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Roux-en-Y gastric bypass surgery triggers rapid DNA fragmentation in vagal afferent neurons in rats

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Previous studies have shown that Roux-en-Y gastric bypass (RYGB), one of the most effective weight loss treatments for obesity, results in neurodegenerative responses in vagal afferent gut-brain connection reflected by microglia activation and reduced sensory input to the nucleus tractus solitarius (NTS). However, it is not known whether RYGB-induced microglia activation is the cause or an effect of the reported neuronal damage. Therefore, the aim of this study was to establish the order of neurodegenerative responses in vagal afferents after RYGB in the nodose ganglia (NG) and NTS in male and female rats. Sprague-Dawley rats were fed regular chow or an energy-dense diet for two weeks followed by RYGB or sham surgery. Twenty-four hours later, animals were sacrificed and NG and NTS were collected. Neuronal cell damage was determined by TUNEL assay. Microglia activation was determined by quantifying the fluorescent staining against the ionizing calcium adapter-binding molecule 1. Reorganization of vagal afferents was evaluated by fluorescent staining against isolectin 4. Results of the study revealed significantly increased DNA fragmentation in vagal neurons in the NG when observed at 24 h after RYGB. The surgery did not produce rapid changes in the density of vagal afferents and microglia activation in the NTS. These data indicate that decreased density of vagal afferents and increased microglia activation in the NTS likely ensue as a result of RYGB-induced neuronal damage.

Key words: obesity, neurodegeneration, vagus, nodose ganglion, hindbrain, microglia, Roux-en-Y gastric bypass

INTRODUCTION

In 2016, the prevalence of obesity was 39.8% in adults and 18.5% among children and adolescents in the United States (Hales et al., 2017). Worldwide, more than 1/3 of adults are overweight or obese (Engin, 2017). Obesity is a multifactorial, chronic inflammatory disease associated with increased risk of developing metabolic syndrome, cardiovascular disease, and type II diabetes (Heron, 2018). In addition, increased adiposity has been shown to have a positive correlation with levels of inflammatory markers, including tumor necrosis factor α (TNFα), interleukin 6 (IL-6), and high sensitivity C-reactive protein (Bahceci et al., 2007; Ferrante 2007; Marques-Vidal et al., 2012).

Roux-en-Y gastric bypass (RYGB) is currently one of the most common procedures, along with vertical sleeve gastrectomy (Kizy et al., 2017, Mulla et al., 2018). Even though the hormonal changes following bariatric surgery are well understood (Meek et al., 2016; Alamuddin et al., 2017), its effects on reorganization of the vagal gut-brain connections are largely unknown (Gautron et al., 2013). Understanding neuroanatomical changes that underlie the beneficial weight-loss effect and/or other side effects of bariatric surgery is of paramount importance for the development of non-surgical therapeutic procedures.

Sensory information from the stomach to the brainstem is carried by gastric vagal afferents (Altschuler et al., 1989; Berthoud and Powley, 1992), and the significance of the integrity of this system for the

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control of ingestive behavior has been previously described (Norgren, 1983; Schwartz, 2000; Ritter, 2004). Approximately 70-80% of vagal fibers are sensory fibers whose cell bodies are located bilaterally in the inferior ganglia of the vagus nerve (nodose ganglia; NG) (Czaja et al., 2006). These afferents innervate the abdominal viscera (Prechtl and Powley, 1990; Berthoud and Powley, 1992) and make their first synapse in the nucleus tractus solitarius (NTS) (Berthoud and Powley, 1992). In turn, NTS neurons project to dorsal motor nucleus of the vagus (DMV) cells to provide preganglionic control of cholinergic excitatory as well as non-adrenergic, non-cholinergic inhibitory postganglionic neurons (Broussard and Altschuler, 2000). The efferent innervation to the stomach originates from the DMV (Kirchgessner and Gershon, 1989; Berthoud et al., 1991; Moran et al., 1997) and the majority of DMV neurons project to the myenteric plexus, with the highest density of efferent fibers terminating in the stomach (Berthoud et al., 1991). During the RYGB procedure, the stomach is cut to form a gastric pouch causing unavoidable damage to afferent and efferent gastric vagal branches close to their origin from the esophageal plexus (Skandalakis et al., 1980), making the procedure a partial vagotomy. Thus, it is likely that sensory input from the gastrointestinal (GI) tract, operating via the vagus nerve to selectively influence food intake, is altered after the procedure (Berthoud et al., 2011).

Previous studies from our laboratory have revealed that subdiaphragmatic vagotomy triggers transient withdrawal and remodeling of central vagal afferent terminals in the NTS observed ten days post-surgery (Peters et al., 2013). Moreover, Ballsmider et al. (2015) showed that ten days after RYGB surgery the density of vagal afferents is dramatically reduced and microglia activation is significantly increased in the NTS. Furthermore, 14 days following subdiapgragmatic vagotomy, we observed increase microglia activation, an inflammatory marker, in both the hindbrain and NG (Gallaher et al., 2012). These observations collectively suggest that the effects of RYGB surgery may be modulated, in part, due to alterations of the anatomical integrity of vagal innervation between the GI tract and the hindbrain feeding centers. However, it is not known whether RYGB-induced microglia activation is the cause or an effect of the reported neuronal damage. Therefore, the aim of this study was to establish the order of neurodegenerative responses in vagal afferents after RYGB in the NG and NTS in male and female rats. We tested the hypothesis that RYGB surgery induces neurodegeneration initially in the periphery (vagal perikarya located in the NG) rather than more central locations, i.e. NTS. We further hypothesized that RYGB triggers rapid withdrawal of vagal afferents and increases microglia activation in the NTS. Because previous studies have shown that markers of injury and neuronal death are expressed early after injury (Shortland et al., 2006; Czaja et al., 2008), we aimed to evaluate neuronal damage in vagal afferent gut-brain connection 24 h after RYGB surgery.

METHODS

Animals

Sprague-Dawley male (n=25; 300g) and female rats (n=20; 250 g; Envigo, Indianapolis, IN) were housed individually in conventional polycarbonate shoe-box cages in a temperature-controlled vivarium with ad libitum access to standard pellets of rat chow (PicoLab rodent diet 20, product #5053, Fort Worth, TX) and water. Rats were maintained on a 12: 12 h light: dark cycle with lights on at 07: 00 h and allowed to acclimate to laboratory conditions for one week prior to starting experiments. All animal procedures were approved by the University of Georgia Institutional Animal Care and Use Committee and conformed to National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Food intake, body weight, and body composition

Following the acclimation period, half of the animals (males n=13, females n=10) were switched to an energy-dense diet (45% calories from fat, Research Diet #D12451, New Brunswick, NJ). The remaining animals were kept on standard pellets of rat chow. Food intake and body weight were measured twice a week for two weeks. Briefly, pre-weighed food (~50 g) was provided in standard stainless steel hoppers. Twenty-four hours later, the amount of food remaining in the food hopper, including spillage, was recorded. A Minispec LF 110 BCA Analyzer (Bruker Corp., The Woodlands, TX) was used to measure body composition in minimally restrained, non-anesthetized animals. The Minispec is a body composition analyzer based on time-domain nuclear magnetic resonance technology, which provides absolute masses for fat, lean tissue, and water (Künnecke et al., 2004).

Surgery

General anesthesia was used for all surgical procedures. The RYGB operation was performed as previously

described (Hajnal et al., 2010). The day before surgery, rats were fasted overnight. On the day of surgery, rats were weighed and then anesthetized with isoflurane (3% for induction, 1.5% for maintenance). The animals were kept on a temperature-controlled surgical board (38°C) in dorsal recumbency. Ceftriaxone 100 mg/kg IM (Roche, Nutley, NJ) was given as a prophylactic antibiotic. Surgical incisions were injected with 0.5 mL of 0.25% bupivacaine to minimize postoperative discomfort. Under sterile conditions, a midline laparotomy was performed. Next, the stomach was divided by using an ENDOPATH ETS 35 mm straight endocutter (Ethicon Endo-Surgery, Inc.). The staple line on the lesser curvature was placed 2-3 mm below the gastroesophageal junction. On the greater curvature, it was placed such that the resulting gastric pouch represented ~20% of the original stomach size. The abdominal incision was closed with 3-0 polyglycolic acid interrupted sutures in two layers (muscles and skin). All rats were injected subcutaneously with normal saline (50 mL/kg immediately after the operation).

Rats undergoing a sham operation were used as controls. The sham operation consisted of midline laparotomy and intestinal manipulation without stomach/gut resection followed by abdominal closure.

Tissue processing

Twenty-four hours after the operation, rats were anesthetized with CO_2 and transcardially perfused with 0.1 M phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde. Hindbrains and nodose ganglia were harvested, post-fixed in 4% paraformaldehyde for 2 h, and immersed in 18% sucrose and 0.1% NaN3 (Sigma-Aldrich; pH 7.4) in PBS overnight. Hindbrains were cryosectioned (Leica CM1950, Leica Biosystems, Wetzlar, Germany) at 20 µm throughout the caudal to rostral extent of the NTS (between bregma – 14.64 and – 12.96 mm). Nodose ganglia were cyrosectioned at 15 µm. Tissue sections were stained for selected antigens (Table I).

DNA fragmentation in nodose ganglia

To allow for proper identification of neurons and detection of early stages of apoptosis, nodose ganglia sections were incubated overnight with a primary antibody against neurofilament medium (NFM, Abcam cat # ab179870, UniProtKB – P12839) followed by Alexa-488 secondary antibody (ThermoFisher Cat#A-21206, RRDI: AB_141708). After, TUNEL assay (In Situ Cell Death Detection Kit, TMR red, Roche) was performed according to manufacturer's instructions. This assay is based on detection of single- and double-stranded DNA breaks that occur in the early stages of apoptosis. The total number of neurons exhibiting NFM staining and total number of TUNEL positive nuclei were manually counted under a fluorescent microscope.

Microglia activation and fiber density

We used standard immunofluorescence to determine microglia activation and vagal afferent density in the hindbrain. Hindbrain sections were incubated overnight with a primary antibody against ionized calcium binding adaptor molecule 1 (Iba1, Wako Cat#019-19741, RRDI: AB_839504) followed by Alexa-488 secondary antibody for 2 h to visualize microglia activation as previously described (Gallaher et al., 2012). In addition, hindbrain sections were incubated with GSL I - isolectin B4 biotin-conjugated (IB4, Vector Laboratories Cat#B-1205, RRDI: AB_2314661) overnight followed by ExtrAvidin-CY3 (Sigma-Aldrich Cat#E-4142) for 2 h to visualize primary unmyelinated vagal afferents innervating the GI tract as previously described (Shehab, 2009). Sections were mounted in ProLong (Molecular Probes, OR).

Table I. List of antibodies used in this study.

Primary antibody/reagent target	Source	Catalog no.	Application (dilution)	Secondary Antibody
Biotinylated Griffonia (Bandeirafa) Simplicifolia Lectin I Isolectin B4	Vector Laboratories	B-1205	IF: 1: 400	ExtrAvidin-Cy3 Conjugate (Sigma; cat # E 4142) 1: 600 dilution
Ionized Calcium Binding Adaptor Molecule 1 (Iba1)	Wako	019-19741	IF: 1: 1000	Alexa 488 Donkey Anti-Rabbit (Invitrogen by Thermo Fisher Scientific; cat # A21206) (1: 400)
150 kDa Neurofilament Medium antibody – C-terminal	Abcam	AB179870	IF: 1: 1000	Alexa 488 Donkey Anti-Rabbit

Immunofluorescence quantification

Sections were examined under a Nikon 80-I fluorescent microscope. The area fraction of Iba1 and IB4 immunofluorescence was analyzed using Nikon Elements AR software as previously described (Peters et al., 2013). For each studied region, three representative sections from each animal were used to calculate an average exposure time and background fluorescence level as determined by the pixel intensity of stained tissue regions that were negative for each stain. Subsequently, 20x-stitched images of the hindbrain (sections/hindbrain; evenly spaced throughout the rostro-caudal extent of the NTS) were created using this fixed/standardized exposure time. In hindbrain sections, regions of interest (ROIs) were drawn to isolate the NTS.

Statistical analysis

GraphPad Prism 7 (GraphPad Software, Inc.) was used to conduct statistical analyses. Data are expressed as mean ± SEM and were analyzed using ANOVA followed by Holm-Sidak multiple comparisons test. Alpha value for statistical significance was set at 0.05. Tissues from male and female rats were processed at different times. Thus, to avoid erroneous interpretation of results that could have arisen as a result, data from males and females was not subjected to direct comparison.

RESULTS

Energy-dense diet consumption increased body weight and body fat accumulation

Group means for caloric intake per 100 g of body weight, body weight, and body fat mass are shown in Fig. 1. In males, two-way RM ANOVA on daily caloric intake per 100 g of body weight showed a main effect of time ($F_{1, 23}$=39.6, P<0.00) and a significant interaction between time and diet ($F_{1, 23}$=192.1, P<0.0001) (Fig. 1A). Male rats on chow ate significantly more calories during week two than week one. In contrast, animals on E-D diet consumed significantly more calories during week one compared to week two. During week one, males on E-D diet ate significantly more compared to those on chow (P<0.0001), but during week two they ate significantly less than the chow group (P<0.0001). Two-way RM ANOVA on body weight showed a main effect of time ($F_{2, 46}$=227.4, P<0.0001) and a significant interaction ($F_{2, 46}$=11.8, P<0.0001) (Fig. 1B). Compared to baseline (day 0), body weight was significantly higher at the end of weeks one and two, independent of diet. Male rats fed the E-D diet were significantly heavier than those fed chow at the end of week one and week 2, but not day 0. Two-way RM ANOVA on body fat percent showed a main effect of time ($F_{2, 46}$=3.4, P=0.043), diet ($F_{1, 23}$=5.7, P=0.026), and a significant interaction between time and diet ($F_{2, 46}$=17.9, P<0.0001) (Fig. 1C). Compared to baseline, males on chow had significantly lower body fat percent at the end of week two. Males on E-D had significantly higher body fat percent compared to baseline at the end of week one. Animals on E-D diet had a significantly higher body fat percent at the end of weeks one and two compared to animals on chow.

In females, two-way RM ANOVA on daily caloric intake per 100 g of body weight showed a main effect of time ($F_{1, 18}$=239, P<0.0001), diet ($F_{1, 18}$=50.9, P<0.0001), and a significant interaction ($F_{1, 18}$=98.7, P<0.0001) (Fig. 1D). Females on chow and E-D diet ate significantly more calories during week one than week two. During week one, animals on E-D diet ate significantly more compared to those on chow (P<0.0001). There was no significant difference in intake during week 2. Two-way RM ANOVA on body weight showed a main effect of time ($F_{2, 36}$=15.7, P<0.0001), diet ($F_{1, 18}$= 8.6, P<0.0001), and a significant interaction ($F_{2, 36}$=8.1, P=0.0012) (Fig. 1E). In females maintained on chow, there was no significant change in body weight. Animals on E-D diet were significantly heavier at the end of weeks one and two compared to day 0. Female rats on E-D diet were significantly heavier at the end of weeks one and two compared to those on chow. Two-way RM ANOVA on body fat percent showed a main effect of time ($F_{2, 36}$=16.5, P<0.0001), diet ($F_{1, 18}$=17.3, P=0.0006) and a significant interaction ($F_{2, 36}$=24.6, P<0.0001) (Fig. 1F). Female rats on E-D diet had a significantly higher body fat percent at the end of weeks one and two compared to day 0, and to animals on chow.

Roux-en-Y gastric bypass surgery induced DNA fragmentation in the nodose ganglia

Twenty-four hours after RYGB surgery, there was a significantly higher percentage of TUNEL positive nuclei in the nodose ganglia of both male and female rats, independent of diet (Fig. 2). One-way ANOVA revealed a significant difference between RYGB and sham rats in males ($F_{4, 30}$= 2.6, P<0.0001) and females ($F_{4, 35}$=24.3, P<0.0001). Results showed that in RYGB male rats maintained on chow, 50.4% of the total number of nuclei were TUNEL positive compared to 22.4% in sham animals (P=0.001). In RYGB male rats maintained on E-D diet, 59.8% of the total number of nuclei were TUNEL positive compared to 28.7% in sham animals (P<0.0001). Similarly, in RYGB female rats maintained on chow, 56.6% of the total number of nuclei were TUNEL positive compared to 28.4% in sham rats (P=0.0006). In RYGB female rats maintained on E-D diet,

61.7% of the total number of nuclei were TUNEL positive compared to 27.6% in sham animals ($P<0.0001$).

Roux-n-Y gastric bypass surgery significantly decreased the density of vagal afferents in female rats fed an energy-dense diet

Analysis of binary area fraction of IB4-labeled afferents in the caudal (C-NTS), intermediate (I-NTS), and rostral NTS (R-NTS) revealed that the density of vagal afferents was affected by RYGB surgery in female rats fed an E-D diet, but not in lean females or males (Figs 3-5). Two-way ANOVA on binary area fraction in caudal NTS of males revealed no significant difference between the groups. Twenty-four hours after RYGB, IB4 labeling in C-NTS of chow fed RYGB males was 0.29% + 0.08% compared to 0.53% + 0.2% in sham rats. In E-D fed males, the density of vagal afferents was 0.54% + 0.09% in RYGB rats compared to

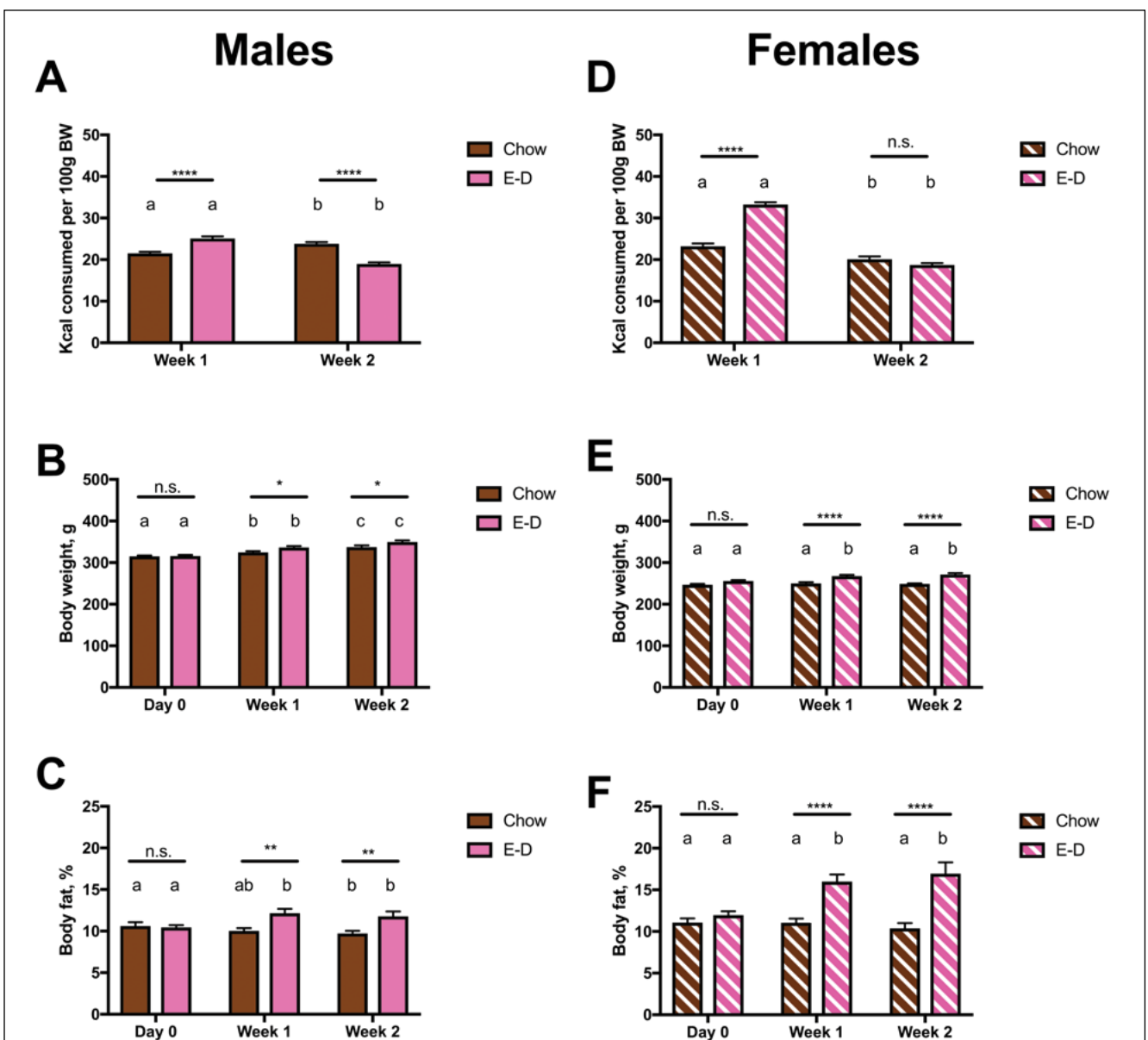

Fig. 1. Food intake, body weight, and fat mass of male and female rats on chow (brown bars) or energy-dense diet (E-D; pink bars). Shown are mean + SE kcal consumed per 100 g of body weight (A, D), body weight (B, E), and body fat (C, F) for males (left column; n=12 chow and n=13 E-D; solid bars) and females (right column; n=10 for chow and E-D; striped bars). Consumption of E-D diet increased body weight and fat mass in male and female rats within one week of diet consumption. Letters (a, ab, b) denote statistical significance between time points within the diet groups. Asterisks indicate statistical significance between diet groups. P<0.05.

0.19% + 0.04% in sham rats (Fig. 3). Two-way ANOVA on binary area fraction in intermediate NTS of males showed a main effect of surgical procedure ($F_{1, 15}$=4.9, P=0.043), but no effect of diet or interaction. IB4 labeling in I-NTS of chow fed RYGB males was 1.96% + 0.5% compared to 2.24% + 0.7% in sham rats. In E-D

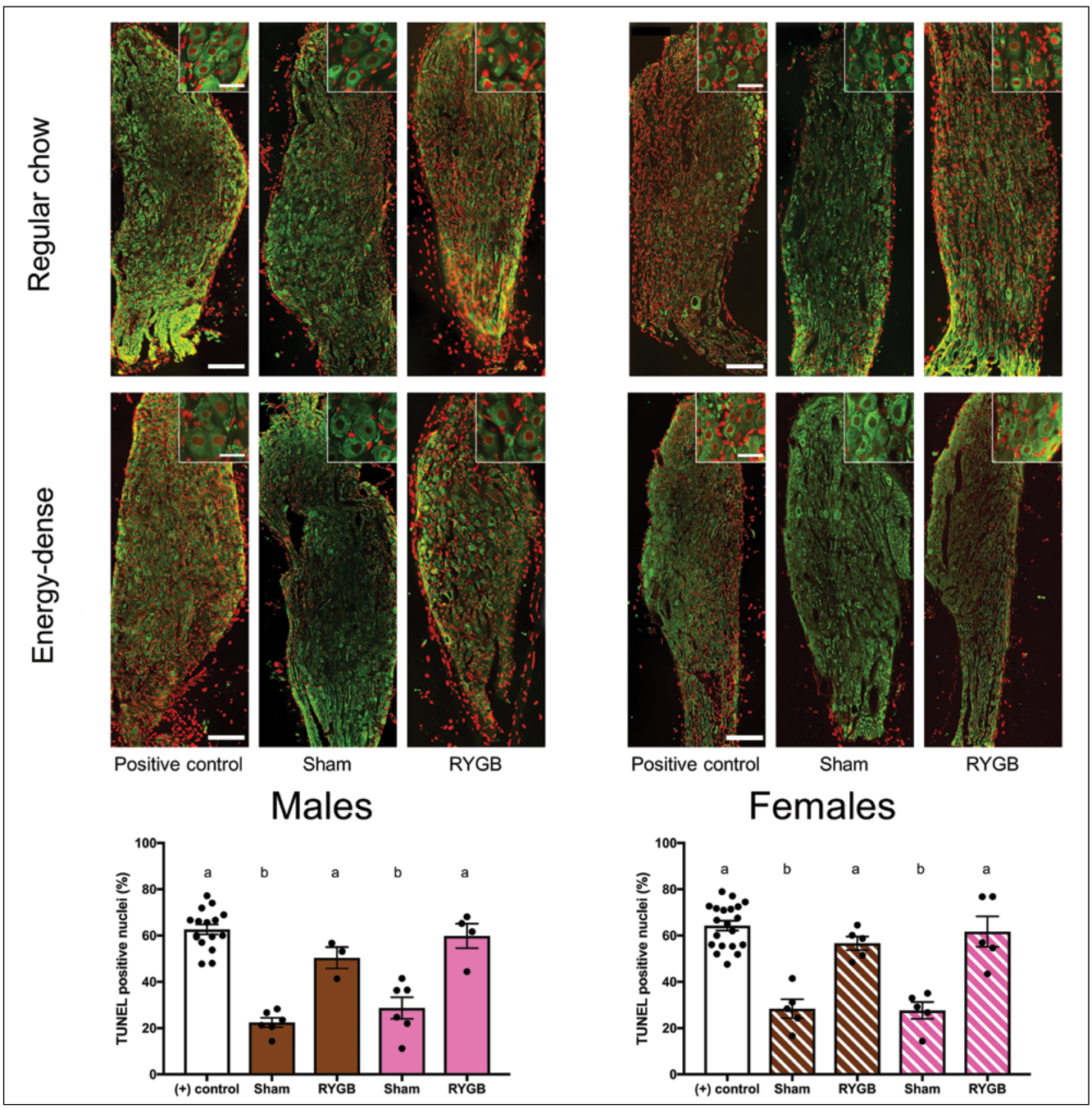

Fig. 2. Roux-en-Y gastric bypass (RYGB) surgery induced rapid DNA fragmentation in the nodose ganglion neurons. Representative sections of the nodose ganglion of animals fed regular chow (top row, brown bars) and an energy-dense diet (bottom row, pink bars) are shown. Analysis of DNA fragmentation by TUNEL staining of male (left panel, solid bars) and female (right panel, striped bars) rats. The positive control section was treated with DNase to induce DNA fragmentation and asses the validity of the assay. Double staining was performed using polyclonal primary antisera against neurofilament medium. Scale bar=200 μm (insets: scale bar=50 μm). Graphs show mean + SEM percentage of the total number of neurons in the nodose ganglia that were TUNEL positive in regular chow (brown bars) and energy-dense (pink bars) fed animals. The percentage of TUNEL positive nuclei was significantly higher in male and female rats that underwent RYGB surgery compared to sham-operated rats independent of diet. Bars denoted with different letters (a, b) differ significantly (P<0.05).

fed males, the density of vagal afferents was 0.99% + 0.3% in RYGB rats compared to 2.97% + 0.4% in sham rats (Fig. 4). Two-way ANOVA on binary area fraction in rostral NTS of males revealed a significant interaction between diet and surgical procedure ($F_{1, 18}$= 5.2, P=0.035), but no main effect. IB4 labeling in R-NTS of chow fed RYGB males was 0.75% + 0.3% compared to 0.48% + 0.1% in sham rats. In E-D fed animals, the density of vagal afferents was 0.68% + 0.1% in RYGB rats compared to 1.71 + 0.4% in sham rats. The density of vagal afferents was significantly higher in E-D fed sham males than in chow fed sham males (P=0.041) (Fig. 5).

In females, similar results were observed. Two-way ANOVA on binary area fraction in caudal NTS of females showed a main effect of diet ($F_{1, 15}$=5.9, P=0.028), but no effect of surgical procedure or interaction Twenty-four hours after RYGB, IB4 labeling in C-NTS of chow fed RYGB females was 1.15% + 0.4% compared to 1.72% + 0.4% in sham rats. In E-D fed females, the density of vagal afferents was 0.62% + 0.1% in RYGB rats compared to 0.75% + 0.2% in sham rats (Fig. 3). Two-way ANOVA on binary area fraction in intermediate NTS of females showed a main effect of diet ($F_{1, 15}$=11.7, P=0.0038), but no effect of surgical procedure or interaction. IB4 labeling in I-NTS of chow fed RYGB females was 6.05% + 0.9% compared to 7.38% + 1.1% in sham rats. In E-D fed females, the density of vagal afferents was 2.95% + 0.3% in RYGB rats compared to 4.27% + 0.8% in sham rats. The density of afferents was significantly lower in E-D fed RYGB rats than in chow fed sham animals (P=0.026) (Fig. 4). A similar trend was observed in males, but it failed to reach significance. Two-way ANOVA on binary area fraction in rostral NTS of females revealed no significant difference between the groups. IB4 labeling in R-NTS of chow fed RYGB females was 1.49% + 0.7% compared to 1.71% + 0.2% in sham rats. In E-D fed females, the density of vagal afferents was 1.51% + 0.4% in RYGB rats compared to 1.11% + 0.2% in sham rats (Fig. 5).

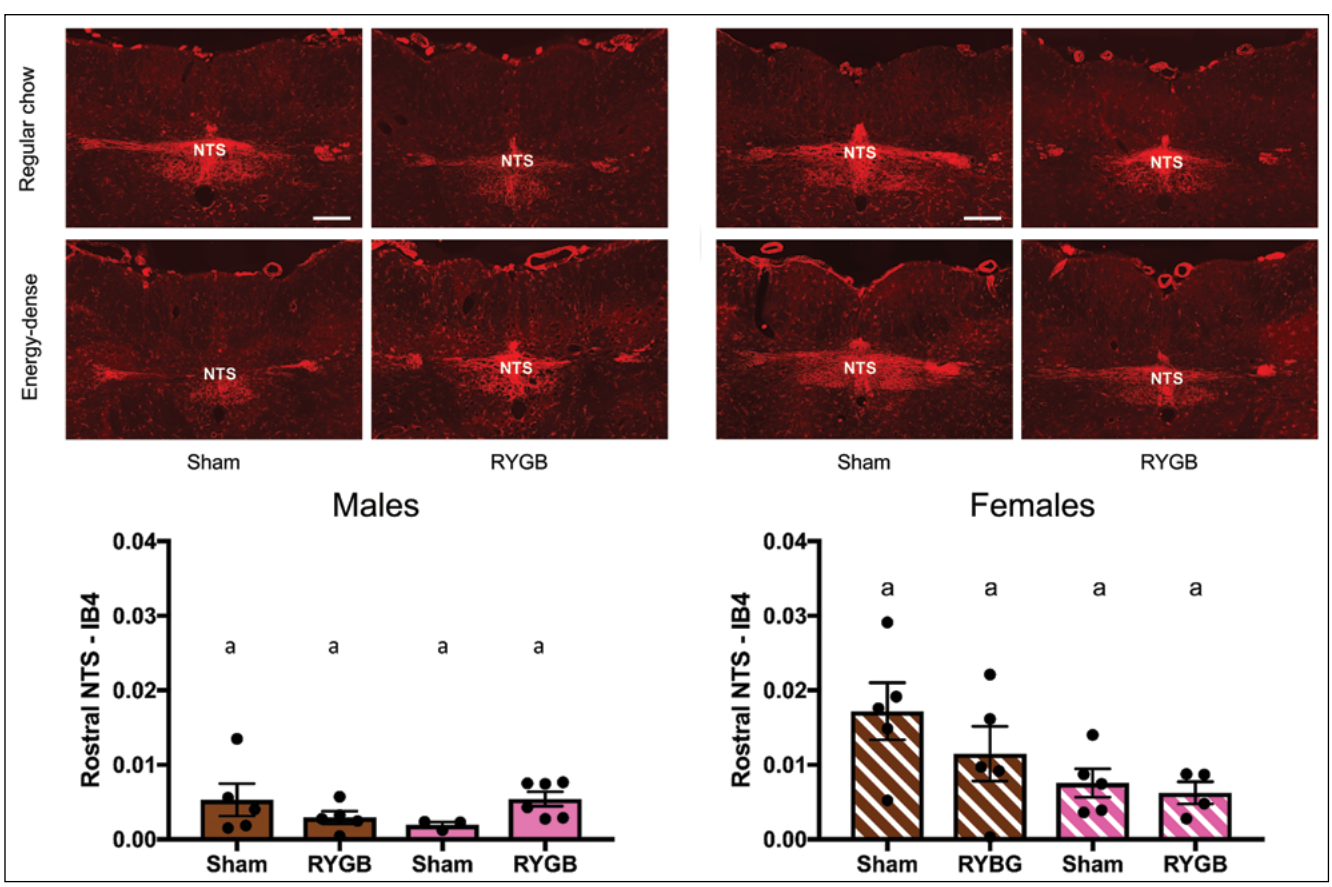

Fig. 3. Roux-en-Y gastric bypass surgery did not affect the density of vagal afferents in the caudal NTS. Representative sections of the caudal NTS of animals fed regular chow (top row, brown bars) and an energy-dense diet (bottom row, pink bars) are shown. Binary analysis of the area fraction of IB4-labelled vagal afferents of male (left panel, solid bars) and female (right panel, striped bars) rats revealed no significant differences in the density of labelled afferent terminals between rats that underwent RYGB surgery compared to sham-operated rats independent of diet. Graphs show mean + SEM. NTS=nucleus tractus solitarius. Scale bar=200 μm. Bars denoted with different letters (a, b) differ significantly (P<0.05).

Consumption of an energy-dense diet, but not Roux-n-Y gastric bypass surgery, triggered microglia activation in the caudal NTS

Immunostaining against Iba1 showed that RYGB surgery did not trigger microglia activation in the C-NTS, I-NTS, and/or R-NTS in male and female rats (Figs 6-8). Two-way ANOVA on binary area fraction in caudal NTS of males showed a main effect of diet ($F_{1, 17}$=9.1, P=0.0077), but no effect of surgical procedure or interaction. In C-NTS, the binary area fraction of fluorescent staining against Iba1 in chow fed RYGB males was 0.00025% + 0.00025% compared to 0.0069% + 0.0044% in sham males. In E-D fed RYGB males, the area fraction of fluorescent staining was 0.049% + 0.02% *vs.* 0.094% + 0.02% in sham males (Fig. 6). Two-way ANOVA on binary area fraction in intermediate NTS of males revealed no significant difference between the groups. In I-NTS, the binary area fraction of Iba1 in chow fed RYGB males was 0.27% + 0.1% compared to 0.34% + 0.1% in sham rats. In E-D fed RYGB males, the area fraction of fluorescent staining was 0.49% + 0.2% *vs.* 0.79% + 0.2% in sham males (Fig. 7). Two-way ANOVA on binary area fraction in rostral NTS of males revealed no significant difference between the groups. In R-NTS, the binary area fraction of fluorescent staining against Iba1 in chow fed RYGB males was 0.22% + 0.1% compared to 0.42% + 0.1% in sham males. In E-D fed RYGB males, the area fraction of Iba1 was 0.36% + 0.1% *vs.* 0.70% + 0.2% in sham males (Fig. 8). There was a trend towards decreased microglia activation in RYGB male rats, but it failed to reach significance.

In females, two-way ANOVA on binary area fraction in caudal NTS of females showed a main effect of diet ($F_{1, 15}$=8.4, P=0.01) and a significant interaction ($F_{1, 15}$=6.2, P=0.0247). In C-NTS, the binary area fraction of fluorescent staining against Iba1 in chow fed RYGB females was 0.022% + 0.01% compared to 0.012% + 0.009% in sham females. In E-D fed RYGB females, the area fraction of fluorescent staining was 0.032% + 0.006% *vs.* 0.15% + 0.04% in sham females (Fig. 6). Two-way ANOVA on binary area fraction in intermediate NTS of females

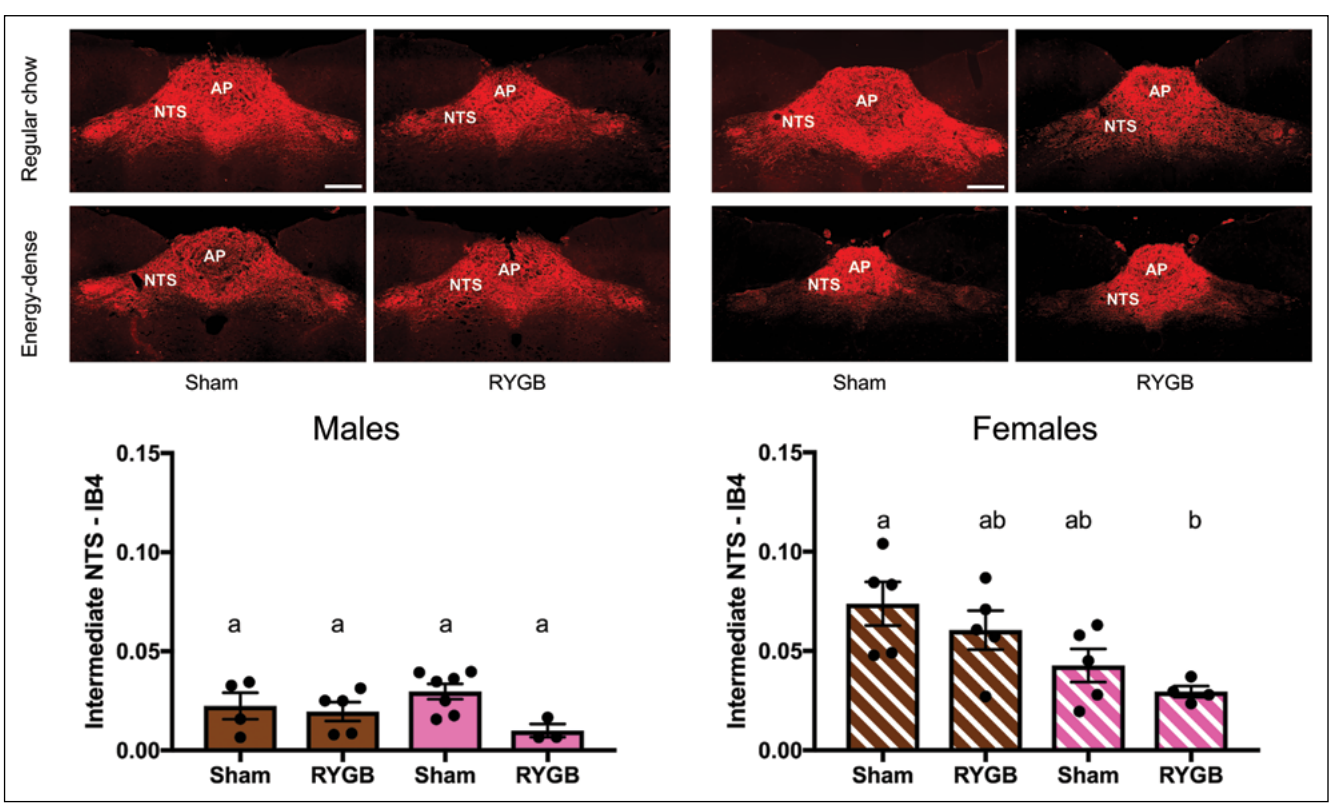

Fig. 4. Roux-en-Y gastric bypass surgery significantly decreased the density of vagal afferents in the intermediate NTS in female rats fed an energy-dense diet. Representative sections of the intermediate NTS of animals fed regular chow (top row, brown bars) and an energy-dense diet (bottom row, striped bars) are shown. Binary analysis of the area fraction of IB4-labelled vagal afferents of male (left panel, solid bars) rats revealed no significant differences in the density of labelled afferent terminals between rats that underwent RYGB surgery compared to sham-operated rats independent of diet. In females (right panel, striped bars), animals fed energy-dense diet and underwent RYGB surgery exhibited significantly less IB4-labelled terminal afferents than chow fed, sham-operated rats. Graphs show mean + SEM. Bars denoted with different letters (a, b) differ significantly (P<0.05). NTS=nucleus tractus solitarius; AP=area postrema. Scale bar=200 μm.

showed a main effect of diet ($F_{1, 15}$=14.8, P=0.0016), but no effect of surgical procedure or interaction. In I-NTS, the binary area fraction of Iba1 in chow fed RYGB female rats was 0.07% + 0.04% compared to 0.28% + 0.1% in sham females. In E-D fed RYGB females, the area fraction of fluorescent staining was 0.49% + 0.2% *vs.* 0.48% + 0.1% in sham female rats (Fig. 7). Two-way ANOVA on binary area fraction in rostral NTS of females showed a main effect of surgical procedure ($F_{1, 14}$=4.8, P=0.0466), but no effect of diet or interaction In R-NTS, the binary area fraction of fluorescent staining against Iba1 in chow fed RYGB females was 0.10% + 0.03% compared to 0.29% + 0.1% in sham females. In E-D fed RYGB female rats, the area fraction of fluorescent staining was 0.22% + 0.1% *vs.* 0.37% + 0.1% in sham females (Fig. 8).

DISCUSSION

In summary, results from this study showed that caloric intake increased significantly immediately after introduction of an E-D diet and leveled off within a week in both male and female rats. Consequently, rats on an E-D diet gained significantly more body fat than chow-fed rats. In addition, female rats fed an E-D diet gained significantly more body weight than their chow-fed counterparts did. Next, we evaluated neuroanatomical changes in the nodose ganglia and hindbrain 24 h after RYGB surgery. Our data showed that RYGB surgery induced rapid DNA fragmentation in nodose ganglia neurons independent of diet. However, RYGB did not affect the density of vagal afferents in the NTS. Furthermore, consumption of an E-D diet, but not RYGB significantly increased microglia activation in the caudal NTS. A similar trend was observed in the intermediate and rostral NTS, but it failed to reach significance.

Consistent with prior reports (Woods et al., 2003; Sen et al., 2017; Vaughn et al., 2017), our data showed that females and males consume significantly more calories when fed an E-D diet compared to chow. This increased intake is more pronounced during the first few days after the diet is introduced. Following acclimation to the E-D diet, caloric intake of E-D fed female rats was similar to chow fed female rats. In males, caloric intake during the second week of E-D feeding was significantly lower than in chow fed rats. This adjustment of caloric intake in rats fed an E-D diet has been previously reported (De Lartigue et al., 2011). Con-

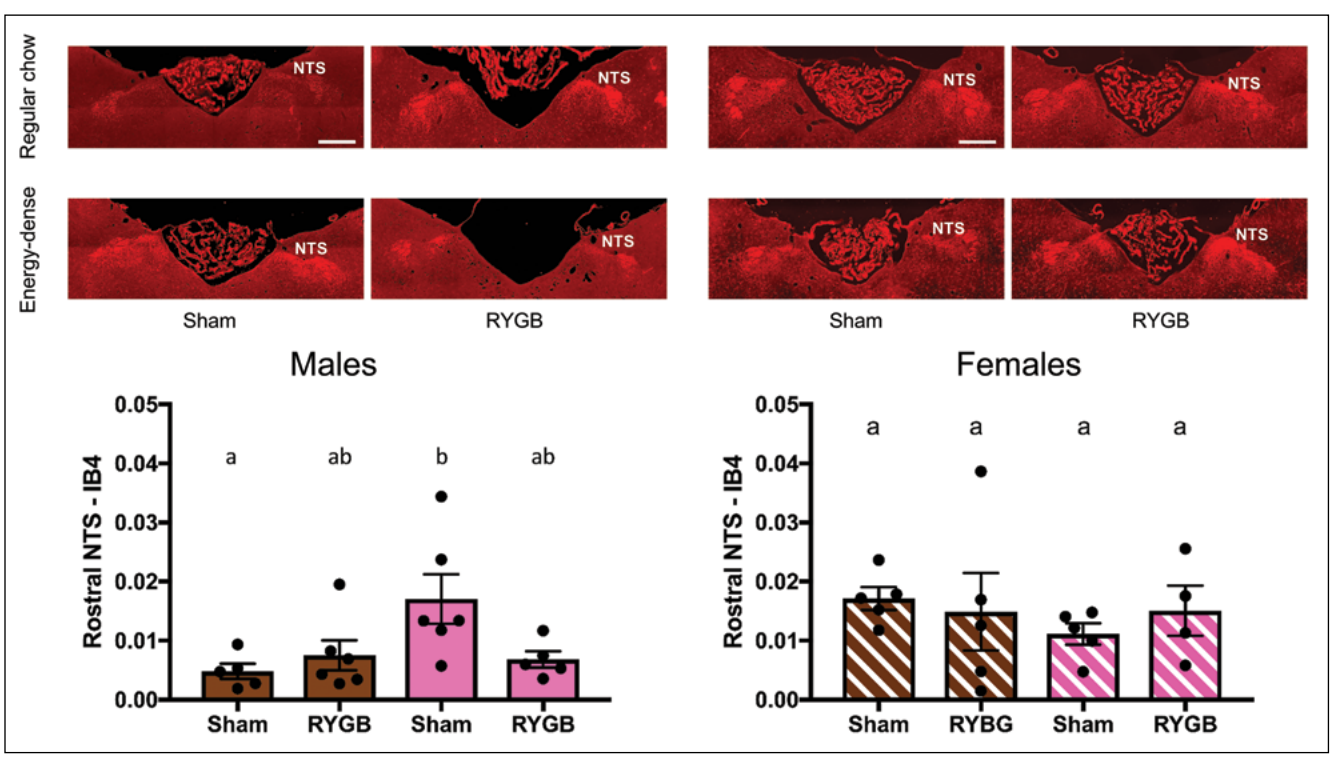

Fig. 5. Roux-en-Y gastric bypass surgery did not affect the density of vagal afferents in the rostral NTS. Representative sections of the rostral NTS of animals fed regular chow (top row, brown bars) and an energy-dense diet (bottom row, pink bars) are shown. Binary analysis of the area fraction of IB4-labelled vagal afferents of male (left panel, solid bars) rats showed that energy-dense fed, sham-operated rats had significantly density of IB4-labelled afferent terminals compared to chow fed, sham-operated animals. In females (right panel, striped bars), we observed no significant differences in the density of labelled afferent terminals between rats that underwent RYGB surgery compared to sham-operated rats independent of diet. Graphs show mean + SEM. Bars denoted with different letters (a, b) differ significantly (P<0.05). NTS=nucleus tractus solitarius. Scale bar=200 μm.

currently, we observed a significant increase in body weight and fat mass accumulation. Our data further shows that when fed chow, females and males consume roughly the same amount of calories per gram of body weight. However, when fed an E-D diet, the initial increase in intake is larger in females than in males (33 *vs.* 25 kcal/100 g BW, respectively). This difference in intake is likely responsible for the higher fat gain observed in females (12% → 17%) compared to males (10% → 12%) over the two-week feeding period.

Increased body fat accumulation has been shown to induce changes in vagal signaling (Daly et al., 2011; Kentish et al., 2013; 2014). In addition, we have previously reported that consumption of an E-D diet induces reorganization of vagal afferents in the NTS within seven days of introducing the diet (Sen et al., 2017; Vaughn et at. 2017). Also, while the majority of patients undergoing bariatric surgery are females (80%) (Kochkodan et al., 2018), most research investigating the effect of RYGB surgery is conducted in males. Thus, the present study was performed on female and male rats fed regular chow or an E-D diet in an attempt to dissect diet-induced and RYGB-induced vagal reorganization.

Peripheral nerve lesions typically result in long-lasting modifications and reorganization of the signaling pathway (Kaas, 1991; Wall et al., 2002; Kass and Collins, 2003; Navarro et al., 2007). We have previously reported that damage of vagal afferents via capsaicin administration or subdiaphragmatic vagotomy results in neuronal death observed as early as 24h post injury followed by neural proliferation and restoration of the neuronal population in the NG of rats (Czaja et al., 2008; Ryu et al., 2010; Gallaher et al., 2011). The current study revealed that partial damage to vagal afferents by RYGB surgery triggered a significant increase in DNA fragmentation, a marker of apoptosis, in vagal neurons located in the NG within 24 h. This response was observed in female and male, chow and E-D fed rats.

Our research group has conducted several studies to characterize the effects of consumption of energy-dense diets, subdiaphragmatic vagotomy, sleeve gastrectomy, and RYGB surgery on the density of vagal

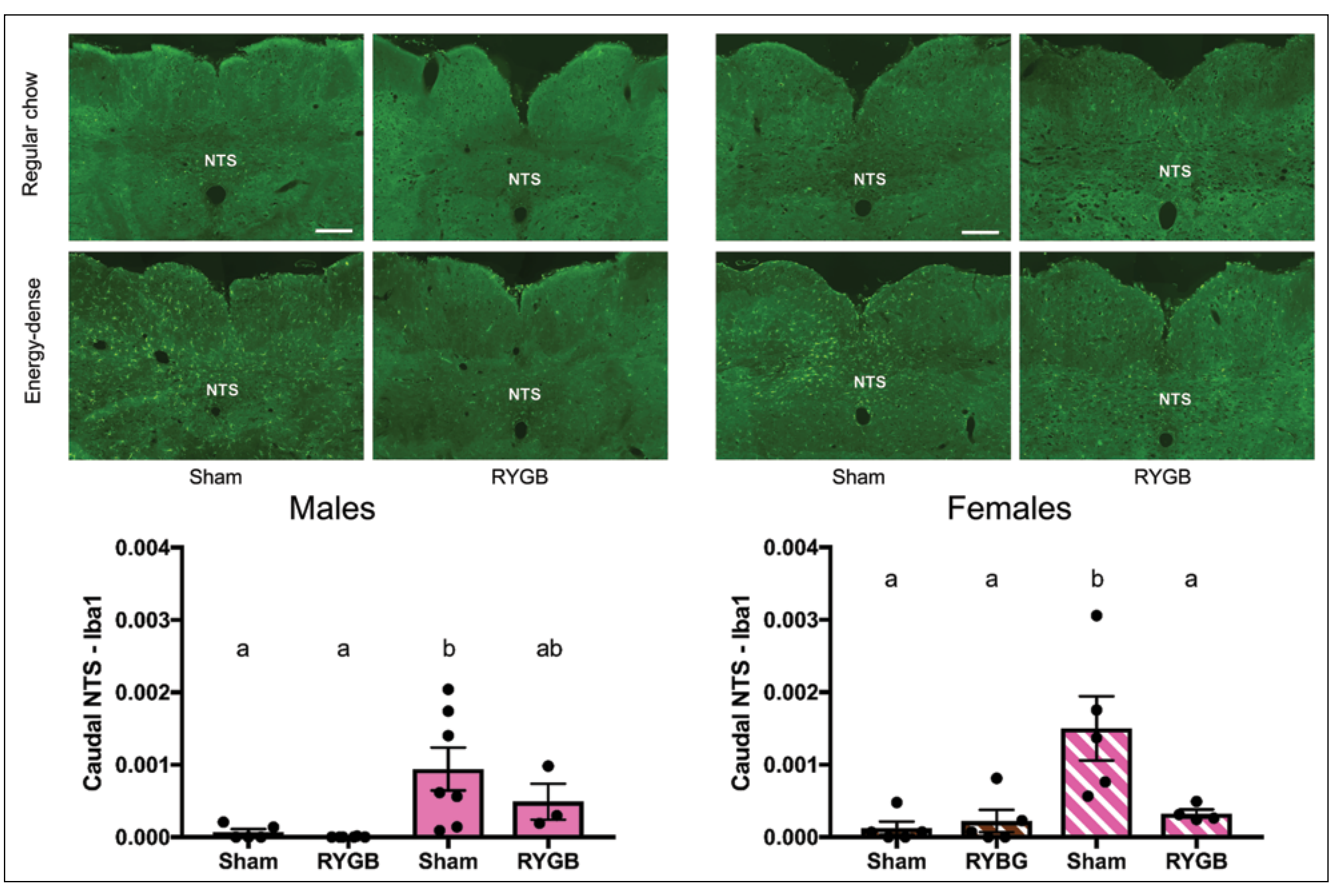

Fig. 6. Consumption of an energy-dense diet significantly increased microglia activation in the caudal NTS. Representative sections of the caudal NTS of animals fed regular chow (top row, brown bars) and an energy-dense diet (bottom row, pink bars) are shown. Binary analysis of the area fraction of Iba1 immunoreactivity revealed that chow fed female and male rats had significantly less microglia activation than energy-dense fed sham-operated animals. In female rats fed an energy-dense diet, RYGB significantly decreased microglia activation (right panel, striped bars). Graphs show mean + SEM. Bars denoted with different letters (a, b) differ significantly ($P<0.05$). NTS=nucleus tractus solitarius. Scale bar=200 μm.

afferent innervation and microglia activation in the intermediate NTS. Seven days after introduction of an E-D diet, there is a significant decrease in the density of vagal afferents in the NTS (Vaughn et al., 2017). Sen et al. (2017) reported that after four weeks of consuming a 45% fat, E-D diet, there were significantly less IB4-immunoreactive fibers in the NTS of rats. In addition, ten days post subdiaphragmatic vagotomy and RYGB surgery there is a significant decrease in the density of vagal afferents and increased microglia activation in the intermediate NTS (Peters et al., 2013; Ballsmider et al., 2015). In the current study, we quantified the density of vagal afferents in the caudal NTS (bregma -14.64 to -14.40) and at the level of the area postrema (intermediate NTS; bregma -14.28 to -13.68 mm) since most vagal afferents from the GI tract make their synapse at this level. We also quantified at the level of the rostral NTS (bregma -13.20 to -12.96) given that at this level there is an overlap between gustatory nerve fibers and vagal afferents (Hamilton and Norgren, 1984). Our data revealed no significant difference in the density of afferent fibers in the caudal, intermediate, and rostral NTS between RYGB and sham rats 24 h post-surgery. Furthermore, RYGB did not induce significant microglia activation in the intermediate and rostral NTS. Nonetheless, there was substantial individual variability in microglia activation observed in the hindbrain.

CONCLUSION

In conclusion, this study provides evidence that RYGB surgery triggers rapid plasticity in the vagal system, the primary route through which nutritional information travels from the GI tract to the NTS. Similar results were observed in both male and female rats. Although there could be many effects from non-vagal mediated mechanisms, e.g. inflammation, microbiota/nutrient byproducts acting directly on the NTS, the present data supports a neuronal ascending insult. We show that RYGB induced rapid and significant DNA fragmentation in the NG in male and female rats. Thus, taking these results and prior observations together, we conclude that the reorganization of the

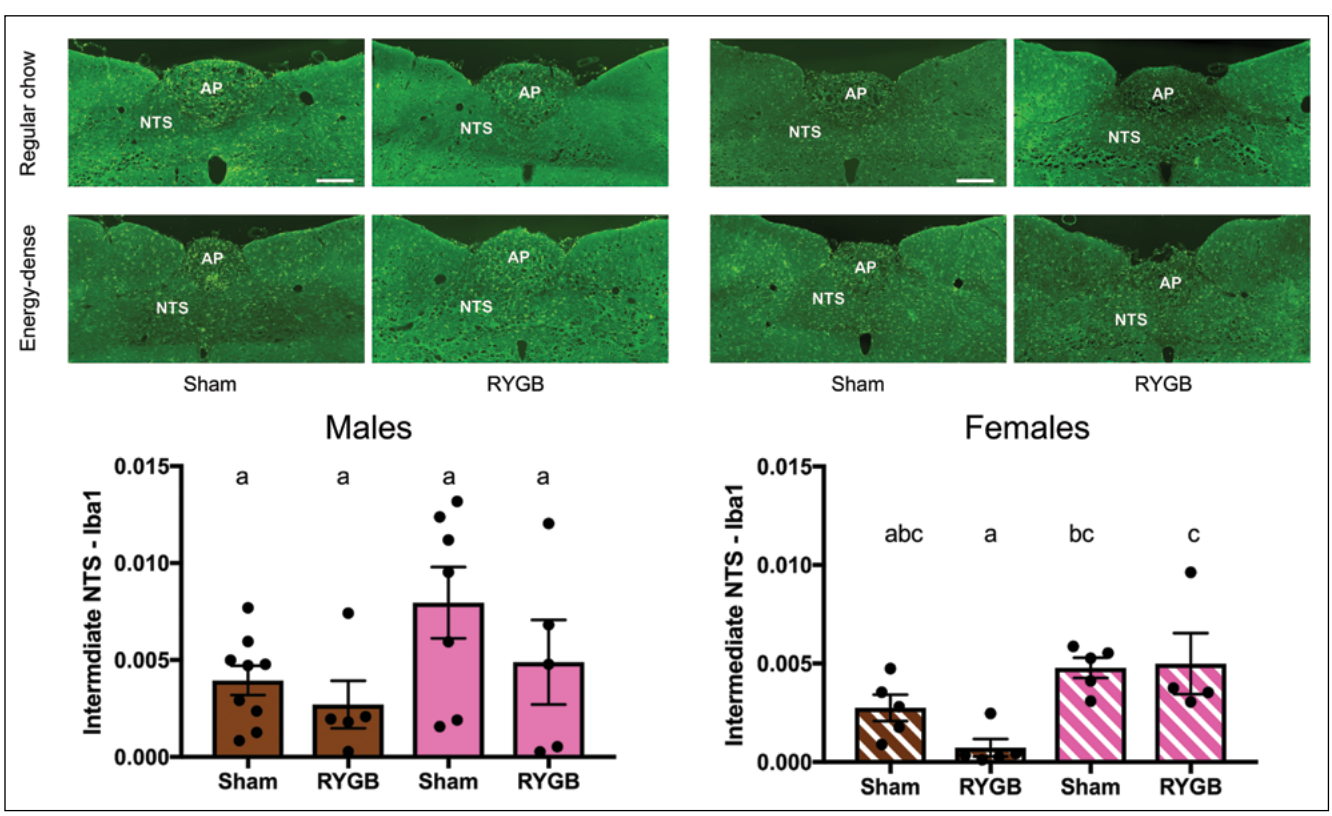

Fig. 7. Roux-en-Y gastric bypass surgery did not increase microglia activation in the intermediate NTS. Representative sections of the intermediate NTS of animals fed regular chow (top row, brown bars) and an energy-dense diet (bottom row, pink bars) are shown. Binary analysis of the area fraction of Iba1 immunoreactivity revealed that in male (left panel, solid bars) rats there were no significant differences in microglia activation between rats that underwent RYGB surgery compared to sham-operated rats, independent of diet. In females (right panel, striped bars), animals fed energy-dense diet and underwent RYGB surgery exhibited significantly more microglia activation than chow fed, RYGB-operated rats. Graphs represent mean + SEM Iba1 intensity. Bars denoted with different letters (a, b) differ significantly ($P<0.05$). NTS=nucleus tractus solitarius; AP=Area Postrema. Scale bar=200 μm.

vagal gut-brain communication system observed after RYGB is a multi-step cascade of events that develops progressively over the course of several weeks. During the first 24 h, RYGB induces DNA fragmentation in the vagus nerve perikarya located in the NG. This, in turn, triggers withdrawal of vagal afferents and increased microglia activation in the NTS, observed ten days post-surgery. It is likely that this reorganization of the gut-brain signaling partially underlies the observed phenotypical changes observed in subjects who undergo RYGB surgery. In future work, it is necessary to investigate the sequence of development of the degenerative response by evaluating the same endpoints at additional time points, i.e. 3, 5, and 7 days post-RYGB. In addition, it is fundamental to elucidate the physiological consequences, i.e. changes in synapse signaling, that ensue subsequent to the neuroanatomical reorganization.

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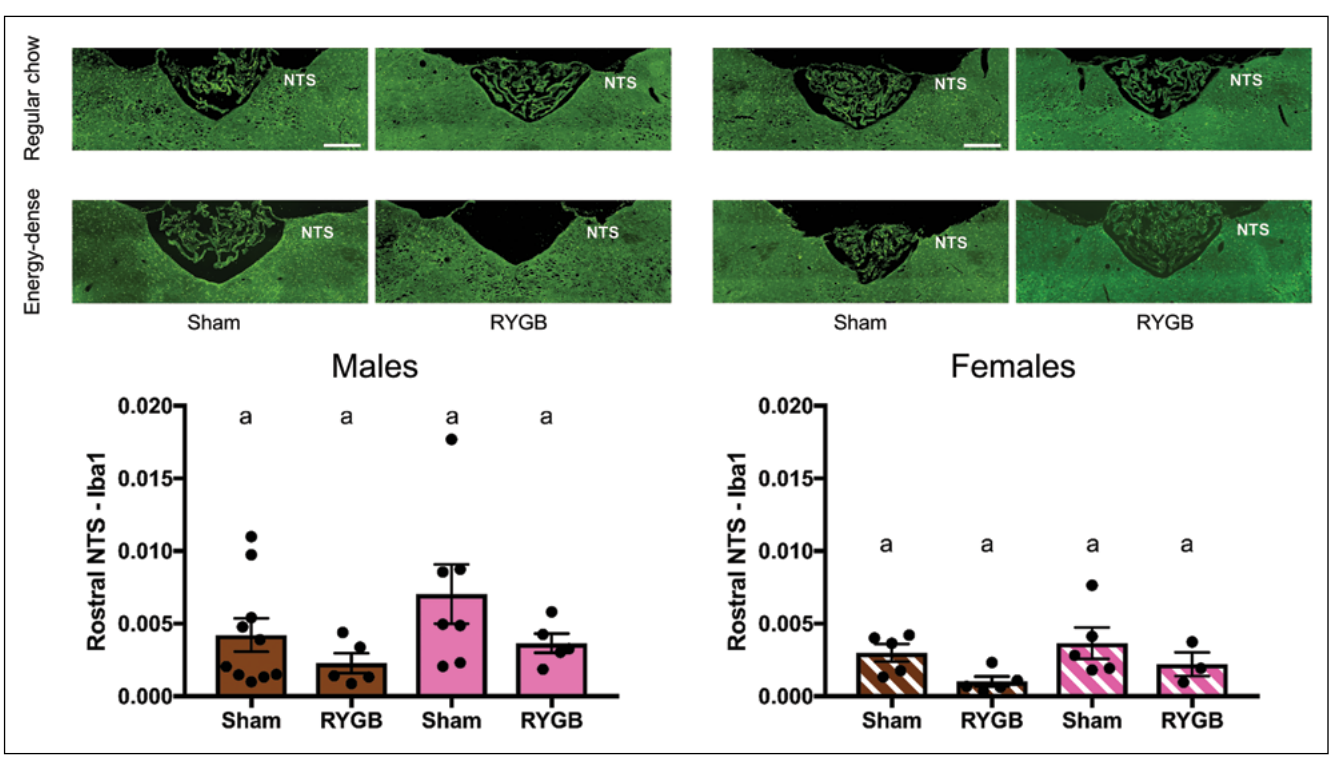

Fig. 8. Roux-en-Y gastric bypass surgery did not affect increase microglia activation in the rostral NTS. Representative sections of the rostral NTS of animals fed regular chow (top row, brown bars) and an energy-dense diet (bottom row, pink bars) are shown. Binary analysis of the area fraction of Iba1 immunoreactivity of male (left panel, solid bars) and female (right panel, striped bars) rats revealed no significant differences in Iba1 intensity between rats that underwent RYGB surgery compared to sham-operated rats, independent of diet. Graphs show mean + SEM. Bars denoted with different letters (a, b) differ significantly ($P<0.05$). NTS=nucleus tractus solitarius. Scale bar=200 μm.

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