One-day high-fat diet induces inflammation in the nodose ganglion and hypothalamus of mice

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Abstract

A high-fat diet (HFD) induces inflammation in systemic organs including the hypothalamus, resulting in obesity and diabetes. The vagus nerve connects the visceral organs and central nervous system, and the gastric-derived orexigenic peptide ghrelin transmits its starvation signals to the hypothalamus via the vagal afferent nerve. Here we investigated the inflammatory response in vagal afferent neurons and the hypothalamus in mice following one day of HFD feeding. This treatment increased the number of macrophages/microglia in the nodose ganglion and hypothalamus. Furthermore, one-day HFD induced expression of Toll-like receptor 4 in the goblet cells of the colon and upregulated mRNA expressions of the proinflammatory biomarkers *Emr1*, *Iba1*, *Il6*, and *Tnfα* in the nodose ganglion and hypothalamus. Both subcutaneous administration of ghrelin and celiac vagotomy reduced HFD-induced inflammation in these tissues. HFD intake triggered inflammatory responses in the gut, nodose ganglion, and subsequently in the hypothalamus within 24 h. These findings suggest that the vagal afferent nerve may transfer gut-derived inflammatory signals to the hypothalamus via the nodose ganglion, and that ghrelin may protect against HFD-induced inflammation.

Keywords: high-fat diet, inflammation, ghrelin, nodose ganglion, vagotomy

Highlights:

- One-day HFD feeding causes inflammation in the nodose ganglion and hypothalamus.
- Vagal afferents transmit HFD-induced inflammatory signals from the gut to the brain.
- Ghrelin administration ameliorates HFD-induced inflammation.

1. Introduction

A high-fat diet (HFD) constitutes one of the major risks for developing obesity, diabetes, atherosclerosis, and other feeding-related disorders [1, 2]. Consumption of a HFD induces inflammation in the adipose tissue, liver, and skeletal muscle by increasing endotoxin lipopolysaccharide (LPS) levels in the intestinal lumen, as well as by enhancing LPS action via Toll-like receptor 4 (TLR4) [2, 3]. This inflammation plays a critical role in the development of obesity and insulin resistance [4, 5]. Obesity-associated inflammation in the hypothalamus was first reported in 2005 [6], and many investigators have since replicated this finding [7]. A recent study demonstrated that inflammation in the hypothalamic arcuate nucleus, a center of feeding regulation, develops 1 to 3 days after initiation of HFD feeding [8]. HFD-induced activation of microglia results in production of a variety of proinflammatory cytokines, thereby provoking inflammatory responses that cause neuronal death in the arcuate nucleus [8].

The nodose ganglion is a constellation of vagal afferent neurons that transmit meal-related signals from the gastrointestinal tract to the nucleus of the tractus solitaries (NTS) in the medulla oblongata [9]. Vagal afferent neurons synthesize receptors for gastrointestinal peptides and transport them to the afferent endings in the gastrointestinal tract. Ghrelin, a 28–amino acid peptide produced in gastric endocrine cells, stimulates food intake by transmitting hunger signals to the NTS via the vagal afferent nerve [10, 11]. Consumption of a HFD alters the composition of Gram-negative gut microbiota, which are a major source of LPS production [12]. Vagal afferent neurons express TLR4, and their axonal terminals innervating the gut are in close proximity to sites of LPS release that could be exposed to high concentrations of LPS

under HFD conditions [13, 14]. Ghrelin can suppress LPS-induced release of proinflammatory cytokines from macrophages/microglia [15].

In this study, we found that very short-term HFD (one day of HFD feeding) resulted in inflammation in the colon, nodose ganglion, and hypothalamus in mice. HFD-induced inflammatory responses were abolished by a single peripheral administration of ghrelin before the initiation of HFD feeding. Celiac vagotomy also suppressed inflammatory responses in the nodose ganglion and hypothalamus of HFD-fed mice. Thus, very short-term HFD causes inflammation in the vagal afferent system, which may transmit inflammatory signals to the hypothalamus. Ghrelin could exert a beneficial effect by suppressing HFD-induced inflammatory processes.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice (6-week-old; 20–21 g; Charles River Laboratories, Yokohama, Japan) were maintained in individual cages under controlled temperature (21–23°C) and light (light on: 08:00–20:00) conditions. Mice were maintained on a chow diet (CD) (12.3% fat, 59.2% carbohydrate, 28.5% protein, 3.4 kcal/g; CLEA Rodent Diet CE-2, CLEA Japan, Tokyo, Japan) with free access to food. All animal experiments were approved by the Animal Care and Use Committee of University of Miyazaki.

2.2. Experimental design

Mice were fed either a CD or HFD (60% fat, 20% carbohydrate, 20% protein, 5.2 kcal/g; no.D12492; Research Diets, New Brunswick, NJ) for a 24-h period from 10:00

to 10:00 on the following day. They received a subcutaneous administration of saline or ghrelin (60 nmol/kg body weight (BW), 50 μl saline; Peptide Institute, Minoh, Japan) just before the start of CD or HFD intake.

2.3. Celiac branch vagotomy

Mice (5-week-old males, n = 8 per group) were anesthetized by intraperitoneal administration of sodium pentobarbital (Abbot Laboratories, Chicago, IL). A midline incision was made to provide wide exposure of the upper abdominal organs. Bilateral celiac branches of the vagal nerve were split and cut. In the sham operation, the celiac branches of mice were only exposed and they were returned to their home cages. Mice were accustomed to experimental conditions by daily handling for 7 days before the experiment. Only mice exhibiting progressive weight gain after surgery were used in experiments.

2.4. Measurements of blood parameters

Blood was collected by tail-prick 24 h after the start of experiment. Glucose was measured using a glucometer (Terumo, Tokyo, Japan). Plasma insulin level was determined using a mouse Insulin EIA Kit (Morinaga Institute of Biological Science, Yokohama, Japan). Serum triglycerides were determined with a L-Type Triglyceride H kit (WAKO Pure Chemical Industries, Osaka, Japan), and non-esterified fatty acids (NEFAs) were measured using an NEFA-SS 'EIKEN' kit (EIKEN Chemical, Tokyo, Japan).

2.5. Immunohistochemistry

Mice (n = 6 per group) were anesthetized by intraperitoneal administration of sodium pentobarbital (5 mg/kg BW) and transcardially perfused with ice-cold phosphate buffer (PB) followed by 4% paraformaldehyde/ phosphate buffer saline

(PBS) solution. Nodose ganglion, brain, and distal colon were immersed in 4% paraformaldehyde/PBS for 24 h at 4°C, and subsequently cryoprotected in 0.1 M PB containing 20% sucrose. The nodose ganglion and distal colon, embedded in Tissue-Tek OCT compound (Sakura Finetek Japan, Tokyo, Japan), were cut into 8-μm slices using a cryostat (Leica CM3050S; Leica, Nussloch, Germany) chilled at −20°C. Hypothalamic sections of 40 μm thickness were prepared on a freezing microtome (Komatsu Electronics, Kanagawa, Japan). Sections were blocked in Serum-Free Protein Block (Dako, Carpinteria, CA) for 5 min at room temperature, and then incubated overnight at 4°C with rabbit anti-Iba1 (1:10,000; Wako Pure Chemicals), rat anti-CD86 (1:100; Abcam, Cambridge, UK), Alexa Fluor 488–conjugated mouse monoclonal anti-NeuN (1:200; Millipore, Temecula, CA), goat anti-TLR4 (1:100; Santa Cruz Biotechnology, Dallas, TX), or rabbit anti-mucin 2 (MUC2) (1:50; Santa Cruz Biotechnology). Slices were washed with PBS and incubated with Alexa Fluor 488–labeled anti-rabbit, Alexa Fluor 594–labeled anti-rat, Alexa Fluor 594–labeled anti-goat, or Alexa Fluor 594–labeled anti-rabbit (all used at 1:400; Invitrogen, Carlsbad, CA) at room temperature for 1 h. Nuclei in colon sections were identified by staining with 4′, 6-diamidino-2-phenylindole (DAPI) (Millipore). Images were visualized on an OLYMPUS AX-7 fluorescence microscope (Olympus, Tokyo, Japan). Cells immunostained with Iba1 or CD86 antibody were counted manually with Olympus cellSens imaging software (Olympus). Quantitative histological analysis of Iba1, CD86, TLR4, and MUC2 pixel intensities were automatically performed on three sections from each animal using the cellSens Dimension imaging software (Olympus). Quantitation was performed in a blinded fashion.

2.6. Real-time polymerase chain reaction (RT-PCR)

Nodose ganglion, hypothalamus, liver, epididymal fat, and mesenteric fat were removed from anesthetized CD- or HFD-fed mice. Colons were flushed with PBS/RNAlater (Life Technologies, Carlsbad, CA), and then the epithelial cells of the luminal side were scraped off and collected. All samples were placed in a tube containing autoclaved glass beads (425–600 μm) (Sigma-Aldrich, St. Louis, MO), and vortexed for 6 min with TissueLyser (Qiagen). Total RNA was extracted using the RiboPure kit (Ambion, Austin, TX). RT-PCR was performed on a LightCycler system (Roche Diagnostics, Mannheim, Germany) using SYBR Premix Ex Taq (2×) (TaKaRa Bio, Otsu, Japan) and the following primer sets: *Iba1*, AGCTGCCTGTCTTAACCTGCATC and TTCTGGGACCGTTCTCACACTTC; *Il6*, CCACTTCACAAGTCGGAGGCTTA and CCAGTTTGGTAGCATCCATCATTTC; *Tnfα*, TATGGCCCAGACCCTCACA and GGAGTAGACAAGGTACAACCCATC; *Itgam* (CD11b), CCACTCATTGTGGGCAGCTC and GGGCAGCTTCATTCATCATGTC; *Itgax* (CD11c), AGGTCTGCTGCTGCTGGCTA and GGTCCCGTCTGAGACAAACTG; *Mpo*, CTGCCTCATTGGCACTCAGTTTA and GGTGATGCCAGTGTTGTCACAG; *F4/80*: CTCTGTGGTCCCACCTTCAT and GATGGCCAAGGATCTGAAAA; *Emr1*, GAGATTGTGGAAGCATCCGAGAC and GACTGTACCCACATGGCTGATGA; *Tlr4*, GGAAGTTCACATAGCTGAATGAC and CAAGGCATGTCCAGAAATGAGA; *Tbp*, CATTCTCAAACTCTGACCACTGCAC and CAGCCAAGATTCACGGTAGATACAA; and glyceraldehyde phosphate dehydrogenase (*Gapdh*), TCAAGAAGGTGGTGAAGCAG and TGGGAGTTGCTGTTGAAGTC. mRNA levels were normalized against the level of *Gapdh* or *Tbp* in the same sample.

2.7. Statistical analysis

Statistical analyses were performed by one-way ANOVA followed by Bonferroni's post-test for multiple comparisons, as appropriate. When two mean values were compared, analyses were performed by unpaired *t*-test. All data are expressed as means ± SEM. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Characteristics of mice

Table 1 shows characteristics and blood parameters of CD- and HFD-fed mice. One-day HFD caused significant increases in body weight, food intake amount, energy intake, epididymal fat weight, and plasma insulin. All characteristics and parameters of sham HFD- and vagotomized HFD-fed mice were comparable.

3.2. Iba1- and CD86-positive cells in the nodose ganglion and hypothalamus, and TLR4 expression in the distal colon

Both the number and size of macrophages stained with Iba1 or CD86 antibody in the nodose ganglion were significantly greater in HFD-fed mice than in CD-fed mice (Fig. 1A–H). Approximately 30% of $Iba1^+$ macrophages in the nodose ganglion of HFD-fed mice expressed CD86-immunoreactivity (Fig. 1F). In addition, in the hypothalamus, both the number and size of $Iba1^+$ microglia were significantly greater in HFD-fed mice than in CD-fed mice, whereas the number and size of $CD86^+$ microglia were comparable in the two groups (Fig. 1I–P). TLR4 and MUC2 expression levels in the distal colon, as determined by immunohistochemistry, were significantly higher in

HFD-fed mice than in CD-fed mice (Fig. 1Q–R). Approximately 29% of $MUC2^+$ cells in HFD-fed mice were immunoreactive for TLR4.

3.3. mRNA analysis of inflammatory markers

mRNA levels of *Emr1, Iba1, Il6*, and *Tnfα* in both nodose ganglion and hypothalamus were significantly higher in HFD-fed mice than in CD-fed mice (Fig. 2A–B). mRNA levels of *Tlr4, Itgax,* and *Tnfα* in the distal colon (Fig. 2C), *Mpo* in the liver and epididymal fat (Fig. 2D–E), and *Itgam*, *Tnfα,* and *Mpo* in the mesenteric fat (Fig. 2F) were significantly higher in HFD-fed mice than in CD-fed mice.

3.4. Suppression of inflammatory response by ghrelin administration or vagotomy

The numbers of macrophages/microglia in both nodose ganglion and hypothalamus were significantly higher in HFD-fed mice than in CD-fed mice (Fig. 3A–D). Macrophages/microglia of HFD-fed mice were morphologically rounded and more ramified than those of CD-fed mice. A single subcutaneous administration of ghrelin to HFD-fed mice before the initiation of HFD feeding significantly reduced the number of $Iba1^+$ macrophages/microglia in these tissues (Fig. 3A–D). However, ghrelin treatment did not alter TLR4 expression in the goblet cells of HFD-fed mice (data not shown). mRNA levels of *Iba1, Il6*, and *Tnfα* in both nodose ganglion and hypothalamus were significantly higher in HFD-fed mice than in CD-fed mice (Fig. 3E–F). Ghrelin administration abolished the upregulation of these mRNAs in both tissues (Fig. 3E). Ghrelin administration to CD-fed mice did not affect expression of any mRNAs tested. Celiac vagotomy significantly reduced upregulation of *Iba1, Il6*, and *Tnfα* mRNA, as well as the numbers of $Iba1^+$ macrophages/microglia in both nodose ganglion and hypothalamus (Fig. 3F–J).

4. Discussion

Obesity-associated inflammation is characterized by immune cell infiltration and high levels of proinflammatory molecules in both the periphery and the hypothalamus. The consumption of a HFD activates microglia and the expression of inflammatory cytokines in the hypothalamus, thereby disrupting the hypothalamic neuronal routes that maintain energy homeostasis [8, 16]. We observed that the inflammatory effects of HFD were rapid, appearing in the nodose ganglion and hypothalamus one day after exposure to a HFD.

Macrophages/microglia are classified into classically activated macrophages (M1) representing CD86 and alternatively activated macrophages (M2) [17]. In a recent study, we showed that 12-week HFD feeding (60% of calories as fat) induced accumulation and activation of M1 ($Iba1^+/CD86^+$) macrophages both in the nodose ganglion and hypothalamus of mice [18]. In the one-day HFD study described here we observed elevated recruitment of M1 macrophages in the nodose ganglion but not in the hypothalamus, suggesting that the nodose ganglion receives inflammatory signals before the hypothalamus.

Intestinal mucins are secreted from goblet cells and function against gut inflammation and infection [19]. Administration of LPS to the human goblet cell line HT29-MTX upregulates mucin secretion [20]. Increases in goblet cell number and MUC2 production in 12-week HFD-fed mice might interrupt LPS absorption from the gut lumen to the systemic circulation [21]. In this study, elevation of TLR4 and MUC2 expression in goblet cells in response to one-day HFD may have served as a protective mechanism in the colon. In the classical immune response to HFD feeding, neutrophils infiltrate adipose tissue prior to macrophage infiltration [22]. In this study, increased

mRNA expressions of *Itgam*, *Tnfα*, and *Mpo* in mesenteric fat indicated neutrophil infiltration and initiation of acute inflammatory responses. The higher levels of *Mpo* mRNA expressions in the liver and epididymal fat may also represent initiation of acute inflammatory responses.

Ghrelin plays important roles in suppressing inflammation and regulating immune function [23]. For example, ghrelin binds to a receptor expressed on activated macrophages and attenuates LPS-induced expressions of IL-6 and TNF-α [24]. Thaler *et al.* demonstrated that the HFD-induced inflammatory response in the rat hypothalamus exhibits a complex "on–off–on" pattern, where the initial response declined to baseline after 7 days [8]. Acute inflammatory cytokine production is crucial for the initiation of adaptive immune responses and allows the host to adapt to pathological conditions [25]. In this study, we found that ghrelin administration prior to HFD feeding suppressed macrophages/microglia infiltration and proinflammatory cytokines production in the nodose ganglion and hypothalamus. Thus, ghrelin may protect against HFD-induced inflammation.

Vagal afferents transmit chemical, physiological, and feeding-related signals from the gut to the NTS [26]. Sub-diaphragmatic vagotomy attenuated LPS-induced IL-1β mRNA expression in the mouse hypothalamus [27], suggesting that the vagal afferent nerve transmits LPS-induced inflammatory signals to the brain. Here, we showed that celiac vagotomy suppressed one-day HFD–induced $Iba1^{+}$ macrophages/microglia accumulation, as well as production of the inflammatory cytokines *Il6* and *TNFα*, in both nodose ganglion and hypothalamus. Thus, the vagal afferent nerve may transfer gut-derived inflammatory signals to the hypothalamus via the nodose ganglion. Our results suggest a novel important role for the vagal afferent nerve in the progress of

HFD-induced obesity, and raise the possibility that ghrelin may have a therapeutic potential as a means to prevent HFD-induced inflammation.

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Figure Legends

Fig. 1. Histochemical analyses of activated macrophages in nodose ganglion (A–F) and hypothalamus (I–N) of CD- or HFD-fed mice. Quantitation of $Iba1^+$ and $CD86^+$ cells in nodose ganglion (G–H) and hypothalamus (O–P). $Iba1^+$ (green) cells: A, D, I, and L; $CD86^+$ (red) macrophages/microglia: B, E, J, and M. Arrows in F: co-localization of CD86 with Iba1. (Q) Immunohistochemistry of TLR4 (red), MUC2 (green) and DAPI (blue) in distal colon of CD- or HFD-fed mice. (R) Relative TLR4 and MUC2 expression levels in the distal colon. Values are means ± SEM. $^*P < 0.05$ vs CD. Scale bars, 50 μm.

Fig. 2. Effect of one-day HFD feeding on inflammatory gene expression. In the nodose ganglion (A), hypothalamus (B), distal colon (C), liver (D), epididymal fat (E), and mesenteric fat (F) of CD- or HFD-fed mice. mRNAs are quantitated relative to *Gapdh* or *Tbp* mRNA and presented as fold change relative to CD. Values are means ± SEM. $^*P < 0.05$ vs CD. NS, not significant.

Fig. 3. Effects of ghrelin administration or celiac vagotomy on HFD-induced inflammation. Immunohistochemical detection and quantitation of Iba1 in the nodose ganglion (A, C) and hypothalamus (B, D) of CD- or HFD-fed mice. (E, F) Relative expression levels of *Iba1, Il6,* and *Tnfα.* Immunohistochemical detection and quantitation of Iba1 in the nodose ganglion (G, I) and hypothalamus (H, J) of sham or vagotomized CD- or HFD-fed mice. $Iba1^+$ cells (red) and nuclei of neurons (green): A, B, G, and H. Values are means ± SEM. $^*P < 0.05$ vs CD-saline, $^{\#}P < 0.05$ vs HFD-

saline, $^{\$}P < 0.05$ vs sham CD, $^{\dagger}P < 0.05$ vs sham HFD. Scale bars, 50 μm.

Table 1

Characteristics and parameters of CD-fed or HFD-fed mice

	CD	HFD	sham HFD	vagotomy HFD
Initial body weight (g)	21.70 ± 0.32	21.88 ± 0.34	21.92 ± 0.17	21.87 ± 0.34
Final body weight (g)	21.92 ± 0.31	23.25 ± 0.30 *	22.94 ± 0.17	22.88 ± 0.42
24-h food intake (g)	3.26 ± 0.14	3.83 ± 0.11 *	3.91 ± 0.05	3.85 ± 0.06
24-h energy intake (kcal)	11.09 ± 0.48	20.05 ± 0.58 *	20.50 ± 0.25	20.20 ± 0.31
Epididymal fat weight (g)	0.30 ± 0.01	0.41 ± 0.01 *	0.39 ± 0.01	0.38 ± 0.01
Blood glucose (mmol/l)	10.33 ± 0.53	10.57 ± 0.49	10.11 ± 0.33	10.19 ± 0.25
Plasma insulin (ng/ml)	0.77 ± 0.05	1.76 ± 0.20 *	1.84 ± 0.20	1.90 ± 0.15

Data are expressed as means ± SEM (n = 5–18). *P < 0.05 vs CD

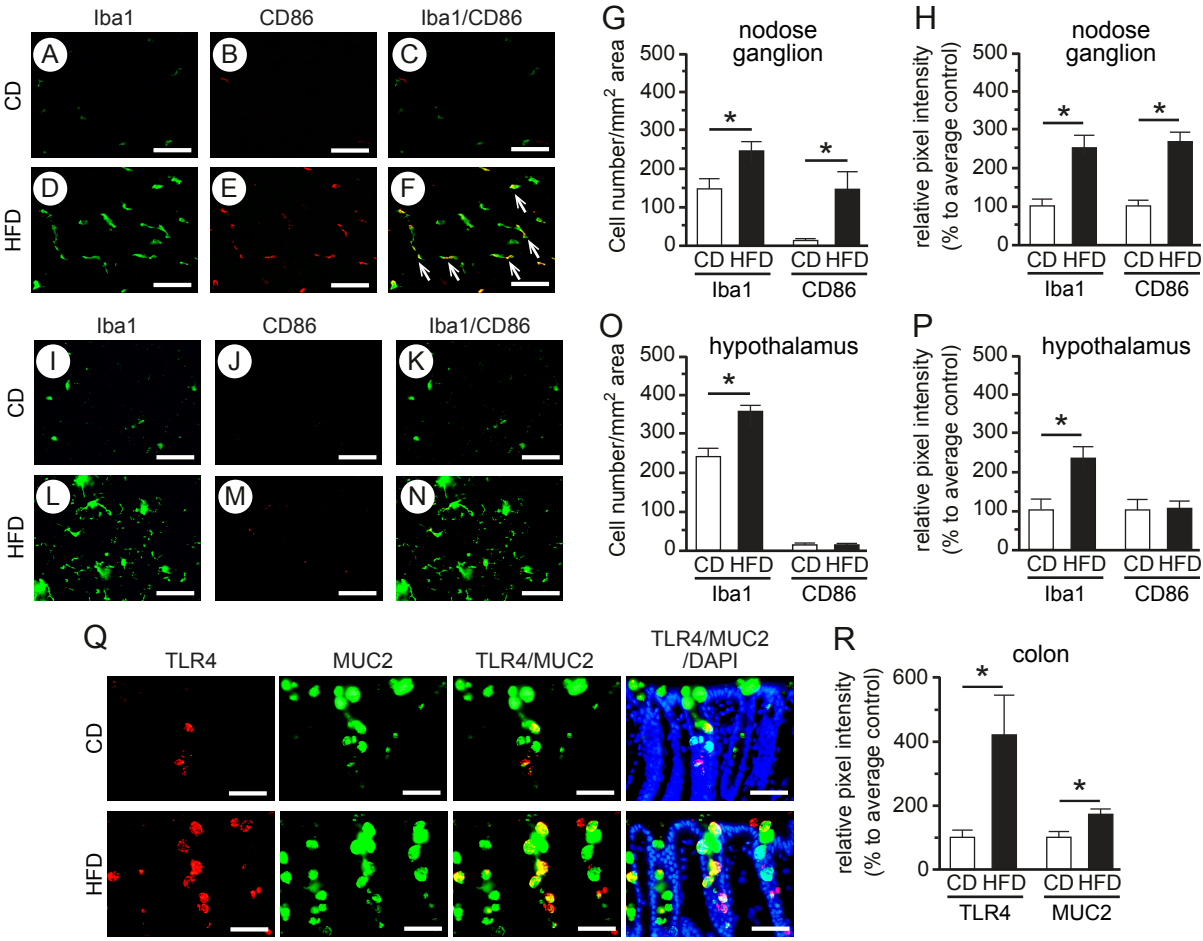
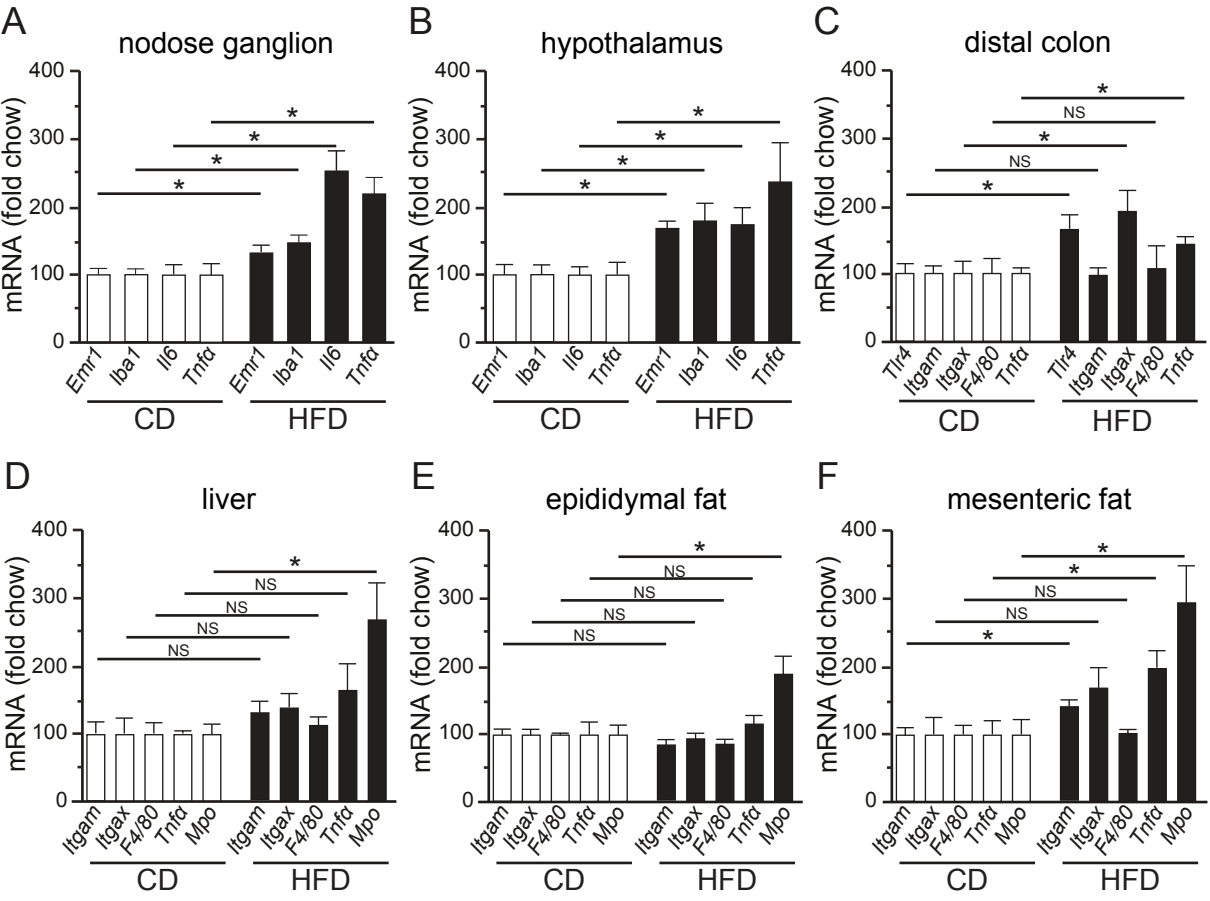


Fig. 1. Waise *et al.*



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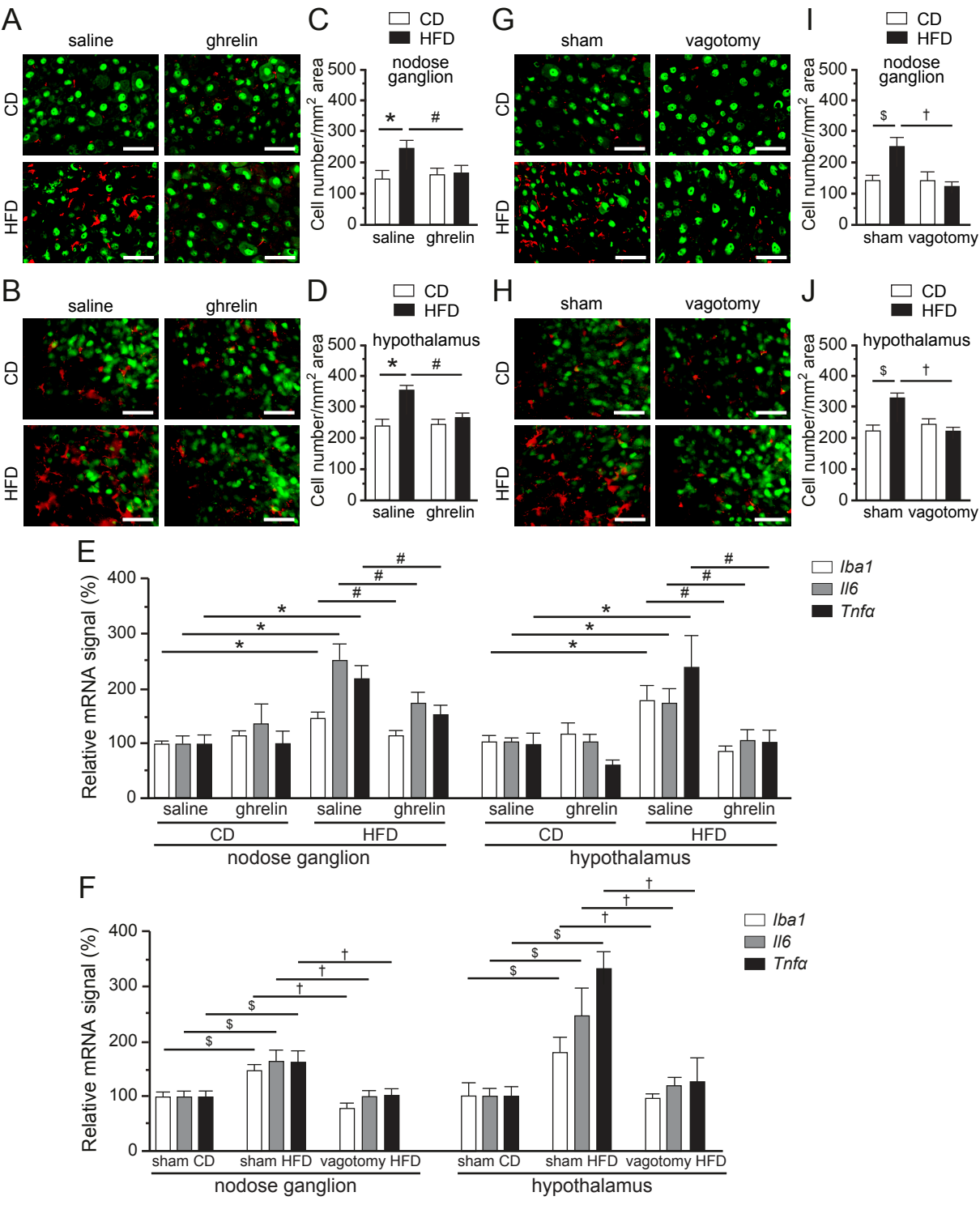

Fig. 3. Waise *et al.*