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Chronic stress induces hypersensitivity of murine gastric vagal afferents

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ABSTRACT

Background: Stress exposure is known to trigger and exacerbate functional dyspepsia (FD) symptoms. Increased gastric sensitivity to food related stimuli is widely observed in FD patients and is associated with stress and psychological disorders. The mechanisms underlying the hypersensitivity are not clear. Gastric vagal afferents (GVAs) play an important role in sensing meal related mechanical stimulation to modulate gastrointestinal function and food intake. This study aimed to determine whether GVAs display hypersensitivity after chronic stress, and whether its interaction with leptin was altered by stress.

Methods: Eight-week-old male C57BL/6 mice were exposed to unpredictable chronic mild stress or no stress (control) for 8 weeks. The metabolic rate, gastric emptying rate, and anxiety- and depression-like behaviors were determined. GVA mechanosensitivity, and its modulation by leptin, was determined using an *in vitro* single fibre recording technique. QRT-PCR was used to establish the levels of leptin and leptin receptor mRNA in the stomach and nodose ganglion respectively.

Key Results: The stressed mice had lower body weight and food intake, and increased anxiety-like behavior compared to the control mice. The mechanosensitivity of mucosal and tension sensitive GVAs was higher in the stressed mice. Leptin potentiated mucosal GVA mechanosensitivity in control but not stressed mice. The expression of leptin mRNA in the gastric mucosa was lower in the stressed mice.

Conclusions & Inferences: In conclusion, chronic stress enhances GVA mechanosensitivity, which may contribute to the gastric hypersensitivity in FD. In addition, the modulatory effect of leptin on GVA signaling is lost after chronic stress exposure.

KEYWORDS

Chronic stress, functional dyspepsia, gastric hypersensitivity, gastric vagal afferents, leptin

Key Points

- Stress exposure triggers and exacerbates functional dyspepsia, which is associated with enhanced gastric sensitivity to distension. The mechanisms for this hypersensitivity remain unclear.

- Chronic stress increases the sensitivity of gastric vagal afferents to food related mechanical stimuli, and alters the modulatory effect of leptin on gastric vagal afferents.
- Our findings suggest that chronic stress induces hypersensitivity of gastric vagal afferents, which may be responsible for the gastric hypersensitivity observed in functional dyspepsia.

1 INTRODUCTION

Functional dyspepsia (FD) is a common gastrointestinal disorder defined by chronic gastroduodenal symptoms in the absence of an identifiable organic cause.[1] It can be classified into two subtypes, postprandial distress syndrome, characterised by postprandial fullness and early satiation, and epigastric pain syndrome, characterised by gastric pain or burning.[2]

Population studies suggest an important role for stress in the aetiopathogenesis of FD.[3] The evidence includes the high prevalence of stressful life events exposure and psychiatric disorders in FD patients,[4,5] the association of psychological disorder with FD symptoms,[3,6,7] the onset or exacerbation of FD symptom caused by stressful events,[3] and the reduction of dyspepsia symptoms following psychological treatment.[8] In rodents, chronic stress has been shown to induce anxiety- and depression-like behaviors,[9,10] as well as gastrointestinal disorders, such as visceral hypersensitivity, and abnormal motility and immune response in the gastrointestinal tract.[11]

FD is associated with multiple gastroduodenal pathophysiological changes. Among them, gastric hypersensitivity has been considered to be a major mechanism.[12] Gastric hypersensitivity refers to an increased perception of gastric distension. It is observed in 34-66% of FD patients, and is associated with a higher prevalence of belching, postprandial pain, and weight loss.[12,13] In FD patients, gastric distension increases the activity of brain areas involved in the regulation of pain and feeding behaviour,[14] which indicates that gastric hypersensitivity may contribute to both pain and non-pain related FD symptoms. However, the origin and mechanisms of gastric hypersensitivity remains unclear.

Gastric vagal afferents (GVAs) are important sensory nerves innervating the stomach. They sense meal related stimuli and convey information to the brain to modulate gastric function, behavioral responses and sensations such as fullness, nausea and pain.[15] There are two types of mechanosensitive GVAs, mucosal receptors which respond to mucosal stroking, and tension receptors which respond to gastric distension.[16] Previous studies indicate that GVAs demonstrate a high degree of plasticity in response to different physiological states[17,18] and

can be malfunctional in pathological conditions. For example, in chronic high-fat diet-induced obesity there is a reduction in the GVAs mechanosensitivity, constituting a contributing mechanism for the increased food intake.[17] We hypothesize that GVAs may be responsible for the gastric hypersensitivity observed in FD, which can be induced by chronic stress.

Leptin, a hormone released from adipose tissue, as well as the stomach, is known for its role in the regulation of food intake.[19] Serum leptin levels are higher in dysmotility-like FD patients compared to healthy controls.[20] Leptin modulates GVA mechanosensitivity, an effect dependent on nutritional status.[21] However, it is unknown whether the effect of leptin on GVA signalling is altered by chronic stress exposure, contributing to gastric hypersensitivity.

In the current study, GVA mechanosensitivity was determined in mice exposed to unpredictable chronic mild stress, to ascertain whether GVAs display hypersensitivity after chronic stress exposure, and furthermore, whether the modulatory effect of leptin on GVA mechanosensitivity is also altered after chronic stress.

2 MATERIALS AND METHODS

2.1 Ethics

All experimental studies were approved by the animal ethics committee of the South Australia Health and Medical Research Institute.

2.2 Mice study design

Eight-week-old male C57BL/6 mice were used in this study. Female mice were excluded because the feeding behavior and gastric function of female mice can be affected by the oestrus cycle.[22,23] Mice were kept under a 12:12-h light dark cycle with lights on at 7 am. Mice had *ad libitum* access to water and a standard laboratory diet comprising 18%, 24% and 58% of energy from fat, protein and carbohydrate respectively (Teklad Rodent Diet 2018, ENVIGO, Wisconsin, USA). Prior to commencement of the stress protocol, the baseline measurements for metabolic rate, gastric emptying rate and behavior tests for anxiety- and depression-like behaviors were obtained. Mice were then randomly assigned to a control group (N = 10 mice) or a stress group (N = 18 mice). The stress group was then single housed and underwent unpredictable chronic mild stress protocol for 8 weeks. The control mice were pair housed with no stress treatment during this 8-week period, unless otherwise indicated. Body weight and sucrose preference were assessed weekly in both control and stress groups. After 8 weeks, metabolic rate, gastric emptying rate and anxiety-and depression-like

behaviors were re-determined. The stress protocol was maintained until the day of the electrophysiology experiment when the mice were anaesthetized with isoflurane, exsanguinated via the abdominal aorta, and decapitated between 7 - 9 am. Stomach (dorsal stomach) with attached nerves were dissected for single fibre recording. Plasma, nodose ganglia, and gastric mucosal scrapings (from ventral stomach) were collected and snap frozen in liquid nitrogen and stored at -80°C until further processing.

2.3 Metabolic rate monitoring

Mice were individually housed in metabolic monitoring cages (Promethion; Sable Systems International) to determine 24-hour food and water intake patterns, movement, and energy expenditure.

2.4 Breath test

Gastric emptying rate of a solid meal was determined using a breath test as previously described.[24,25] Briefly, after an overnight fast (4 pm -9 am), a baseline breath sample was taken after which the mice were given a baked egg yolk (0.1 g) containing 1 μg mL^{-1} of ^{13}C-labelled octanoic acid (99% enrichment, Cambridge Isotope Laboratories) to consume within 1 minute. Breath samples were collected at intervals between 5 -150 minutes and analyzed for $^{13}CO_2$ content with an isotope ratio mass spectrometer (ABCA 20/20 Europa Scientific, Crewe, UK). The gastric half-emptying time (t ½) was analyzed based on $^{13}CO_2$ content by nonlinear regression analysis for curve fitting.

2.5 Behavior tests

All the behavior tests were performed between 2 - 5 pm to avoid circadian effects. Tests were performed at least 24 hours apart to minimize the impact of previous test. Anxiety-like behavior was evaluated using an open field test and an elevated plus maze. Depression-like behavior was evaluated using a sucrose preference test and forced swim test.

Open field test. Mice were singularly placed in a 45 cm x 45 cm open arena for 10 minutes. The movements of the mice were recorded using a video tracking software Noldus EthoVision XT (Noldus Information Technology, Netherlands). The time spent in the center, percentage of distance travelled in the center (distance travelled in the center/total distance travelled x 100) and number of entries into the center were used to evaluate the anxiety-like behavior.

Elevated plus maze. Mice were placed at the intersection of a maze, consisting of two open and two closed arms, and left for 5 minutes. Movement was recorded using the video tracking software Noldus EthoVision XT. The amount of time spent in the open and closed arms, the distance travelled in the arms, and the number of entries to the arms were studied to evaluate anxiety-like behavior.

Sucrose preference test. Mice were individually housed during the test. At the beginning of the study, mice were given two bottles of 1% sucrose solution for a day to get accustomed to the sucrose solution and trained for 2 days with a free choice of one bottle of 1% sucrose solution and one bottle of water. The position of the bottles was switched every 12 hours to prevent possible effects of side preference on drinking behavior. On the fourth day, the consumption of both solutions was measured over 24 hours and the sucrose preference was determined. Weekly sucrose preference tests, over 24 hours, were performed on the same day each week. Sucrose preference was calculated (sucrose water consumed/total liquid consumed x 100) and lower preference indicates anhedonia and a more depressive-like behaviour.[26]

Forced swim test. Mice were singularly placed in a cylindrical tank (30 cm height and 20 cm diameter) containing 18 cm of water at 23°C for 5 minutes. Mice movement was recorded and analyzed by the video tracking software Noldus EthoVision XT. The immobility time was calculated when the mouse was floating with the absence of any movement of the body and head except for those necessary for keeping the nose above water. Increase in immobility time indicates a more depressive-like behavior.

2.6 Unpredictable chronic mild stress

A modified unpredictable chronic mild stress mouse protocol was used.[27] In brief, the stress group was exposed to one or two randomly scheduled stressors each day between 9 am and 5 pm unless otherwise indicated. The stressors included a) restraint stress in a polypropylene mouse restrainer for 2 hours, b) removal of bedding and nesting material for 8 hours, c) wet bedding for 8 hours, d) overnight fasting, e) light/dark cycle alterations for 2 hours, f) light/dark cycle reversal over 24 hours, g) 45 degree cage tilting on the long side of the cage for 2 hours, h) predator odour stress by introducing rat faecal pellets in the cage, and i) social stress by pair housing with a mouse from a different litter for 2 hours. The stressors are randomly scheduled day by day over the 8 weeks, therefore they are unpredictable to the mice. No stressor was applied for at least 24 hours before the metabolic analysis or behavior tests. No stressor was applied on the day of the electrophysiology study.

2.7 Quantification of circulating glucose and corticosterone levels

After collecting blood from the abdominal aorta, the blood glucose levels were determined using a glucometer (Accu-chek, NSW, Australia). Plasma was separated by centrifugation at 1000 rpm for 15 minutes at 4 °C. Plasma corticosterone levels were determined using corticosterone ELISA kit (ADI-900-097, Enzo Life Sciences, NY, USA)

2.8 In vitro single fibre recording preparation

The *in vitro* single fibre recording preparation was performed as described previously.[28] In brief, the stomach with attached vagal nerves was dissected in a modified Krebs solution at 4°C, and placed into an organ bath where it was superfused with a modified Krebs solution to maintain tissue viability. The vagal nerve was extended into another paraffin oil filled chamber. The nerve fibres were teased apart into small bundles and, one by one, the small bundles were placed onto an electrode for single fibre recording.

2.9 Gastric vagal afferent mechanosensitivity

GVA mechanosensitivity was determined as described previously.[25,28] The mucosal receptors respond to mucosal stroking but not circular stretch and the tension receptors respond to both mucosal stroking and circular stretch. The mechanosensitivity of gastric mucosal receptors was determined by stroking across the receptive field with calibrated von Frey hairs (10 - 1000 mg). The response of tension receptors to stretch was determined by applying a tension stimulus to the stomach. A claw connected to a cantilever system was attached to the stomach adjacent to the receptive fields. Weights (0.5 - 5 g) were placed at the opposite end of the cantilever system for 1 minute intervals with at least 1 minute recovery period between each weight.

To determine the effect of leptin on GVA mechanosensitivity, after the mechanosensitive GVA was identified, leptin (1 nM) was added to the superfusing Krebs solution and allowed to equilibrate for 20 minutes. The response of mucosal or tension receptors, to mucosal stroking or circular tension respectively, was determined before and after leptin treatment. Afferent impulses (action potentials) were amplified with a biological amplifier (DAM 50, World Precision Instruments, Sarasota, FL, USA), filtered (band-pass filter 932, CWE, Ardmore, PA, USA) and analyzed using SPIKE 2 software (Cambridge Electronic Design, Cambridge, UK).

2.10 Quantitative real-time PCR

Total RNA was extracted from nodose ganglion using a Purelink RNA micro kit (Life technologies, VIC, Australia), and from gastric mucosa using a Purelink RNA mini kit (Life technologies), respectively, in accordance with the manufacturer's instruction. Total RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific).

Quantitative real-time PCR reactions (qRT-PCR) were performed using a 7500 Fast Real-time PCR System (Life technologies) and Express One-Step SuperScript qRT-PCR Kit (11781-200, Life technologies). All primers were predesigned Taqman gene expression assays (4331182, Life technologies) and targeted leptin (Mm00434759_m1), leptin receptor (Mm00440181_m1), β-tubulin (Mm00727586_s1), B2M (Mm00437762_m1) and β-actin (Mm00607939_s1). QRT-PCR reactions were performed as described in detail previously.[29] Each assay was run in triplicate. Negative controls were carried out substituting RNase-free water for template RNA. Relative RNA levels were calculated using the delta CT method as described previously.[30]

2.11 Statistical analysis

Data are expressed as mean ± SEM with n = number of individual afferents and N = number of mice in all cases. The mechanosensitivity of GVAs were averaged for each mouse. Repeated-measures two-way ANOVA with Sidak post hoc test (Prism 7, Graphpad, CA, USA) was performed for analysis of body weight gain, weekly sucrose preference and GVA mechanosensitivity. All other data was analyzed using unpaired *t*-test. $P < 0.05$ was considered statistically significant.

3 RESULTS

3.1 Mouse body weight and circulating hormones levels

Body weight gain, final body weight and circulating hormone levels are illustrated in Figure 1. Body weight gain was lower in stressed mice compared to control mice over the 8-week stress period ($F(1, 26) = 20.2$, $P < 0.001$, repeated-measures two-way ANOVA; Fig. 1A). At the end of the 8-week stress period, the body weight of the stressed mice (29.51 ± 10.40 g) was significantly lower than the body weight of control mice (31.18 ± 0.74 g; $P = 0.038$ ($T = 2.18$, $df = 26$), unpaired *t*-test; Figure 1B). Plasma corticosterone and blood glucose levels were not different between control and stressed mice ($P > 0.05$, unpaired *t*-test; Figure 1C).

3.2 Mouse metabolic data and gastric emptying rate

There was no difference in the baseline metabolic data and gastric emptying rate between the control and stress groups (data not shown). Food and water intake after the stress period are illustrated in Figure 2. The stressed mice had lower 24-hour food intake than control mice after the stress period (Stress: 3.05 ± 0.12 g, Control: 3.65 ± 0.25 g; $P = 0.024$ (T = 2.4, df = 26), unpaired *t*-test; Figure 2A). The number of meals consumed in 24 hours was lower in the stressed mice compared to the control mice (25.75 ± 0.83 vs.35.65 ± 2.25 respectively; $P < 0.001$ (T = 4.94, df = 26), unpaired *t*-test; Figure 2B). There was no difference in the average meal size between the control and stressed mice (Control: 0.10 ± 0.01 g, Stress: 0.12 ± 0.01 g; $P > 0.05$, unpaired *t*-test; Figure 2C). The 24-hour water intake, number of water intake episodes in 24 hours, and average water intake size in 24 hours were not different between control and stressed mice ($P > 0.05$, unpaired *t*-test; Figure 2D-F).

In stressed mice, energy expenditure and locomotor activity was significantly lower in comparison to control mice ($P = 0.039$ (T = 2.18, df = 26) and $P = 0.013$ (T = 2.68, df = 26) respectively, unpaired *t*-test; Table 1). There was no difference in the resting energy expenditure and the respiratory exchange ratio between control and stressed mice ($P > 0.05$, unpaired *t*-test; Table 1). The gastric half-emptying time of a solid meal was not different between control and stressed mice ($P > 0.05$, unpaired *t*-test; Table 1).

3.3 Anxiety and depression-like behaviors

There was no difference in baseline behavior parameters between control and stress groups. In the open field test, stressed mice spent significantly less time in the center of the field ($P = 0.016$ (T = 2.57, df = 26), unpaired *t*-test; Figure 3A). The percentage of total distance travelled in the center of the field and number of entries to the center were not different between the control and stressed mice ($P > 0.05$, unpaired *t*-test; Figure 3B-C). In the elevated plus maze, control and stressed mice spent similar amount of time in the open and closed arms ($P > 0.05$, unpaired *t*-test; Figure 3D). The stressed mice tended to move more distance in the closed arms ($P = 0.05$ (T = 2.01, df = 26), unpaired *t*-test; Figure 3E) and have more entries into the closed arms ($P = 0.048$ (T = 2.07, df = 26), unpaired *t*-test; Figure 3F) compared to the control mice. There was no difference in the moving distance in the open arms or entries to the open arms between control and stressed mice ($P > 0.05$, unpaired *t*-test; Figure 3D-F).

In the weekly sucrose preference tests, stressed mice displayed a similar preference to sucrose as control mice over the 8-week stress period ($F(1, 26) = 1.27$, $P > 0.05$, repeated-measures

two-way ANOVA; Figure 4A). There was no difference in the sucrose preference in stressed (80.27% ± 2.82%) and control mice (79.11% ± 2.72%) at the end of the stress period ($P > 0.05$ (T = 0.27, df = 26), unpaired *t*-test). In the forced swim test, the immobility time of the stressed mice (227.1 ± 8.51 seconds) was not different from the immobility time of control mice (205.3 ± 16.23 seconds; $P > 0.05$ (T = 0.96, df = 26), unpaired *t*-test; Figure 4B).

3.4 Gastric vagal afferent mechanosensitivity

The response of GVA mucosal and tension receptors to mucosal stroking and circular stretch respectively is illustrated in Figure 5. The response of gastric mucosal receptors to mucosal stroking was significantly higher in stressed mice compared to control mice (F(1, 22) = 4.65, $P = 0.042$, stress effect; F(3, 66) = 93.6, $P < 0.001$, von Frey hair effect; no interaction; repeated-measures two-way ANOVA; Figure 5A). The response of gastric tension sensitive vagal afferents to circular tension was significantly higher in stressed compared to control mice (F(1, 25) = 8.91, $P = 0.006$, stress effect; F(6, 150) = 36.73, $P < 0.001$, tension effect; $P = 0.008$, interaction; repeated-measures two-way ANOVA; Figure 5B).

3.5 Effect of leptin on gastric vagal afferent mechanosensitivity

In control mice, leptin potentiated the response of mucosal receptors to mucosal stroking (F(1, 8) = 15.93, $P = 0.004$ vs. no leptin control, leptin effect, no interaction, repeated-measures two-way ANOVA; Figure 6A). However, in stressed mice, leptin had no effect on the mechanosensitivity of mucosal receptors ($P > 0.05$ vs. no leptin control, leptin effect, repeated-measures two-way ANOVA; Figure 6B). In both control and stressed mice, leptin had no effect on the response of gastric tension receptor to stretch ($P > 0.05$ vs. no leptin control, leptin effect, repeated-measures two-way ANOVA; Figure 6C, D).

3.6 Leptin and leptin receptor mRNA levels

QRT-PCR data revealed that leptin mRNA levels in the gastric mucosa were reduced in the stressed mice in comparison with control mice ($P = 0.015$ (T = 2.88, df = 11), unpaired *t*-test; Figure 7A). There was no difference in leptin receptor mRNA levels in the nodose ganglion between control and stressed mice ($P > 0.05$ (T = 1.26, df = 9), unpaired *t*-test; Figure 7B).

4 DISCUSSION

In this study, GVAs were hypersensitive to food related mechanical stimuli after mice were exposed to unpredictable chronic mild stress. Furthermore, the leptin-induced potentiation of GVA mucosal receptor mechanosensitivity, observed in control mice, was lost after chronic

stress. Therefore, both GVA mechanosensitivity and its interaction with leptin are altered in chronic stress conditions.

Tension sensitive GVAs play an important role in generating satiety signalling and controlling food intake.[31,32] In the current study, after chronic stress, the response of tension sensitive GVAs to distension was increased. Consistent with this hypersensitivity, the stressed mice consumed less food and gained less body weight compared to the control mice. This was due to reduced meal numbers rather that meal size. This could be related to the specific eating pattern of mice, which consume multiple small meals mainly in the dark phase. Thus, the distension of stomach and feeling of satiety occur gradually and depend more on the accumulation of meal numbers rather than a single meal. It should be noted that the stressed mice had reduced energy expenditure, therefore, the reduced body weight was due to a decrease in food intake rather than an increase in energy expenditure.

Chronic stress also induced increased mechanosensitivity of GVA mucosal receptors. As mechanosensitive mucosal receptors detect light stroking from the consumed food passing over the receptive field, they may also constitute part of the mechanisms of gastric hypersensitivity. The roles of mechanosensitive GVA mucosal receptors are not clear. Evidence suggests that they may recognize food particle size within the stomach and prevent gastric emptying to ensure food is sufficiently churned.[33] Therefore, the hypersensitivity of mucosal GVAs may contribute to the delayed gastric emptying observed in 20-50% of FD patients.[12] However, we did not detect any significant change in the gastric emptying rate in the stressed compared to control mice. Therefore, the hypersensitivity of GVA mucosal receptors in the stressed mice may be related to other functions, which requires further investigation.

In FD patients, gastric hypersensitivity is associated with a history of abuse and a variety of psychological disorders,[6,34] suggesting a contributory role of stress in the development of gastric hypersensitivity. Similarly, in rodents, chronic stress has been shown to induce gastric hypersensitivity to distension.[35] In the current study, chronic stress enhanced GVA mechanosensitivity to distension. The increased GVA hypersensitivity may contribute to the stress-induced gastric hypersensitivity in FD, which is supported by other physiological changes in the stressed mice. Firstly, in the current study, the stressed mice have reduced food intake and body weight compared to the control group mice. Similarly, in FD patients, reduced food intake and body weight are commonly observed and associated with gastric

hypersensitivity.[13,36,37] Furthermore, the stressed mice displayed greater anxiety-like behavior. It is reported that FD patients have higher prevalence of anxiety and depression.[3] More specifically, psychological changes and anxiety are more associated with postprandial distress syndrome, rather than epigastric pain syndrome.[38] Thus, the increased sensitivity of GVAs, reduced food intake and body weight, early satiety, and heightened anxiety-like behavior indicate that the unpredictable stress mouse model may represent a good model of postprandial distress syndrome. However, we cannot exclude that this stress mouse model may also represent epigastric pain syndrome. In the current study, the modulation of chronic stress on GVA mechanosensitivity was only studied in male mice. However, it is well established that the prevalence of FD-like symptoms is higher in female compared to male patients.[39] It is known that GVA mechanosensitivity is dependent on the stage of the estrus cycles in mice.[40] Furthermore, the receptors for estradiol are expressed in the cell bodies of vagal afferents,[41] and estradiol was found to increase GVA mechanosensitivity,[40] and inhibit vagal-dependent gastric functions, including food intake[23] and gastric motility.[22] Therefore, it is possible that estradiol can enhance chronic stress-induced hypersensitivity of GVAs and, subsequently, enhance the prevalence of FD-like symptoms in females. However, this is highly speculative and requires further investigation.

Gastric hypersensitivity in FD patients is also associated with postprandial pain.[14] It has been demonstrated that chronic stress exposure, in rats, is associated with hypersensitivity of greater splanchnic afferents to gastric distension.[42] Therefore, it is likely, that spinal afferent hypersensitivity contributes to the postprandial pain in FD. However, it is known that vagal afferent inputs can result in modulation of central pain pathways[15] and therefore a role of GVAs in the pain symptoms associated with FD cannot be totally excluded.

The mechanisms leading to increased GVA mechanosensitivity are currently unknown. One possibility is that the ion channel transient receptor potential vanilloid 1 (TRPV1) is involved. Hypersensitivity to the TRPV1 agonist capsaicin has been demonstrated in about 50% of FD patients.[43,44] Further, upregulation of TRPV1 channels has been observed in the gastric mucosa of FD patients.[45] TRPV1 channels are expressed in rodent gastrointestinal vagal afferents [46-49] and activation of TRPV1, by oleoylethanolamide (OEA), causes depolarisation of nodose neurones and decreased short-term food intake in mice.[50] Therefore, there is the possibility that TRPV1 can also play an indirect role in the observed hypersensitivity of GVAs. However, this is highly speculative and requires further investigation. Another possibility is that there are immune changes[51] in the gut, associated with the stress response,

driving hypersensitivity of GVAs. Immune responses in the gut have been shown to be increased by stress.[52] Evidence suggest that these altered immune responses may contribute to visceral hypersensitivity, which occur through the sensitization of afferent nerve fibres in the gut.[53]

In control mice, the sensitivity of mucosal GVAs was upregulated by leptin, as observed in a previous study.[21] However, this effect of leptin was lost in stressed mice. It should be noted that the stress-induced hypersensitivity of the gastric mucosal receptors was at a similar level to the leptin-induced hypersensitivity observed in the control mice. Therefore, it is possible that no further increase in the hypersensitivity can be obtained by leptin after the stress-induced hypersensitivity, however, this needs to be further investigated. In the current study, we observed a reduced gastric leptin transcription level after chronic stress. This is consistent to the reduced gastric leptin protein level observed in water-immersion stressed rats.[54] In FD patients[20] and various stress rodent models,[54-56] leptin levels in the circulation are increased. Therefore it is logical to speculate that elevated circulating leptin levels lead to a compensatory downregulation of gastric leptin, however, this requires further investigation. The leptin receptor transcription level in the nodose ganglion was not changed. However, it should be noted that the transcription level in the nodose ganglion does not necessarily reflect its expression in the subpopulation of GVA cell bodies or its protein levels. The reduced expression of leptin, along with the blunted effect of leptin on gastric mucosal vagal afferents, may constitute a compensatory adaptation of the body in response to the stress-induced gastric hypersensitivity.

In conclusion, we have established that chronic stress significantly increased the mechanosensitivity of gastric mucosal and tension sensitive vagal afferents. This hypersensitivity would lead to increased satiety and hypersensitivity of the stomach to meal related stimuli, which could constitute a mechanism for gastric hypersensitivity in FD.

Conflict of interest: The authors have no competing interests.

Author Contributions

HL designed and performed the study, analyzed and interpreted the data, and drafted the manuscript. FB interpreted the data. MN, CF and GH conducted experiments. AI, ML and MW provided assistance for designing animal model and data interpretation. SC analyzed the metabolic data. SK analyzed gastric emptying data. AP designed and performed the study, and analyzed and interpreted the data. All authors contributed to review of the manuscript.

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Abbreviations

FD, functional dyspepsia; GVA, gastric vagal afferents; OEA, oleoylethanolamide; TRPV1, ion channel transient receptor potential vanilloid 1

Table 1. Metabolic and gastric emptying rate in control and stressed mice

	Control	Stress
Energy intake/energy expenditure	1.09±0.05	1.03±0.06

Energy expenditure (kcal hour-1)	0.43±0.01	0.40±0.01 *
Resting energy expenditure (kcal hour-1)	0.32±0.01	0.30±0.01
Locomotor activity/24 hour (meter)	206.7±17.69	160.6±8.26 *
Respiratory exchange ratio	0.86±0.01	0.88±0.01
Gastric half-emptying time (minutes)	77.01±5.42	81.84±8.36

Data is mean ± SEM. Significance between control and stressed mice was determined using an unpaired *t*-test. * $P < 0.05$ versus control mice.

FIGURE LEGENDS

Figure 1. Mouse body weight and circulating hormones levels. A, Weekly body weight gain of control mice (○; N = 10) and stressed mice (●; N = 18). B-D, Final body weight (B), plasma corticosterone levels (C), and blood glucose levels (D) in control (N = 10) and stressed (N = 18) mice after stress treatment. *** $P < 0.001$ versus control mice, repeated-measures two-way ANOVA. * $P < 0.05$ versus control mice, unpaired *t*-test.

Figure 2. Food and water intake patterns of control and stressed mice. A-C, 24-hour food intake (A), meal number in 24 hours (B) and average meal size in 24 hours (C) in control (N = 10) and stressed mice (N = 18). D-F, 24-hour water intake (D), number of water intake episodes in 24 hours (E) and average water intake size in 24 hours (F) in control (N = 10) and stressed mice (N = 18). * $P < 0.05$, *** $P < 0.001$ versus control mice, unpaired *t*-test.

Figure 3. Anxiety-like behavior in control and stressed mice. A-C, Time in center area (A), percentage of total distance moved in the center (B) and number of entries to center (C) in the open field test in control and stressed mice. D-F, Time spent in open or closed arms (D), distance travelled in open or closed arms (E) and number of entries to open or closed arms (F) in elevated plus maze in control and stressed mice. * $P < 0.05$ versus control, unpaired *t*-test.

Figure 4. Depression-like behavior in control and stressed mice. A, Weekly sucrose preference in control (○; N = 10) and stressed mice (●; N = 18). B, Immobility time in the forced swim test of control (N = 10) and stressed mice (N = 18).

Figure 5. Response of gastric vagal afferents to mechanical stimulation in control and stressed mice. A, The response of mucosal receptors to mucosal stroking in control mice (○; n = 28, N = 10) and stressed mice (●; n = 38, N = 14). B, The response of tension receptors in control

mice (○; n = 24, N = 10) and stressed mice (●; n = 42, N = 16). * $P < 0.05$, ** $P < 0.01$ versus control, repeated-measures two-way ANOVA. # $P < 0.05$, ## $P < 0.01$ versus control, Sidak post hoc test.

Figure 6. Leptin modulation of gastric vagal afferent mechanosensitivity in control and stressed mice. A, B, The response of mucosal receptors in the absence (○) and presence of leptin (1 nM, ●) in control (n = 5, N = 5; A) and stressed mice (n = 9, N = 8; B). C, D, The response of tension receptors in the absence (○) and presence of leptin (1 nM, ●) in control (n = 9, N = 6, C) and stressed mice (n = 10, N = 9, D). ** $P < 0.01$ versus absence of leptin, repeated-measures two-way ANOVA. # $P < 0.05$ versus absence of leptin, Sidak post hoc test.

Figure 7. Leptin mRNA levels in the gastric mucosa and leptin receptor mRNA levels in the nodose ganglion of control and stressed mice. A, Leptin mRNA levels in the gastric mucosa in control (N = 6) and stressed mice (N = 7). B, Leptin receptor mRNA levels in the nodose ganglion in control (N = 6) and stressed mice (N = 5). * $P < 0.05$ versus control mice, unpaired *t*-test.