic nerve terminals concentration-dependently upregulates NGF, which proportionately increases the visceromotor response to gastric distention. Neonatal programming concurrently increases anxiety-like behavior in GHS-rats.
Functional dyspepsia (FD) is a complex, heterogeneous, biopsychosocial functional gastrointestinal disorder that is comprised of a cluster of symptoms, including postprandial epigastric pain/discomfort, impaired gastric emptying and early satiety in the absence of any apparent structural, biochemical or organic abnormality (14, 45, 49). Experimental clinical studies found that gastric sensitivity to distention is increased in a subset of FD patients (3, 9, 11, 12, 25, 34, 39, 44) and it contributes to the sensory symptoms, such as postprandial pain/discomfort, fullness and early satiety. The cellular mechanisms of gastric hypersensitivity (GHS) in FD patients remain incompletely understood, primarily because of lack of availability of live visceral tissues from human subjects. In addition, ethical and safety considerations preclude the use of interventional experiments, such as induction of inflammation and severe chronic stress, in human subjects. However, epidemiological studies found that adverse early-life experiences (AELE), including gastrointestinal infections, abuse and psychological stress, are risk factors for the development of FD (2, 16, 24, 33, 41, 42).

Using clues from the epidemiological and experimental clinical findings, preclinical models of gastric hypersensitivity show that either gastric (29, 30) or colon inflammation (50) during neonatal development induces gastric hypersensitivity in adult-life. We called these “gastric hypertensive rats (GHS-rats)” (50). Cellular and neurohormonal findings revealed that neonatal colonic inflammation elevates basal plasma norepinephrine (NE), which makes a significant contribution to the induction of GHS; the blockade of \( \alpha_1/\alpha_2 \), and \( \beta_1/\beta_2 \) adrenergic receptors suppressed the visceromotor response (VMR) to gastric distention in GHS-rats (50). The increase in plasma NE induced GHS by upregulating the expression of nerve growth factor (NGF) in the muscularis externa of the gastric fundus, which increased the expression of brain-derived growth factor (BDNF) in the thoracic dorsal root ganglia (DRG) and spinal cord. The neutralization of NGF by its antibody suppressed the elevation of BDNF and GHS (50). There is evidence for increased sympathetic activity at baseline and in response to stress or a meal in FD patients (10). However, the source of increase in sympathetic activity in FD patients remains unknown.
Several, but not all, epidemiological studies found a greater prevalence of anxiety in a subset of FD patients than in healthy controls (48). However, the correlation between anxiety and the symptoms of FD has remained contentious. We tested the hypothesis that neonatal programming triggered by neonatal colon inflammation elevates the expression of tyrosine hydroxylase in the celiac ganglia to increase sympathetic nervous system (SNS) activity in the gastric fundus as well as anxiety-like behavior.
METHODS

Animals: Litters consisting of 10 five days old male Sprague Dawley pups with a nurturing, primiparous mother were delivered from Harlan, Houston, TX. The litters were a combination of the mother’s own pups and fostered pups. The pups were weaned at 22 days of age and were housed 3-4 per cage until they weighed 250 gm each at which time they were housed 2 per cage. The IACUC at UTMB approved all procedures performed on these animals.

Neonatal colonic inflammation: The rat pups received 0.2 ml of 130 mg/kg trinitrobenzenesulfonic (TNBS) acid in 10% ethanol in saline through a catheter inserted 2 cm into the distal colon on postnatal day (PND) 10 without anesthesia. The rats were placed on their backs with their four legs restrained by one hand of the investigator for about 2 minutes during the administration of TNBS. The control pups received saline only in a similar manner. Six-to-eight weeks later, when these rats were adults, they were evaluated for gastric hypersensitivity and anxiety-like behavior and tissues were collected for molecular experiments. Previous publications show that there is no residual colon or gastric inflammation six to eight weeks after the induction of neonatal colon inflammation (7, 50). We validated previously that the delayed gastric hypersensitivity in adult rats results when they were exposed to colon inflammation for the first time as neonates, but not when they were exposed to first time colon inflammation as adults (50). One group of pups was treated daily with 16 ug/kg s.c. GR antagonist RU486 from PND 9 to 17 or vehicle.

Implantation of gastric balloon and evaluation of gastric sensitivity to gastric distention:

The following procedures were performed under isoflurane anesthesia. Gastric balloon implantation: A two cm long balloon was prepared from a condom and attached to PE240 tubing. A 2 cm incision was made below the chest cavity to the right of the abdominal midline. The stomach was exteriorized and wrapped in gauze wetted with saline. The balloon was inserted through a small incision at the tip of the fundus; it was secured to the fundus wall with suture; the opening was closed.
left the lower esophageal sphincter and pylorus unobstructed. The stomach was returned to the abdominal cavity and the muscle wall was closed with suture. The tubing tethering the balloon was pushed under the skin and externalized at the nape of the neck. The skin was closed. Electrodes implantation to record electromyographic (EMG) activity: A small incision was made at the nape of the neck to expose the acromeotrapezious muscle. Bipolar electrodes were sutured to the acromeotrapezious muscle and the skin closed.

The rats were allowed to recover in their home cage. Each rat received twice daily s.c. injection of buprenorphine (0.03mg/kg) for up to three or four days. Health was monitored by ensuring that the rats were maintaining or gaining weight and absence of lack of activity, such as lethargy and grooming. Seven-to-ten days later, gastric sensitivity to fundus distention was measured. The rats received a series of 20-second gastric balloon distention of increasing intensity: 30, 40, 50, 60, 80, 100, and 120 mmHg using a sphygmomanometer with a 2 min interval between successive distentions. The rats were awake and freely moving during gastric balloon dissentions. The pressures were applied in duplicate. A Biopac (Biopac, Goleta, CA) amplifier (sample rate 2000 per second; high-pass cut-off 1Hz, low-pass cut-off 500 Hz) was used to record EMG activity. The traces were visualized and analyzed using Acknowledge (Biopac, Goleta, CA). EMG was rectified and the area under the curve (AUC) calculated for each 20-second distention period. Baseline activity, 20 seconds before distention, was subtracted from the EMG induced by distention. Data were displayed as EMG activity in volts x seconds (VxS) as a function of distention pressure. The area under the pressure-EMG curve for each rat was calculated and significance was assessed with unpaired t-test.

Plasma norepinephrine (NE) levels: Blood was drawn from the saphenous vein under isoflurane anesthesia into tubes containing EDTA and spun at 10,000 x g for 10 min at 4°C. NE was measured by ELISA purchased from Rocky Mountain Diagnostics, Colorado Springs, CO.
Adrenalectomy and 6-hydroxy dopamine (6-OHDA) treatment: Gastric sensitivity was measured in 24 GHS-rats, which were then randomly assigned to one of three experimental groups (n=8 each): Adrenalectomy (ADx), sham surgery and 6-OHDA. A 1.5 to 2 cm dorsal incision was made over the center line behind the rib cage. One cm incisions were made in the muscle layers to the left and the right of the rib cage. The adrenal glands were externalized from the abdominal cavity and a ligature was placed below each adrenal gland. The adrenal glands were excised. Sham surgery consisted of making the incisions without removing the adrenal glands. ADx rats were given 0.9% saline in place of drinking water. 6-OHDA (150 mg/kg in 0.1% ascorbic acid) was administered i.p. once per day for 3 consecutive days. The rats were allowed to recover for 10 days; gastric sensitivity to fundus distention was measured again. Fundus muscularis externa tissue was saved for the measurement of nerve growth factor (NGF). Figure 1 illustrates the time-line of the experiments.

Heart rate variability (HRV): Twelve GHS and twelve control rats were tested. A pair of electrodes was attached to the muscle layer under the skin overlying the chest and was externalized behind the nape of the neck. ECG activity was recorded for 30 min using a Biopac Systems data acquisition unit MP100A-CE, EG100C, universal interface module UIM100C, high frequency (HF) and low frequency (LF) components; the LF/HF ratio was calculated with Acknowledge software. The RR intervals were extracted from the ECG signal (modified Pan-Tompkins QRS detector). Frequency information was extracted from the RR intervals. A Welch periodogram was used to generate the Power Spectral Density (in units of sec²). The efferent vagal activity is a major contributor to the high frequency component and the low frequency component is considered a marker of sympathetic modulation. Data were expressed as the LF/HF ratio which mirrors sympathetic-vagal balance.

Elevated plus maze (EPM) test for anxiety-like behavior: A total of 36 rats in 4 groups (Ctr., Ctr.+stress, GHS and GHS+stress) were tested. The rats were stressed by the application of a heterotypic intermittent chronic stress (HeICS) protocol that consisted of a random sequence of three
types of stressors, one hour water avoidance stress (WAS), 45 min cold restraint stress (CRS) or 20 min forced swim stress (FSS) for nine days (8, 51). One stressor was applied in the morning (9-11 AM) and the other in the afternoon (3-5 PM) in a random order, as described previously (51). The EPM tests were performed 1 day after the last stressor. Anxiety-like behavior was assessed on an Elevated plus maze (San Diego Instruments) consisting of two open arms (38 cm X 5 cm) and two closed arms (38 cm X 5 cm X 15 cm) with a central open intersection (5 cm X 5 cm) elevated 75 cm above the ground. Each rat was tested for 5 minutes. At the beginning of the test, each rat was placed individually into the central platform facing an open arm. The rat's movement through the maze was followed with a video camera and data were analyzed by AnyMaze flexible video tracking software (Stoelting, Wood Dale, IL). The time spent in the open and closed arms, the number of entries into open and closed arms and the total distance traveled were measured.

**Cold stress-induced defecation and social interaction:** 12 Ctr. and 12 GHS rats were tested. In cold stress defecation, the rats were placed in individual novel polycarbonate cages on ice, and the numbers of fecal pellets counted over a 20 min period (19). For the social interaction test (13, 19), the test rat’s behavior in its home cage with a novel rat of similar size was recorded for 10 minutes. The total time spent in active interaction, defined as sniffing, close following, and allo-grooming was recorded. The observer was blinded to treatment when monitoring behaviors.

**RT-PCR/Western blot:** Celiac ganglia and fundus muscularis externa tissue were removed and frozen in liquid nitrogen. RNA was prepared from the frozen tissue with a MicroRNeasy kit (Qiagen, Valencia, CA). RT was performed using a Superscript III kit (Invitrogen). Quantitative PCR was performed on an Applied Biosystems Step One Plus Real-time PCR. TAQMAN primer/probe sets for each gene were purchased from Applied Biosystems. Western blots were performed as described (50).
**Norepinephrine release:** Freshly dissected fundus muscularis externa tissue strips were incubated in oxygenated Krebs, (in mmol/l) 126 NaCl, 5 KCl, 2.5 NaH2PO4, 1.2 MgCl2, 2.5 CaCl2, 11 glucose, 25 NaHCO3, bubbled with 95% O2-5% CO2 + 30 µM pargyline, a monoamine oxidase inhibitor + 50 µCi $^{3}$Hnorepinephrine (0.2 µmol/l, 42 Ci/mmol, Amersham, Piscataway, NJ) for 1 hr. at 37°C. The strips were washed three times and transferred either to Krebs to measure basal release or to Krebs with 70 mM KCl for stimulus-evoked release. The buffer was changed every 5 min over a period of 15 min. The buffer containing the released NE contained 30 µM pargyline and a norepinephrine transport inhibitor, 20 µM maprotiline. At the conclusion of NE release measurements, the tissues were blotted dry, weighed, and solubilized with 1 ml Soluene-350 tissue solubilizer (Perkin-Elmer, Waltham, MA) and neutralized with 50 µl glacial acetic acid. The solubilized tissue was then added to 3 ml scintillation fluid and the samples were analyzed to determine the total residual tritium content in each tissue sample. Counts remaining in the tissue + counts released = total counts, which were normalized to tissue wet tissue weight. The NE release was expressed as a percentage of total uptake.

**Statistics:** Data were expressed as mean ± SEM. Paired or unpaired t-test, one or two-way ANOVA were used where appropriate. Tukey test was used to compare individual means where a significant main effect was obtained by ANOVA; p<0.05 was considered significant.
RESULTS

The source of NE to upregulate gastric fundus NGF and its correlation with VHS

We reported previously that the basal plasma level of NE is significantly elevated in GHS-rats and it underlies the upregulation of NGF in the fundus muscularis externa tissue, which contributes to the enhancement of sensitivity to gastric fundus distention (50). Here, we investigated the predominant source of norepinephrine to upregulate NGF and hence gastric hypersensitivity, i.e. sympathetic neuronal overflow or adrenal gland. We performed chemical sympathectomy with 6-OH-dopamine or bilateral adrenalectomy in separate groups of GHS-rats (Fig. 1). 6-OH-dopamine treatment significantly suppressed the gastric sensitivity to fundus distention in GHS-rats (Fig. 2A, p<0.05, n=6). By contrast, adrenalectomy or sham surgery had no significant effect on the sensitivity to fundus distention (Fig. 2B and C, p>0.05, n=6 each). 6-hydroxy-dopamine treatment also suppressed NGF expression in the fundus muscularis externa tissue vs. the baseline values in GHS-rats, but adrenalectomy had no significant effect (Fig. 2D). To evaluate the effects of 6-OHDA treatment on fundus sympathetic innervation, we measured TH expression by western blot. 6-hydroxy- dopamine treatment nearly ablated tyrosine hydroxylase (TH) expression in the gastric fundus, but adrenalectomy had no significant effect (Fig. 2E). We found greater than 90% decrease in TH expression in all 6-OHDA treated rats.

The measurement of heart rate variability is often used as a non-invasive test to evaluate the balance between the sympathetic and vagal tones. We investigated whether the increase of sympathetic tone in GHS-rats reflects changes in heart rate variability. The measurement of heart rate variability showed a significant increase in the LF/HF ratio in GHS vs. the control rats (Fig. 2F). This increase reflected both an increase in the low-frequency (LF) component (0.57±0.04 vs. 0.36±0.04, GHS vs. Ctr.) and a decrease in the high-frequency component (HF) (0.43±0.03 vs 0.64±0.05, GHS vs. Ctr.). These data were consistent with increased sympathetic tone and a decrease in vagal tone.
Functional dyspepsia is a heterogeneous disorder where the intensity of symptoms varies among patients. We investigated whether biological variables, such as plasma NE and NGF expression in the gastric fundus, may relate to the intensity of gastric sensitivity and therefore with the symptoms related to it, such as epigastric pain and discomfort. We reported previously that even though all neonate rats were subjected to the same TNBS inflammatory insult, only about half of them developed gastric hypersensitivity (50). Here, we investigated whether the level of NGF expression in the fundus correlates with the intensity of gastric hypersensitivity. We found that the intensity of visceromotor response (VMR) to gastric distention was linearly correlated with the expression of NGF in the fundus muscularis externa ($r^2=0.66; F=27.1, p=0.0001, n=5$ control and $n=11$ neonatal inflammation; Fig. 3A). Consistent with this finding, we found that the plasma NE levels in 14/26 adult rats (identified by dark shaded diamonds in Fig. 3B) subjected previously to neonatal colon inflammation were greater than two standard deviations above the mean of controls ($1.03\pm0.05, n=14$ responder rats vs. $0.64\pm0.04, n=12$ non-responder rats and $0.53\pm0.03, n=25$ control rats). We also found a wide scatter in the plasma concentration of norepinephrine among the GHS-rats subjected to the same inflammatory insult as neonates. The scatter and mean concentration of plasma NE were significantly greater in GHS than in vehicle treated control rats (Fig. 3B, $p<0.05$, rank sum test).

**Alterations in the release and uptake of NE from the fundus muscularis externa sympathetic nerve endings in GHS-rats**

We found a significantly greater basal and KCl-evoked release of NE from the fundus muscularis externa strips of GHS-rats than those from the control rats (Fig. 4A). By contrast, the uptake of NE from the fundus strips of the GHS and control rats did not differ (Fig. 4B). To investigate the molecular mechanisms of increase of fundus NE release in GHS-rats, we measured tyrosine hydroxylase (TH), the rate-limiting enzyme for the synthesis of NE, expression levels in the celiac ganglia, which supplies sympathetic innervation to the stomach. The $TH$ mRNA in the celiac ganglia was significantly greater (2.5-fold; $p<0.05$) in GHS than in control rats (Fig. 4C).
Neonatal colon inflammation upregulates *Th* gene transcription

We reported previously that colon inflammation on PND 10 prematurely upregulates the plasma corticosterone by PND 15, which triggers neonatal programming to induce GHS in adult-life (50). In control rats, the increase in plasma corticosterone occurs by PND 17 (50). We found that the blockade of glucocorticoid receptors by RU-486 in neonates prevented the induction of GHS in adult-life (50). Here, we investigated whether neonatal programming targets the *Th* gene to upregulate its transcription in GHS-rats (experimental protocol, Fig. 1B). Treatment of neonates with RU-486 significantly reduced the upregulation of *Th* mRNA in the GHS-rats; it had no effect in vehicle-treated control rats (Fig. 4C). Tyrosine hydroxylase protein was significantly increased 1.3 fold (*p*<0.05) in the celiac ganglia of GHS vs. control rats; this increase was significantly reduced in rats treated with RU-486 as neonates (Fig. 4D).

Increase in trait but not stress-induced anxiety-like behavior in GHS-rats

Since anxiety is often a co-morbid condition in FD patients, we used elevated plus maze, cold stress-induced defecation and social interaction tests to investigate whether GHS-rats displayed anxiety-like behavior. In the elevated plus maze test, we subjected the GHS and control rats to sham stress or a nine-day heterotypic chronic stress (HeICS) protocol (51) (Fig. 1C). Sham-stressed GHS-rats spent significantly less time in the open arm of the elevated plus maze compared to that by the control rats (*p*<0.05) (Fig. 5A). HeICS significantly reduced the time spent in the open arm by the control rats, but it was without additional significant effect on the time spent in the closed arm by the GHS-rats. Ctr.+HeICS rats, GHS-rats, and GHS+HeICS rats made significantly fewer open arm entries compared to the control rats (Fig. 5B). HeICS significantly reduced the number of closed arm entries by GHS rats (Fig. 5B), indicating an increased unwillingness to cross the open center space in the maze. There were no significant differences in the total distance traveled in the open and closed arms (Fig. 5C), suggesting that neonatal inflammatory insult did not alter locomotor activity in GHS-rats.
Cold stress significantly increased the number of fecal pellets in GHS than in control rats (Fig 5D).

Social interaction between GHS rats was significantly lower than that between control rats (Fig. 5E).
DISCUSSION

Several, but not all, clinical studies found an increase in the ratio of sympathetic tone to vagal tone measured by heart rate variability in FD patients (5, 20-22, 37). Our findings in a preclinical model show that neonatal programming triggered by colon inflammation increases the ratio of sympathetic to vagal tone in later-life. We show for the first time that neonatal programming triggered by colon inflammation increases activity of the sympathetic nerves innervating the gastric fundus. The upregulation of tyrosine hydroxylase in the celiac ganglia contributed to an increase in the activity of the sympathetic neurons innervating the gastric fundus. The increase in the expression of tyrosine hydroxylase was due to premature upregulation of plasma corticosterone by neonatal colon inflammation; RU-486 treatment of the neonates blocked the upregulation of tyrosine hydroxylase in the celiac ganglia. Therefore, it appears that neonatal programming triggered by colon inflammation concurrently upregulates the activity of the sympathetic neurons innervating the gastric fundus and the heart. It remains unknown whether neonatal programming triggered by colon inflammation alters the sympathetic activity of the neurons originating from the other sympathetic ganglia, such as the inferior mesenteric ganglia.

We reported previously that neonatal colon inflammation elevates plasma NE as well as the expression of NGF in the fundus muscularis externa, which underlies the induction of GHS (50). The blockade of α1, α2, β1 and β2 adrenergic receptors prevented the upregulation of NGF in the gastric fundus muscularis externa of the GHS-rats. Our present findings show that the upregulation of NGF and the induction of GHS were due to an increase in the release of NE from the sympathetic neurons innervating the fundus muscularis externa, rather than due to contribution of the adrenal medulla to the plasma NE. Chemical sympathectomy, but not adrenalectomy, blocked the upregulation of NGF and gastric hypersensitivity in GHS-rats.
In vitro experiments showed that the increased release of NE in the gastric fundus of the GHS-rats was due to its greater release from the nerve endings, rather than due to impaired uptake. The biological effects of catecholamines are concentration-dependent. The concentration of NE at the neuro-effector junctions and neural synapses is much greater than that in the plasma, which may be the reason that adrenalectomy, which partly decreases the plasma NE, did not affect the upregulation of NGF in the gastric fundus or the induction of GHS. An increase in the concentration of NGF is known to cause sprouting at the sympathetic nerve terminal (6, 23), which may set up a cycle to further enhance the release of NE in the fundus muscularis externa.

We found that even though the plasma NE increased on the average by about 30% in the entire group of GHS-rats, each one of which was subjected previously to the same neonatal inflammatory insults, there was a wide variation in the plasma NE concentration among the individual rats in the group. Our findings also show that the expression of NGF in the gastric fundus muscularis externa in the GHS-rats correlates with the VMR to gastric distention, We reported previously that NE concentration-dependently upregulates the expression of NGF in the fundus muscularis externa (50).

Functional dyspepsia is a heterogeneous disorder; the intensity of symptoms differs among patients and they show temporal variations in the same patient, probably due to environmental and psychosocial factors. Stress stimulates sympathetic neurons to release NE (8). A clinical study found that mental stress aggravates postprandial symptom severity in FD patients (10). In addition, a single subcutaneous dose of clonidine given just prior to a meal significantly suppressed the cumulative meal-related symptoms in FD patients with gastric hypersensitivity (43). Clonidine, a $\alpha_2$-adrenergic receptor agonist (31) inhibits the release of NE from the sympathetic neurons. These clinical findings together with our findings in a preclinical model of functional dyspepsia that plasma NE concentration was significantly elevated in rats with GHS suggest that above normal plasma NE levels may contributor to FD sensation related symptoms. However, the relationships between plasma NE levels
and gastric sensitivity and severity of FD symptoms in patients remain to be investigated. Of course in patients, the psychosocial factors may further modify the intensity of symptoms. Anxiety and depression are prevalent in subsets of patients with chronic diseases, including chronic obstructive pulmonary disease (35, 38), inflammatory bowel disease (18, 36, 40) and functional bowel disorders (1). Psychological stress induced by the morbidity and chronicity of these diseases/disorders, including FD, is an obvious factor in the induction of mood disorders. Our findings and those of others (29) show that adverse early life experiences may induce anxiety-like behavior independent of psychosocial factors. Other types of prenatal and neonatal stress also induce anxiety-like behaviors in rodents (15, 47). However, the underlying mechanisms of the development of anxiety-like behaviors remain unknown. In this study, we did not obtain direct evidence that the increase in sympathetic nerve activity contributes to the development of anxiety-like behavior in GHS-rats. However, experimental clinical evidence from patients with post-traumatic stress disorder (PTSD) suggests that an increase in the sympathetic nervous system activity contributes to anxiety. The SNS activity is chronically upregulated in PTSD patients (15, 26, 27, 32) resulting in elevated urine norepinephrine (32). In addition, the NE concentrations in the cerebrospinal fluid correlate with the severity of PTSD symptoms (17). The blockade of the stellate ganglion or the upper thoracic ganglion that innervate the brain or treatment with prazosin, a α-adrenergic receptor antagonist, reduced anxiety in PTSD patients (4, 28, 46). The role of elevated sympathetic neuronal activity in inducing anxiety in FD patients remains to be investigated.

In conclusion, our findings show that neonatal programming triggered by colon inflammation upregulates tyrosine hydroxylase expression in the celiac ganglia, which increases the release of NE in the fundus muscularis externa tissue. The increase of NE release at the sympathetic nerve terminals upregulates NGF in the muscularis externa, which proportionately increases the visceromotor response to gastric distention. In our preclinical model, both the magnitude of NGF
expression and plasma NE may serve as biological markers of the severity of gastric hypersensitivity.

This correlation remains to be investigated in FD patients. Concurrently, neonatal programming
induces anxiety-like behavior in gastric hypersensitive rats. The targets of neonatal programming in
the brain to induce anxiety-like behavior remain to be investigated.
FIGURE LEGENDS

**Fig. 1** Time line of experimental protocols. (A) TNBS was used to induce colonic inflammation on postnatal day (PND) 10. Six to eight weeks later, gastric sensitivity was measured and the GHS rats were assigned to one of three groups: 6-hydroxy-dopamine treatment, sham surgery and bilateral adrenalectomy (n=8 each). Ten days later gastric sensitivity was measured again in each group of rats. (B) TNBS inflammation was induced on PND10, as above (n=5). In addition, these rats were treated once a day with 16 mg/kg RU486 from PND 9 to PND 17 to block the glucocorticoid receptor. Experiments were performed six to eight weeks later. (C) Adult GHS rats subjected previously to TNBS neonatal colon inflammation on PND 10 and age-matched controls (Ctr., n=6, GHS, n=9 and GHS+RU486, n=10) were subjected to 9-day heterotypic intermittent chronic stress protocol; experiments were performed 24-hours after the end of the stress protocol.

**Fig. 2** Chemical sympathectomy suppressed gastric hypersensitivity (GHS) and nerve growth factor (NGF) expression in the gastric fundus. (A-D) A. Graphs showing the visceromotor response to gastric distention (electromyographic activity in volts x sec) as a function of distention pressure in GHS-rats, before and after 6-OH-dopamine treatment (*p<0.01); (B) after adrenalectomy (ADx) and (C) after sham surgery (each comparison in A, B and C used t-test, n=8 each group). (D) Panels and bar graph illustrating the results of Western blotting measuring NGF in the fundus muscularis externa normalized to β-actin. The significant increase in NGF in GHS-rats was normalized by 6-hydroxy-dopamine (6-OHDA) treatment, but not by ADx (*p<0.05, n=8; ANOVA). (E) Bar graph showing that 6-OHDA treatment almost completely ablated the expression of tyrosine hydroxylase (TH) in the gastric fundus muscularis externa, while ADx had no significant effect (*p<0.05, n=8; ANOVA). (F) Bar graph showing a significant increase in the ratio of sympathetic to vagal activity in GHS-rats compared to controls rats (*p<0.05, n=12 each; t-test).
**Fig. 3** Plots representing gastric funds NGF vs. sensitivity to gastric distention and individual plasma NE levels in control (Ctr.) and gastric hypersensitive (GHS) rats. (A) Gastric sensitivity, calculated as area under the distention pressure curve in volts-seconds x mmHg (EMG) was significantly correlated with NGF expression in the muscularis externa of the gastric fundus of the control and GHS rats ($r^2=0.66; F=27.1, p=0.0001, n=16$). (B) The mean values of NE were significantly different between the entire group of GHS and control rats ($p<0.05$, rank sum test, Ctr., $n=25$, GHS, $n=26$). However, the plasma levels of NE showed a wide variation in GHS rats. In 14 out of 26 GHS rats (shown by dark diamonds), each individual plasma level of NE was more than two standard deviations greater than the mean in control rats — responder rats. The mean value of plasma NE in non-responder rats (shown by lightly shaded diamonds) was not different from that in control rats ($n=25$).

**Fig. 4** Effects of neonatal colon inflammation on celiac tyrosine hydroxylase expression and gastric NE release. (A) Bar graph showing NE release in vitro in the presence of re-uptake and mono-oxygenase inhibitors from fundus muscularis externa tissue isolated from GHS and control (Ctr.) rats in Krebs or high KCl Krebs expressed as a percentage of the total tissue uptake ($n=5; \ast p>0.05$ GHS vs Ctr.; $\# p>0.05$ KCl vs Krebs; 2 way repeated measures ANOVA). (B) Bar graph showing no significant difference in NE uptake, expressed as cpm/100 mg tissue weight, by fundus muscularis externa strips from Ctr. and GHS rats ($p>0.05$, $n=5$, t-test). (C) Bar graph showing the levels of $Th$ mRNA expression normalized to (protein gene product) PGP9.5 in the celiac ganglia of adult control and GHS rats that were treated with RU486 during the neonatal period ($n=6; \ast p>0.05$ vs Ctr.+Veh.; $\# p>0.05$ vs Ctr.+RU486; 2 way ANOVA). (D) Western blot panel and bar graph showing tyrosine hydroxylase and β-III-tubulin expression in celiac ganglia extracts from Ctr. ($n=6$), GHS ($n=9$) and GHS+RU486 ($n=10$) rats ($\ast p>0.05$ vs Ctr.; $\# p>0.05$ vs GHS+Veh; ANOVA).

**Fig. 5** Gastric hypersensitive (GHS) rats exhibit anxiety-like behavior in response to HeICS on the elevated plus maze and cold stress relative to controls. (A) Bar graph showing the average times
spent on the closed and open arms of the elevated plus maze by control (Ctr.), Ctr.+HeICS, GHS, and GHS+HeICS rats. GHS rats spent significantly less time in the open arm of the elevated plus maze compared to controls. HEICS (heterotypic intermittent chronic stress) significantly reduced the open arm time of control rats but was without significant effect on the open arm time in GHS rats (*p<0.05; two-way ANOVA, n=12 each). (B) Bar graph showing the average numbers of entries into the open and closed arms. GHS, Ctr.+HeICS and GHS+HeICS rats made significantly fewer open arm entries compared to controls; (*p<0.05; two-way ANOVA, n=12 each). (C) Bar graph showing no significant differences in the average total distance traveled in meters (m) on the elevated plus maze (*p<0.05; two-way ANOVA, n=12 each). (D) Bar graphs showing a significant increase in the number of fecal pellets defecated in response to cold stress in GHS rats compared to controls (*p<0.05, n=12 each; t-test). (E) Bar graphs showing a significant decline in time spent in social interaction by GHS rats compared to controls (*p<0.05, n=12 each; t-test).
REFERENCES


Fig. 1
Fig. 2

A. 

B. 

C. 

D. 

E. 

F. 

Fold Change (NGF/β-actin)

Fold Change (TH/β-actin)

Fold Change (TH/β-actin)

HRV (LF/HF)
Fig. 3

A. B.

AUC (VxSxmmHg)

NGF (pg/mg)

Ctr. GHS

Norepinephrine (ng/ml)

*
A. Time spent (sec) for each condition in closed and open arms.

B. Number of entries into each arm for different conditions.

C. Distance traveled (m) for each condition.

D. Number of fecal pellets produced for control and GHS conditions.

E. Time spent (sec) for control and GHS conditions.

* indicates significant difference.