Published in final edited form as:

Exp Physiol. 2016 January 1; 101(1): 81–99. doi:10.1113/EP085427.

Sex-related differences in small intestinal transit and serotonin dynamics in high fat diet-induced obesity in mice

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Abstract

Obesity alters gastrointestinal (GI) motility and 5-hydroxytryptamine (5-HT) signaling. Altered 5-HT signaling disrupts control of GI motility. Levels of extracellular 5-HT depend on enterochromaffin (EC) cell release and serotonin transporter (SERT) uptake. We assessed GI transit and 5-HT signaling in the jejunum of normal and obese mice. Male and female mice were fed a control diet (CD, 10% kcal fat) or a high-fat diet (HFD, 60% kcal fat). GI transit was increased in male HFD-fed and female CD-fed compared to male CD-fed. The 5-HT₃ receptor blocker, alosetron, increased gastric emptying in male CD-fed, but decreased transit in female CD-fed mice. 5-HT-induced jejunal longitudinal muscle contractions *in vitro* were similar in all mice. In contrast to male CD-fed, 5-HT uptake (measured using continuous amperometry *in vitro*) in male HFD-fed was fluoxetine-insensitive, yet sensitive to cocaine and the dopamine transporter (DAT) blocker GBR 12909. DAT-immunoreactivity was present in the mucosa and protein levels were greater in male HFD-fed compared to CD-fed. Extracellular 5-HT and mucosal 5-HIAA (5-HT metabolite) were similar in male HFD-fed compared to CD-fed. 5-HT uptake was fluoxetine-sensitive in all females. Greater SERT protein, decreased extracellular 5-HT and greater mucosal 5-HIAA were observed in female HFD-fed compared to CD-fed. Mucosal 5-HT and EC cell numbers were similar in CD-fed and HFD-fed in both sexes; female 5-HT and EC cell numbers

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Competing interests

The authors have no financial conflicts of interest.

Author Contributions

All experiments were conducted in Life Science Building Rooms B308 and B328 on the Michigan State University campus. MF conducted the experiments, analyzed the data and prepared the first draft of the manuscript. ES conducted the longitudinal muscle contraction experiments, analyzed the data and edited the manuscript. GMS helped design the experiments, provided the facilities for construction and verification of the diamond microelectrodes and edited the manuscript. MK helped design the experiments, provided the facilities for western blots and edited the manuscript. JJG helped design the experiments, edited the manuscript and provided the funding.

were increased compared to males. HFD did not alter plasma sex hormone levels in any mice. Overall, obesity alters GI transit and 5-HT signaling in a sex-dependent manner.

Keywords

serotonin; intestine; obesity

INTRODUCTION

Obesity, (body mass index ≥ 30), is a global public health problem as 13% of the world's population was obese in 2014 (<http://www.who.int/mediacentre/factsheets/fs311/en/>). In addition, more women are severely obese compared to men (Ogden *et al.*, 2014). Obesity is associated with altered gastrointestinal (GI) motility as there is a positive association between BMI and diarrhea (Delgado-Aros *et al.*, 2004; Talley *et al.*, 2004a; Talley *et al.*, 2004b; Aro *et al.*, 2005). Indeed, small intestinal transit of a radiolabeled liquid test meal to obese humans is faster compared to lean controls (Wisen & Johansson, 1992). However, studies demonstrating that transit is altered in obese humans are limited and inconsistent. For example, orocecal transit is delayed (Basilisco *et al.*, 1989) or not altered (French *et al.*, 1993) in obese compared to normal weight humans.

Some antidiarrheal drugs target 5-HT signaling. For example, the 5-HT₃ receptor antagonist, alosetron, is used to treat diarrhea-predominant irritable bowel syndrome (IBS-D) in women. 5-HT contributes to the control of intestinal motility by modulating enteric nervous system (ENS) activity and therefore motility and secretomotor function (Jin *et al.*, 1999; Gershon & Tack, 2007; Heredia *et al.*, 2009). 5-HT released from enterochromaffin cells (EC cells) in the mucosa stimulates the mucosal terminals of intrinsic primary afferent neurons (Bertrand *et al.*, 2000) leading to activation of enteric nerve circuits controlling propulsive motility and secretomotor reflexes (Furness *et al.*, 2014). 5-HT is synthesized from tryptophan by the rate-limiting enzyme tryptophan hydroxylase 1 (TPH1) in EC cells. 5-HT is stored in secretory granules until its release into the lumen or lamina propria following EC cell activation by chemical (Kidd *et al.*, 2008; Raghupathi *et al.*, 2013) or mechanical stimuli (Chin *et al.*, 2012). When 5-HT is released from the EC cell, it acts in a paracrine manner by activating 5-HT₃ receptors localized on nerve terminals of IPANS. Extracellular 5-HT levels are predominately regulated by the serotonin transporter (SERT) localized on enterocytes near EC cells (Wade *et al.*, 1996; Gill *et al.*, 2008). 5-HT transported into the enterocyte is metabolized by monoamine oxidase A (MAO_A) to 5-hydroxyindolacetic acid (5-HIAA).

Changes in 5-HT synthesis, release or uptake cause intestinal dysmotility (Coates *et al.*, 2004; Spiller, 2008). Increased extracellular 5-HT levels due to increased EC cell numbers and impaired SERT function, occur in the small intestine of a diet-induced obesity (DIO) rat model (Bertrand *et al.*, 2011). In DIO rodents, small intestinal motility is increased (Izzo *et al.*, 2009) possibly due to abnormal 5-HT levels. We hypothesized that increased small intestinal motility in a DIO mouse model is caused by altered 5-HT signaling and that there are sex-dependent differences in the intestinal 5-HT system in obesity. We measured : 1) high-fat diet (HFD) induced changes in GI transit caused by altered 5-HT signaling in

C57BL/6J mice *in vivo*, 2) HFD induced changes in serotonergic receptor activation and 5-HT clearance by SERT 3) plasma sex hormone levels that may account for altered 5-HT signaling.

METHODS

Ethical Approval

All animal use protocols were approved by the Institutional Animal Care and Use Committee at Michigan State University. The investigators understand the ethical principles under which the journal operates and that their work complies with the animal ethics checklist.

Mice and diets

Male and female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) at 14 weeks of age. These mice had received either a control diet (CD, 10 kcal % as fat overall with 6 kcal % soybean oil and 4 kcal % lard; D12450B) or a HFD (60 kcal % as fat with 6 kcal % soybean oil and 54 kcal % lard) (Research Diets, Inc., New Brunswick, NJ, USA) after weaning at 4–5 weeks of age. For D12450B, carbohydrate comprises 70 kcal % from maltodextrin and sucrose and for D12492, the carbohydrate is 20 kcal % from corn starch, maltodextrin and sucrose. These diets are isocaloric and have identical levels of protein, micronutrients (minerals and vitamins) and cellulose BW200 (12.3 mg/kcal for each). Diet details are available at: <http://www.researchdiets.com/opensource-diets/stock-diets/dio-series-diets>.

After arrival at our animal facility, the mice remained on the CD or HFD for 3 additional weeks before use in our studies. Mice were housed in individual cages and allowed food and water *ad libitum* on a 12 h light/dark cycle. Mice were studied at 17–19 wk of age (13–15 wk on diets). Mice were euthanized using isoflurane anesthesia followed by cervical dislocation. Body weight and abdominal fat pad weights were measured at the time of sacrifice. For all studies, a segment of the jejunum was excised just distal to the ligament of Treitz and placed in oxygenated (95% O₂, 5% CO₂) Krebs buffer (pH 7.4).

Solutions and chemicals

The Krebs buffer used in the measurements consisted of the following composition (mmol L⁻¹): 117 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃, and 11 glucose. The phosphate buffered saline (PBS) consisted of the following composition (mmol L⁻¹): 137 NaCl, 2.7 KCl, 10 Na₂HPO₄ and 1.8 KH₂PO₄. All drugs and reagents were obtained from Sigma Aldrich (St. Louis, MO, USA). For continuous amperometry (CA) and organ bath studies, drugs were stored as aliquots at –20 °C and were freshly prepared in Krebs buffer for each experiment. Alosetron was dissolved in sterile saline solution. Fluorescein isothiocyanate (FITC)-dextran (70 kDa) was freshly prepared for each experiment in tap water.

***In vivo* GI transit**

GI transit was assessed by measuring the distribution of a fluorescent marker along the small intestine. Mice were fasted 12 h prior to gastric gavage of FITC-dextran (0.1 mL/10 mg mL⁻¹) given between 8–9 AM the following day. After 30 min, mice were euthanized and the stomach and small intestine were removed. The small intestine was cut into 6 segments (5 cm in length) with the duodenum as segment #1 and the most distal ileum as segment #6. Each segment was flushed with 1.5 mL of PBS. PBS containing luminal content was then centrifuged (14,000 RPM for 10 min). The fluorescence of the supernatant from each segment was measured (485 nm excitation; 510 nm emission) on a plate reader (Fluoroskan Ascent/FL, Thermo Scientific, Rockford, IL). The fraction of total FITC-dextran in each segment was determined and this was used to calculate the geometric center (GC) of marker distribution (Miller et al., 1981). The GC value does not include the stomach. The GC value was calculated using the following equation:

$$GC = \sum (\text{fraction of FITC per segment}) * (\text{segment number})$$

Gastric emptying was calculated as [(Total fluorescence – fluorescence in stomach)/(Total fluorescence)] × 100.

The 5-HT₃ receptor antagonist alosetron (0.1 mg kg⁻¹) or saline was administered by intraperitoneal (i.p.) injection 30 min prior to gastric gavage of FITC-dextran.

Estrous cycle and sex hormone assays

Vaginal cytology using wet smears was performed to identify estrous cycle stage each day at 9 AM for two weeks prior to GI transit and *in vitro* contractility studies (Caligioni, 2009; Byers *et al.*, 2012; McLean *et al.*, 2012). Transit studies were performed during metestrus because 1) estradiol and progesterone modulate small intestinal motility in rats (Chen *et al.*, 1995) and 2) estradiol and progesterone levels are at a moderate and stable level (Wood *et al.*, 2007).

Estradiol, progesterone and testosterone were measured in blood samples collected by cardiac puncture and centrifuged (4 °C) at 14,000 RPM for 10 min. Serum was removed and stored at – 80 °C until analysis. Estradiol was measured in female plasma samples using a commercial enzyme-linked immunosorbent assay (ELISA) kit (CalBiochem, Spring Valley, CA) (Haisenleder *et al.*, 2011). Progesterone levels were measured in female plasma and testosterone levels were measured in male plasma using commercially available ELISA kits (BioVendor, Asheville, NC; CalBiochem, Spring Valley, CA, respectively) and an Infinite M1000 Pro Microplate Reader (Tecan, Männedorf, Schweiz). Analysis was performed using a four parameter logistic curve (SigmaPlot 11.0, Systat Software, Inc., San Jose, CA, USA).

5-HT₃ reactivity of jejunal segments *in vitro*

5-HT₃ receptor function was determined by measuring the reactivity of jejunal longitudinal muscle to 5-HT. The jejunum was removed from euthanized mice and cut into 4 1 cm segments. Segments were hung in individual organ baths (20 mL) filled with warm

oxygenated (37 °C, 95% O₂ +5% CO₂) Krebs buffer by attaching one end to a glass tissue holder and the other to an isomeric force-displacement transducer (FT03C, Grass Instruments, Inc.) with silk suture thread (LOOK 4-0 SP116). Tissues were placed under 1.0 g of tension and allowed to equilibrate for 20 min. Each organ bath was randomly assigned a drug treatment for the remainder of the experiment. The drug treatments used were: methysergide (1 µmol L⁻¹), a 5-HT₁, 5-HT₂, 5-HT₅ and 5-HT₇ receptor antagonist; 5-HT₃ receptor antagonist, ondansetron (0.1 µmol L⁻¹) + methysergide (1 µmol L⁻¹); and no antagonists. Methysergide was used to isolate 5-HT₃ receptors and methysergide addition alone served as the control. Antagonists were added to a bath 10 min prior to the first 5-HT dose. The 5-HT was applied at different doses (0.01 – 10 µmol L⁻¹) in 10 min intervals. Each 5-HT dose was added for 1 min to achieve maximum contraction followed by 2 rinses with Krebs buffer for washout. After the washouts, antagonists were added to the bath again for 10 min prior to the next 5-HT dose. Maximum longitudinal muscle contraction to acetylcholine (ACh; 100 µmol L⁻¹; 1 min) was measured before and after the 5-HT dose-response curve. The average ACh response in each tissue was used to normalize each 5-HT response per tissue weight (g). 5-HT responses are expressed as a percent of the average ACh response in each tissue. Data were collected and analyzed using a Powerlab system and Chart software, respectively (ADI Instruments, Colorado Springs, CO).

HPLC measurements of 5-HT and 5-HIAA levels

Mucosal whole tissue levels of 5-HT and 5-HIAA and extracellular 5-HT levels were measured using HPLC with electrochemical detection. For whole tissue levels, segments of jejunum were removed and placed in Krebs buffer. Segments were cut along the mesenteric border and the mucosa was rinsed with Krebs buffer. The mucosa (~2 cm) was removed using a sharp scalpel and placed into a tissue homogenizer (2 mL) filled with ice cold 0.1 M perchloric acid (0.5 mL). Samples were centrifuged (4 °C) at 14,000 RPM for 10 min. Supernatant was removed and stored at -80 °C until analysis. Protein analysis was performed using a DC™ Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). For extracellular 5-HT levels, a Hamilton syringe was used to sample the Krebs buffer (150 µL) near to the mucosa surface (~500 µm away) using a micromanipulator in jejunal preparations used for continuous amperometry experiments (see below). Extracellular fluid was quickly mixed with 50 µL of ice cold 0.1 M perchloric acid and then centrifuged (4 °C) at 14,000 RPM for 10 min. The supernatant was then removed for HPLC analysis. Supernatant (20 µL) samples from whole tissue and extracellular fluid were injected using an autosampler (ESA 542, Thermo-Fisher) maintained at 35 °C onto a ESA guard column (70-1972) in-line with a Thermo MD column (150 mm × 3.2 mm, 3 µm particle size) for separation. Detection was accomplished using an ESA 5011A analytical cell set to a potential of +200 mV. The mobile phase consisted of the following composition (mmol L⁻¹): 90 sodium phosphate, 50 citrate, 1.94 sodium octyl sulfate, 0.05 EDTA and 10 % acetonitrile and flowed at a rate of 0.6 mL min⁻¹ (ESA 582). Results are presented as ng per mg protein and nmol L⁻¹ for whole tissue and extracellular analyte levels, respectively.

Western blotting

Segments of jejunum (~1 cm) were removed, placed in Krebs buffer and cut along the mesenteric border. The mucosal surface was rinsed with Krebs buffer, removed using a

sharp scalpel, flash-frozen in liquid nitrogen and stored at -80°C . For western blot analysis, mucosal scrapes were lysed using a mortar and pestle in lysis buffer (62.5 mM Tris pH 6.8, 20% SDS, 5 mM EDTA) containing 10 mM NaF, 1 mM Na_3VO_4 and a commercially available protease inhibitor cocktail (#P8340, Sigma-Aldrich). Samples were sonicated and centrifuged (14,000 RPM for 12 min at room temperature). The supernatant was removed to measure total protein using a DCTM Protein Assay kit (Bio-Rad, Hercules, CA). The remaining pellet was resuspended with 1:1 Laemmli buffer (#1610737, Bio-Rad, Hercules, CA) containing β -mercaptoethanol (5% vol/vol; M6250; Sigma-Aldrich), boiled at $95-100^{\circ}\text{C}$ (5 min), aliquoted and frozen at -20°C until further analysis. Protein (30 μg) separation was accomplished using a 4% SDS-PAGE stacking gel on top of a 10% SDS-PAGE running gel at 30 V for 30 min then increasing to 100 V for 1.5 h. After separation, protein was transferred to a polyvinylidene difluoride (PVDF) membrane (#IPVH00010, pore size 0.45 μm , EMD Millipore, Billerica, MA) overnight at 30 V. The following morning, membranes were blocked with 5–10% w/v non-fat dry milk in T-TBS (1% Tween-20, 20 mM Tris pH 7.5, 200 mM NaCl) for 1 h at room temperature. Membranes were then incubated with primary antibodies for 2 h at room temperature. For SERT detection, a goat anti-SERT primary antibody (sc 1458, Santa Cruz, CA) was used at 1:800 dilution. For DAT detection a rabbit anti-DAT primary antibody (AB 2231, EMD Millipore, MA) was used at 1:2000 dilution. Lactate dehydrogenase (LDH) protein levels were determined to control for loading, transfer and developing. For LDH detection a rabbit anti-LDH primary antibody (SC 33781, Santa Cruz, CA) was used at 1:30,000 dilution. Next, membranes were washed 3 times for 10 min with T-TBS and incubated with horseradish-peroxidase-conjugated secondary antibodies for 1 h at room temperature and finally washed 3 times for 10 min with T-TBS. The secondary antibody used for SERT was donkey anti-goat (sc 2056, Santa Cruz, CA) at a 1:2000 dilution. The secondary antibody for DAT and LDH was donkey anti-rabbit (sc 2313, Santa Cruz, CA) at a 1:8000 and 1:30,000 dilution, respectively. A pre-stained protein ladder (#26616, Thermo Fisher Scientific Inc., Waltham, MA, USA) provided apparent molecular weights. Immunoreactivity was detected using an enhanced chemiluminescence kit (#RPN2232, GE Healthcare, Buckinghamshire, UK). Densitometry was used to measure protein levels normalized to LDH and images were analyzed using ImageJ 1.48v software (<http://imagej.nih.gov/ij>).

SERT mRNA Expression

Quantitative real-time PCR (qRT-PCR) was used to quantify mRNA encoding SERT in jejunal mucosal scrapes. Segments of jejunum (~1 cm) were removed and cut along the mesenteric border. The mucosa was rinsed with Krebs buffer, removed using a sharp scalpel, flash-frozen in liquid nitrogen and stored at -80°C until further analysis. Tissue digestion and RNA isolation were performed using MELTTM Total Nucleic Acid Isolation System Kit according to the manufacturer's instructions (AM 1983, Ambion, Carlsbad, CA). Nucleic acid concentration and purity was determined using Nanodrop 2000c (Thermo Fisher Scientific, Wilmington, Delaware USA). Samples of cDNA were generated by reverse transcription using a High Capacity RNA-to-cDNA kit according to the manufacturer's instructions (4387406; Applied Biosystems, Carlsbad, CA). Resultant cDNA was used for qRT-PCR and was performed using TaqMan reagents on a 7500 FAST Real-time PCR System (Applied Biosystems, Carlsbad, CA). Amplifications were performed in a

final volume of 20 μ L prepared with TaqMan Fast Advanced master mix according to manufactures instructions (4444556, Applied Biosystems). cDNA synthesis reactions were ran with samples that did not contain reverse transcriptase as a control for genomic DNA contamination. Samples and controls were run in triplicate with TaqMan Gene Expression assays (Rn01462137_m1 for SERT, Rn01775763_g1 for GAPDH, Life Technologies). Measurements were analyzed using the C_T method. SERT mRNA levels were expressed as a fold change and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH is a housekeeping gene that was found not to be altered from an obesity-causing diet (Bertrand *et al.*, 2011).

Immunohistochemistry and quantification of EC cells

Segments of jejunum were cut along the mesenteric border, stretched gently ($\sim 1 \text{ cm}^2$) and pinned with the mucosal surface facing upwards in a Sylgard® lined Petri dish. The segment was gently washed with Krebs buffer and fixed with 10% neutral buffered formalin (pH 6.9 – 7.1) overnight (4 °C). 10% neutral buffered formalin-fixed specimens were embedded in paraffin and 4–5 μ m thick sections were obtained using a rotary microtome. Sections obtained at 40 μ m intervals were placed on slides (4 sections per slide)) coated with 2% 3-aminopropyltriethoxysilane and dried overnight (56 °C). Slides were subsequently deparaffinized in xylene and hydrated through descending grades of ethyl alcohol to distilled water. To adjust pH, slides were placed in Tris Buffered Saline (TBS, pH 7.4) (Scytek Labs, Logan, UT) for 5 min. Following TBS, slides underwent heat induced epitope retrieval utilizing Citrate Plus (pH 6.0) (Scytek Labs, Logan, UT) for 30 min in a vegetable steamer at 100°C followed by 10 min at room temperature and then underwent several changes of distilled water. Endogenous enzyme activity was blocked with a 3% hydrogen peroxide/ methanol solution (1:4) for 30 min followed by tap and distilled water rinses. Following, standard avidin-biotin complex (ABC) staining protocol was performed at room temperature on a DAKO Autostainer. All staining steps were followed by rinses in TBS with Tween 20 (Scytek Labs, Logan, UT). Blocking for non-specific protein was accomplished using normal goat serum (Vector Labs, Burlingame, CA) for 30 minutes. Sections were then incubated with avidin-biotin blocking system for 15 min (Avidin D, Vector Labs, Burlingame, CA; d-Biotin, Sigma, St. Louis, MO). Primary antibody slides were incubated for 1 h with either polyclonal rabbit anti-5-HT diluted at 1:40,000 (S5545, Sigma, St. Louis, MO) or rabbit anti-DAT (Ab2231, Millipore) diluted at 1:1350 in normal antibody diluent (NAD) (Scytek Labs, Logan, UT). Following, slides were incubated for 30 min in biotinylated goat anti-rabbit IgG prepared in NAD (11.5 μ g/ml) and then incubated for 30 min in Ready-to-use Vector Elite Peroxidase Reagent (Vector Labs, Burlingame, CA). Reaction development was performed by incubating slides for 15 min in Vector® NovaRED™ peroxidase chromogen (Vector Labs, Burlingame, CA) followed by counterstain in Gill Hematoxylin (Thermo Fisher, Kalamazoo, MI) for 15 s, differentiation, dehydration, clearing and finally mounting with synthetic mounting media.

Sections were viewed using a Nikon microscope and images were acquired using SPOT Advanced software (Spot Imaging Solutions, Sterling Heights, MI, USA). The number of intact villi and the number of 5-HT positive cells located in the epithelium (crypt-villus axis) in intact villi was counted in the four sections of one slide (4 sections per 1 animal) by a

blinded observer. The ratio of the total number of 5-HT positive cells located in the epithelium of the crypt-villus axis in intact villi to the total number of intact villi was calculated for individual sections. This ratio was averaged over the four sections and that average was used for each animal. The number of EC cells is expressed per 10 villus-crypt axes.

Continuous amperometric (CA) measurements of 5-HT uptake

Details of the preparation and characterization of the diamond microelectrodes have been reported elsewhere (Park *et al.*, 2005; Bian *et al.*, 2007; Patel *et al.*, 2007; Zhao *et al.*, 2010). A segment of jejunum was cut along the mesenteric border ($\sim 1 \text{ cm}^2$), stretched gently and pinned (stainless steel, 0.2 mm diameter, 26002-20, Fine Science Tools, Foster City, CA) with the mucosal surface facing upwards in a Sylgard® lined flow bath ($\sim 6 \text{ mL}$). Oxygenated Krebs buffer solution, kept at room temperature, flowed over the tissue at a rate of 2 mL min^{-1} using a peristaltic pump (Minipuls 2, Gilson, Middleton, WI). Precise positioning of the diamond microelectrode was accomplished using a micromanipulator (MM33, Fine Scientific Tools, Foster City). Villi and diamond microelectrode positioning were visualized using an upright microscope (Model SMZ1000, Nikon, USA). Experiments commenced after a 30 min equilibration period. Measurements were made in a Faraday cage to minimize electrical noise.

For the CA experiments, 5-HT detection was accomplished at $+0.8 \text{ V}$ vs. Ag/AgCl (3 M KCl) using a ChemClamp potentiostat (Dagan Corp., Minneapolis, MN, USA). The analog current from the potentiostat was captured and digitally converted with an e-corder hardware unit (eDAQ Inc., Colorado Springs, CO, USA) at a sampling rate of 200 Hz. The current was low-pass filtered (1 kHz filter) prior to digitization. Current recordings were generated on-line using Chart software. A home-made Ag/AgCl electrode served as the counter and reference in a two-electrode measurement configuration. The reference electrode was positioned in the flow bath somewhat removed from the position of the working electrode and tissue. CA measurements consisted of current approach curves in which the electrode distance in relation to the mucosal surface was decreased in a step-wise fashion. Electrode distances used were 750, 500, 250, 100, 50 and $10 \mu\text{m}$ away from the mucosal surface. In order to determine electrode distance from the mucosal surface, the electrode was first positioned such that it gently touched the villus, then it was retracted to a distance of $2000 \mu\text{m}$ that served as the distance for background current measurements. The background current was measured at $2000 \mu\text{m}$ as at this distance the current was unaffected by 5-HT release due to dilution effects. When the background current was stable, the recording of current-approach curves commenced. For each current measurement, the electrode was positioned at a calibrated distance for ca. 40 – 60 s. Within this time window, a stable current was reached. The current magnitude at each electrode distance was recorded by averaging the current over the last 20 – 30 s of the period and subtracting from this the average background current measured at the $2000 \mu\text{m}$ distance. The natural log ($\ln$) of the current at each electrode distance was plotted as a function of the distance. A linear fit of the data was performed and the slope was determined in order to assess 5-HT uptake as previously described (Marcelli & Patel, 2008, 2010). Fluoxetine ($1 \mu\text{mol L}^{-1}$), a selective 5-HT reuptake inhibitor (SSRI), cocaine ($10 \mu\text{mol L}^{-1}$), a SERT, norepinephrine (NET) and

dopamine transport (DAT) inhibitor, and GBR 12909 ($0.1 \mu\text{mol L}^{-1}$), a DAT inhibitor, were used to assess SERT and DAT function. Fluoxetine, cocaine and GBR 12909 are not electroactive at the potential used for detecting 5-HT (data not shown). A solution containing any of the drugs used was flowed across the tissue for 5 min before a current approach curve was recorded. The drug was also present during the measurement. Measurements were made before (Krebs buffer) during application and after washout (20 min) of the drug. All drugs evoked a reversible increase in the 5-HT oxidation current due to an increase in the 5-HT extracellular concentration. In our studies, SERT function was assessed by comparing the slope of the $\ln$ current (pA) vs. the electrode distance plots (μm) with and without drug addition (Marcelli & Patel, 2010). A decrease in the slope indicated reduced 5-HT uptake (Marcelli & Patel, 2010).

Statistical Analysis

All data analysis was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Two group comparisons were made using an unpaired Student's *t*-test for parametric data. For data not normally distributed, a log transformation of the data or a Mann-Whitney test was performed. For CA experiments, comparisons between measurements made in Krebs buffer and measurements made in the presence of a drug were made using a paired Student's *t*-test. Comparisons of 5-HT concentration response curves were made using half maximal effective concentrations (EC_{50}) values and the maximum percent contraction values. For all comparisons, $P < 0.05$ was considered statistically significant. Data are reported as mean $\pm$ S.E.M.

RESULTS

Male and female C57BL/6J mice developed DIO on HFD

Male (Fig. 1A) and female (Fig. 1B) mice fed a HFD were heavier compared to CD-fed mice at 11–15 weeks spent on the diets (.. HFD-fed male (Fig. 1C) and female (Fig. 1D) mice had greater abdominal fat pad weights at 13–15 weeks compared to CD-fed mice. Gastric emptying was increased in HFD male mice compared to CD-fed males (Fig. 2A).

Gastric emptying was similar between CD-fed and HFD-fed females, but gastric emptying was greater in CD-fed female compared to CD-fed male mice (Fig. 2A). The GC (which measures gastrointestinal transit) was increased in HFD-fed compared to CD-fed males (Fig. 2B). HFD-fed and CD-fed female mice had similar GC values (Fig. 2B), but CD-fed female mice had a greater GC value compared to CD-fed male mice (Fig. 2B). In order to further analyze GI transit, we plotted the fraction of fluorescence intensity vs. intestinal segment number. Marker distribution in CD-fed males had a single peak while the distribution in HFD-fed males was biphasic with a larger fraction of marker present in a more distal segment (Fig. 2C). In HFD-fed females, fluorescence was distributed uniformly along most of the small intestine while ~70% of total fluorescence was found in one segment (#4) in CD-fed females (Fig. 2D).

Sex specific effect of alosetron on GI transit

Alosetron increased gastric emptying in CD-fed males compared to saline-treated CD-fed mice (Fig. 3A) but it did not change gastric emptying in HFD-fed males or in CD-fed or HFD fed female mice. (Fig. 3A). Alosetron did not change GI transit in CD- or HFD-fed male or HFD-fed female mice but it did decrease GI transit in CD-fed female mice (Fig. 3B)..

In addition to calculating the GC we also plotted the distribution of FITC-dextran along the small intestine for each group. Alosetron did not change the distribution of marker in CD-fed (Fig. 3C) or HFD-fed (Fig. 3D) male mice. However, alosetron did shift the marker distribution to the left in HFD-fed compared to the distribution in saline-treated CD-fed male mice (Fig. 3E) This suggests that small intestinal transit was modestly reduced by alosetron in HFD-fed males although the GC did not detect this difference. Alosetron shifted the FITC-dextran distribution to the left in CD-fed female mice (Fig. 3F) indicating delayed transit as detected by the GC (Fig. 3B). FITC-dextran distribution was uniform over much of the small intestine in HFD-fed female mice while alosetron treatment shifted this distribution to the left producing a single peak in the marker distribution (Fig. 3G). This was similar to the distribution detected in CD-fed saline treated mice (Fig. 3H). suggesting that alosetron normalized transit in HFD-fed females.

5-HT₃ receptor function was unchanged in HFD-fed mice

To determine if 5-HT₃ receptor signaling is altered by a HFD we measured 5-HT concentration response curves ($0.01\text{--}10\ \mu\text{mol L}^{-1}$) for longitudinal muscle contraction in jejunal segments in the absence and presence of the 5-HT₃ receptor antagonist ondansetron ($0.1\ \mu\text{mol L}^{-1}$). Ondansetron caused a significant reduction of the 5-HT-induced contractions when non-5-HT₃ receptors were blocked with methysergide ($P < 0.05$) in male (Fig. 4A) and female (Fig. 4B) jejunum. Ondansetron produced a selective reduction in contractions caused by higher 5-HT concentrations ($1\text{--}10\ \mu\text{mol L}^{-1}$) while methysergide inhibited contractions caused by lower concentrations of 5-HT ($< 1\ \mu\text{mol L}^{-1}$). Interestingly, 5-HT-induced muscle contractions in female CD-fed jejunum, but not female HFD-fed jejunum, were insensitive to methysergide. 5-HT ($0.3\ \mu\text{mol L}^{-1}$) produced a greater muscle contraction in the jejunum from male CD-fed compared to female CD-fed mice. 5-HT ($3\ \mu\text{mol L}^{-1}$) produced a greater muscle contraction in the jejunum from female HFD-fed (~60%) compared to CD-fed mice (~40%, $P = 0.051$).

Effects of diet 5-HT uptake and SERT expression

A schematic of the diamond microelectrode and the mucosal preparation used in CA measurements is shown in Figure 5A. Figure 5B shows a representative voltammogram of 5-HT ($10\ \mu\text{mol L}^{-1}$) prepared in Krebs buffer against the voltammogram of Krebs buffer alone. Electrode potential was scanned from -0.2 to $1\ \text{V}$ at a rate of $0.1\ \text{V s}^{-1}$. The peak oxidation potential (E_p^{ox}) of 5-HT is ca. $0.65\ \text{V}$ (arrow in Fig. 5B) where 5-HT released by nearby EC cells is oxidized at a mass-transport controlled rate.

Current-approach curves were generated at $+0.80\ \text{V}$, based on the E_p^{ox} (Fig. 5B). The current increased as the electrode was positioned closer to the mucosal surface ($750\text{--}10\ \mu\text{m}$).

Figure 5C shows current approach curves made before (Krebs buffer, pH = 7.4), during application and after washout of fluoxetine ($1 \mu\text{mol L}^{-1}$, 5 min). Two consecutive current approach curves (only one of which is shown) were made before fluoxetine application. Fluoxetine ($1 \mu\text{mol L}^{-1}$) applied for 5 min caused an increase in the oxidation current at all electrode distances. Two consecutive curves were made in the presence of fluoxetine and both were similar in terms of the current magnitudes. After fluoxetine washout (20 min), oxidation currents returned control levels. Currents were plotted as a function of tissue-electrode distance (Fig. 5D) and the best-fit line to the $\ln$ current vs. electrode distance plot (Fig. 5E) was used to obtain the slope, a measure of 5-HT uptake.

Fluoxetine decreased 5-HT uptake (i.e. slope) in jejunal tissues from male CD-fed (but not HFD-fed mice (Fig. 6A). In jejunal tissues from female CD-fed and HFD-fed mice, fluoxetine decreased 5-HT uptake (Fig. 6A). These findings indicate that SERT function was impaired in the jejunum from male HFD-fed mice, but unchanged in female HFD-fed mice.

We next measured extracellular 5-HT near the mucosa surface, mucosal SERT protein and mRNA and whole-tissue 5-HIAA. Extracellular 5-HT levels were similar between male CD-fed and HFD-fed mice (Fig. 6B). Extracellular 5-HT levels were reduced in female HFD-fed compared to CD-fed mice while extracellular 5-HT levels were greater in tissues from female CD-fed compared to male CD-fed mice (Fig. 6B).

Whole tissue 5-HIAA levels were similar in jejunal mucosa from male HFD-fed and CD-fed mice (Fig. 6C). 5-HIAA levels were not different in the mucosa of female HFD-fed compared to CD-fed mice (Fig. 6C). Regardless of diet, 5-HIAA levels were greater in female compared to male mice (Fig. 6C).

Western blot for SERT protein in jejunal mucosa revealed a single band at the apparent molecular weight of ~ 100 kDa and a LDH band at 35 kDa (Fig. 6D). The 100 kDa SERT band was absent in secondary antibody only controls (data not shown) and was absent in SERT knock-out rat small intestine compared to wildtype controls (data not shown). In male jejunal mucosa, the ratio of SERT to LDH was similar between CD- and HFD-fed (Fig. 6E), but jejunal mucosa from female HFD-fed mice had greater SERT protein levels compared to tissues from female CD-fed mice (Fig. 6E). SERT mRNA expression was similar between jejunal mucosa from female HFD-fed and CD-fed mice (Fig. 6F).

HFD does not alter EC cell number or mucosal 5-HT levels

We measured the number of 5-HT positive EC cells within the epithelial layer along the crypt-villus axis (per 10 villi) in the jejunum of males (Fig. 7A, B) and females (Fig. 7B) to distinguish between 5-HT positive mast cells (Theoharides *et al.*, 1982) located in the lamina propria in stained paraffin-embedded sections. EC cell number was similar in HFD-fed and CD-fed male jejunum and HFD-fed and CD-fed female jejunum (Fig. 7B). CD-fed female mice had more EC cells compared to CD-fed male mice (Fig. 7B).

5-HT levels in jejunal mucosa were similar in HFD- and CD-fed male mice (Fig. 7C) and HFD-fed and CD-fed female jejunum (Fig. 7C). Whole-tissue 5-HT levels were greater in females compared to males regardless of diet (Fig. 7C).

5-HT is cleared by DAT in HFD-fed male jejunum

To determine if other monoamine transporters compensate for impaired SERT function in HFD-fed male mice, we used cocaine ($10 \mu\text{mol L}^{-1}$) in CA experiments (not shown). Cocaine decreased 5-HT uptake (i.e. slope) in jejunal tissues from male CD-fed mice (cocaine = $0.0031 \pm 0.0003 \mu\text{m}^{-1}$ vs. Krebs buffer = $0.0045 \pm 0.0005 \mu\text{m}^{-1}$, respectively, $n = 7$, $P < 0.05$). Cocaine blocked 5-HT uptake in tissues from male CD-fed mice to a similar extent as fluoxetine as determined by the percentage change in the slope from Krebs buffer ($-28.9 \pm 4.1\%$, $n = 9$ vs. $-29.6 \pm 5.2\%$, $n = 7$, respectively) confirming that SERT was mainly responsible for clearing 5-HT. In tissues obtained from male HFD-fed mice, cocaine reduced slightly 5-HT uptake (cocaine = $0.0027 \pm 0.0002 \mu\text{m}^{-1}$ vs. Krebs buffer = $0.0031 \pm 0.0002 \mu\text{m}^{-1}$, $n = 13$, $P < 0.05$). The DAT antagonist GBR 12909 ($0.1 \mu\text{mol L}^{-1}$) did not change 5-HT uptake in tissues from male CD-fed mice (Fig. 8A). Conversely, GBR 12909 reduced 5-HT uptake in jejunal tissues obtained from male HFD-fed mice (Fig. 8A).

We next determined if DAT protein levels were increased in HFD-fed male jejunum mucosa using Western blot. The DAT antibody revealed two predominant bands at the apparent molecular weights of ~60 and 100 kDa (Fig. 8B, *left*). These bands were absent in secondary antibody only controls (Fig. 8B, *right*). DAT expression, particularly the 100 kDa band, was increased in the jejunum of HFD-fed male mice compared to CD-fed male mice (Fig. 8C). The mucosal scrapes did not include the muscularis propria containing the myenteric plexus nor the submucosal plexus as determined by histologic examination of hematoxylin and eosin stained paraffin-embedded sections (data not shown). The ratio of DAT/LDH was greater for the 100 kDa band and for the 60+100 kDa combined (Fig. 8D), but not the 60 kDa band alone in HFD-fed male mice.

Using the same DAT antibody, immunohistochemical analysis revealed DAT-immunoreactivity in paraffin-embedded sections of jejunal mucosa in the lamina propria (*dotted arrow*) and concentrated at the apical side of enterocytes (*solid arrow*) (Fig. 8E). As a positive control, DAT-immunoreactivity was observed in the myenteric and submucosal plexuses (Fig. 8G). DAT-immunoreactivity was absent in sections stained in the absence of primary antibody (Fig. 8F, H).

HFD did not change plasma sex hormone levels in male or female mice

Estradiol and progesterone levels in HFD-fed and CD-fed females were similar (Fig. 9A, B). Testosterone levels in HFD-fed and CD-fed males were also similar (Fig. 9C).

DISCUSSION

Diet composition

We did not compare the purified ingredient CD (D12450B) with a standard chow diet (e.g., Purina 5001) which typically has similar macronutrients (approximately 14 kcal % fat, 58

kcal % CHO, and 28 kcal % protein) but contains a mixture of both soluble/fermentable and nonsoluble/nonfermentable fibers. Fiber composition has recently been shown to be important in adiposity, development of intestinal atrophy and microbiota-mediated intestinal inflammation (Chassaing et al. 2015). In addition, Kim et al. (2012) demonstrated different microbiota composition from stool samples taken from C57BL/6J mice placed on the same purified chow diets (D12450B vs. D12492) used in the present study. Future studies would determine if changes in fiber or microbiota *per se* alter 5-HT dynamics and sex-related differences in small intestinal transit.

Sex-dependent differences in SERT expression in HFD-fed mice

SERT is a key regulator of extracellular 5-HT (Chen *et al.*, 1998; Martel, 2006). We determined if changes in SERT function were present in the jejunum from CD-fed and HFD-fed male and female mice using CA. Impaired SERT function was demonstrated previously using CA where it was found that 5-HT oxidation currents recorded in ileum preparations from Western Diet-fed male rats were fluoxetine-insensitive (Bertrand *et al.*, 2011). We found that 5-HT uptake was fluoxetine-insensitive in the jejunum from HFD-fed male but not female mice suggesting that SERT function is impaired selectively in the jejunum of male HFD-fed mice. This result is important because it demonstrates sex differences in the effects of obesity on 5-HT signaling and is not due to direct effects of the HFD. Bertrand et al. (2011) showed that the Western Diet did not alter 5-HT signaling in male rats until after the rats had become obese. Similarly, Reichardt et al. (2013) found that a HFD increased myenteric neuronal excitability in male rats. Our results are also consistent with data showing that SERT function in the central nervous system is altered in obese humans. For example, obese patients required a greater number of weeks to respond to antidepressant treatment (Kloiber *et al.*, 2007) and greater body weight was found to be a predictor for fluoxetine resistance in patients with major depressive disorder (Papakostas *et al.*, 2005). Intestinal SERT function in obese humans has not been studied. There is also evidence in humans for sex differences in responses to SSRI treatment in obesity. For example, obese women, but not men, respond well to SSRI treatment for major depressive disorder (Khan *et al.*, 2007).

In male HFD-fed mice, impaired SERT function would decrease the amount of 5-HT cleared thereby raising local extracellular 5-HT levels. Moreover, if less 5-HT was cleared by SERT, less 5-HT would be metabolized into 5-HIAA in the enterocyte. We demonstrated that both steady state extracellular 5-HT and whole tissue 5-HIAA levels were similar in tissues from male HFD-fed and CD-fed mice. These results suggest that a compensatory mechanism may clear 5-HT when SERT function is impaired. Possible compensatory mechanisms include increased expression of other monoamine transporters. For example, both NET and DAT are capable of clearing 5-HT in the CNS of MAO_A knockout mice that have high extracellular 5-HT levels (Cases *et al.*, 1998). We found that cocaine, a non-selective monoamine transport blocker (Torres *et al.*, 2003), reduced 5-HT uptake in the jejunum of male HFD-fed compared to CD-fed mice. As DAT is expressed throughout the intestine (Chen *et al.*, 2001; Li *et al.*, 2004), we determined if DAT functions to clear 5-HT. In CA studies, blocking DAT with GBR 12909 reduced 5-HT uptake in the HFD-fed male jejunum, indicating that DAT clears 5-HT when SERT activity is reduced.

We found the decrease in SERT function was not due to decreased SERT expression because jejunal protein and mRNA levels in the mucosa were similar in male HFD-fed compared to CD-fed mice. The calculated MW for SERT is 70 kDa, but the antibody used in our study revealed a single protein band at 100 kDa in extracts from the jejunal mucosa.. The increase in the apparent molecular weight of SERT is likely due to post-translational modifications, including protein glycosylation and phosphorylation (Chamba *et al.*, 2008). SERT glycosylation may be important for function, stabilization, trafficking and orientation in the plasma membrane (Rasmussen *et al.*, 2009). It is possible that a change in function without a reduction in total protein levels could be due to changes in SERT plasma membrane surface expression or protein activity. For example, changes in SERT phosphorylation (e.g. by PKC/PKG), may lead to changes in internalization and sequestering in endosomes/secretory granules (Blakely *et al.*, 1998). It is also possible that in HFD-fed male jejunum SERT remains present at the plasma membrane, but is inactive independent of changes in localization. For example, the inflammatory cytokines interferon gamma (IFN γ) and tumor necrosis factor alpha (TNF α) decreased SERT function in a human intestinal epithelial cell line (Foley *et al.*, 2007). Future studies could be aimed at assessing diet-induced changes in SERT localization.

We determined if GBR 12909-sensitive 5-HT uptake in the jejunum from male HFD-fed mice was due to an increase in DAT protein expression. Western blot studies revealed that the jejunal mucosa from male HFD-fed mice had greater DAT protein levels at the apparent molecular weight of ca. 100 kDa, but not 60 kDa compared to CD-fed males. The calculated MW for DAT is 68.8 kDa. It is possible that the higher molecular weight DAT may arise from glycosylation, although we did not determine this in our study. The band we report was similar to that observed in bovine nasal mucosa (Chemuturi *et al.*, 2006) and to that reported in the rodent myenteric and submucosal plexuses (Li *et al.*, 2004). Dopamine (DA) is present both in gastric (Mezey *et al.*, 1999) and duodenal (Mezey *et al.*, 1996) luminal fluid and has been proposed to play a protective role against mucosal injury as DA receptor agonists aid in healing upper GI ulcers (Sikiric *et al.*, 1991). Consistent with this, DAT-immunoreactivity was present in epithelial cells of duodenal mucosa in rodents (Tian *et al.*, 2008). In our studies, DAT-immunoreactivity was found in epithelial cells concentrated at the apical surface, in strands found in the lamina propria and within the myenteric and submucosal plexuses. Based on our CA studies, it is likely that this higher MW DAT is located on epithelial cells and is responsible for 5-HT uptake we observed in our CA studies.

SERT function was unchanged in female HFD-fed mice as 5-HT uptake was fluoxetine-sensitive in the jejunum from these mice. However, extracellular 5-HT levels were reduced in HFD-fed compared to CD-fed females suggesting increased clearance of extracellular 5-HT in HFD-fed females.. We also found greater SERT protein levels, but not SERT mRNA, in the jejunum of HFD-fed compared to CD-fed female mice suggesting the SERT protein turnover is decreased in HFD-fed female mice.

HFD does not change mucosal 5-HT or 5-HT₃ receptor function

The number of EC cells and mucosal 5-HT levels were similar in HFD-fed and CD-fed mice in both sexes. This suggests that 5-HT synthesis is unaffected by HFD. This result is in

contrast with a previous report where the number of EC cells was greater in the ileum of rats fed a Western diet (Bertrand *et al.*, 2011). These inconsistencies may be due to differences in species, diet composition (CD and HFD), time spent on diet, and region of small intestine. For example, not all mouse strains develop an obese phenotype when placed on a HFD (Lee *et al.*, 2011) while other substrains are more sensitive to changes caused by a HFD (e.g. C57BL/6J versus C57BL/6NJ) (Nicholson *et al.*, 2010). In addition, we used isocaloric CD and HFD whereas other HFD consist of added fat to the regular chow diet.

Our studies revealed similar reductions in 5-HT-induced muscle contractions by blocking 5-HT₃ receptors in CD-fed and HFD-fed jejunum in males and females. These results suggest that 5-HT₃ receptor function remains unchanged in mice fed a HFD and are in agreement with a previous study demonstrating unchanged 5-HT₃ receptor mRNA levels in nodose ganglia HFD-fed mice (Nefti *et al.*, 2009). 5-HT₃ receptor blockade reduced 5-HT-induced jejunal longitudinal muscle contractions at 5-HT concentrations sufficient to activate 5-HT₃ receptors (Buchheit *et al.*, 1985; Kilbinger & Wolf, 1992). Jejunal longitudinal muscle from HFD-fed female mice were more sensitive to high concentrations of 5-HT compared to tissues from CD-fed female mice suggesting that HFD-fed females are more sensitive to 5-HT acting at 5-HT₃ receptors. Likewise, low concentrations of 5-HT (0.3 $\mu\text{mol L}^{-1}$) produced greater muscle contractions in the jejunum of male CD-fed compared to female CD-fed mice suggesting females are less sensitive to lower concentrations of 5-HT that do not activate 5-HT₃ receptors. Interestingly, we found that 5-HT-induced longitudinal muscle contractions of CD-fed female jejunum are methysergide-insensitive while 5-HT-induced contractions in HFD-fed female jejunum are methysergide-sensitive. Thus, there is a gain of function in non-5-HT₃ receptors in the jejunum of HFD-fed female mice.

HFD alters GI transit

HFD increased gastric emptying and small intestinal transit in male mice. Likewise, CD-fed female mice had increased gastric emptying and small intestinal transit compared to CD-fed male mice. Overall, female CD-fed mice have increased GI transit compared to male mice. Although the GC value was similar between CD-fed and HFD-fed females, intestinal transit is altered in HFD-fed females based on the intestinal distribution of FITC-dextran.

Increased intestinal transit in male HFD-fed mice may be a result of increased extracellular 5-HT levels due to impaired SERT function. Indeed, DAT clears 5-HT in HFD-fed male mice as revealed by our CA experiments and direct measurements of extracellular 5-HT. For DAT to clear 5-HT, 5-HT must reach a threshold concentration as DAT has low affinity for 5-HT (Daws, 2009). Therefore, the local 5-HT concentration may be increased in HFD-fed male mice and leading to greater activation of 5-HT₃ receptors increasing motility and small intestinal transit.

The GC was similar between CD and HFD-fed female mice. Transit was assessed in metestrus and since estrogen and progesterone modulate transit in female rats (Chen *et al.*, 1995), it is possible that diet-induced changes in transit may vary with the estrous cycle. Moreover, obesity can alter the estrous cycle in rodents (Balasubramanian *et al.*, 2012) and change plasma sex hormone levels. Although we found no diet related differences in plasma sex hormone levels, HFD-fed female mice may be less responsive to sex hormones. For

example, in a DIO mouse model similar to ours, the authors found similar plasma hormone levels in HFD-and CD-fed female mice. However, HFD-fed mice were less sensitive to estrogen-dependent effects in mammary gland development (Olson *et al.*, 2010).

Increased transit in CD-fed females compared to males could be caused by the elevated extracellular 5-HT levels we detected. Females also have greater tissue 5-HT levels compared to males regardless of diet. This increase does not appear to be related to differences in EC cell number as cell numbers in HFD-fed females were not different from those in HFD-fed males. Thus, it is likely EC cells in female mice produce greater amounts of 5-HT. In CD-fed female mice, greater whole tissue 5-HT levels translated to greater extracellular 5-HT levels due to greater 5-HT release. Our data show that female mice have 2-3X higher intestinal 5-HT concentrations compared to male mice. The increase in 5-HT concentration would most likely result in greater 5-HT₃ receptor activation and increased motility and intestinal transit.

Alosetron treatment increased gastric emptying, but did not change intestinal transit in male CD-fed mice. Gastric emptying is regulated by extrinsic inputs from the CNS that are modulated by vagal afferent activation (Chang *et al.*, 2003). Increased gastric emptying caused by alosetron may occur by blocking glucose-dependent 5-HT activation of vagal afferent 5-HT₃ receptors that decrease gastric emptying by a vagovagal reflex (Raybould *et al.*, 2003). Glucose could come from FITC-dextran metabolized to glucose by intestinal dextranases. The lack of alosetron effects on small intestinal transit in male mice is sex related since alosetron decreased intestinal transit in CD-fed female mice. Alosetron sensitivity observed only in females mimics what is seen in human subjects where alosetron improves IBS symptoms in female but not male patients (Viramontes *et al.*, 2001). In addition, Bush and colleagues (2011) demonstrated *ex vivo* that female mouse ileum was more sensitive to alosetron-dependent decreases in pressure distention evoked peristaltic contractions compared to male mouse ileum. In HFD-fed female mice, the distribution of the fluorescent marker was fairly uniform along the length of the small intestine while in CD-fed females, the majority of marker (~70%) was found in the fourth intestinal segment. Alosetron treatment of HFD-fed female mice changed the uniform distribution to a single peak similar to that seen in saline treated CD-fed female mice. These data suggest that alosetron may change *in vivo* motility from a continuous propulsive pattern in HFD female mice to a periodic propulsive pattern. These data suggest that alosetron may be helpful in treating obesity related intestinal dysmotility in females, but not males.

CONCLUSIONS

A key finding of this study is sex differences in the effects of a HF diet on 5-HT signaling and transit in male and female mice. SERT function was impaired in the jejunum of HFD-fed male but not female mice. In addition, we demonstrated sex differences in the effect of alosetron on intestinal transit. CD and HFD-fed female mice also had greater mucosal 5-HT content that may account for increased transit compared to males and offer an avenue for explanations in sex differences in alosetron effects on gastrointestinal motility.

Acknowledgments

Funding R01DK094932 and T32GM092715 from the National Institutes of Health (USA)

The authors thank Robert Burnett for performing the HPLC measurements. The authors thank Amy S. Porter, HT (ASCP) QIHC and Kathleen A. Joseph, HT (ASCP) QIHC at Michigan State University Investigative Histopathology Laboratory for performing the IHC experiments. The authors thank Laura Osaer for counting EC cells. The authors thank Cameron Cieglo for illustrating the CA flow bath schematic.

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New findings

- We demonstrate that small intestinal transit is altered in male and female obese mice.
- We found sex differences in intestinal 5-HT signaling in the jejunum of male and female obese mice that may underlie the altered intestinal transit.
- Our results reveal important sex differences in 5-HT-linked intestinal dysfunction in obesity.

Figure 1.

Male and female mice (C57BL/6J) developed DIO with time when fed a HFD. HFD-fed (60 kcal %) male and female body weights measured at a number of weeks spent on the diet (11–15 weeks; A and B, respectively $n = 4–10$, $*P < 0.05$) and abdominal fat pad weights at time of sacrifice (13–15 weeks on diet; C and D, respectively) were greater compared to their respective CD-fed (10 kcal %) mice ($n=8$ for each group, $*P < 0.05$). Data are mean $\pm$ SEM.

Figure 2.

In vivo gastric emptying and small intestinal transit was increased in male HFD-fed mice. Gastric emptying and small intestinal transit were assessed 30 min after gastric gavage of FITC-dextran. (A) Gastric emptying was increased in HFD-fed male and CD-fed female compared to CD-fed male mice ($n = 7-8$, $*P < 0.05$). (B) The GC value was greater in HFD-fed male mice compared to CD-fed male mice and in CD-fed females compared to CD-fed males, indicating increased transit in these groups ($n=7-8$, $*P < 0.05$). (C) Fraction of fluorescence as a function of segment number describes FITC-dextran distribution along the length of the intestine where HFD-fed males displayed increased transit compared to CD-fed males ($*P < 0.05$ vs. same segment #). (D) HFD-fed females presented a flattened distribution of fluorescence where FITC-dextran was present in equal amounts within the middle portion of the small intestine compared to CD-fed females where the greater majority of FITC-dextran was found in segment 4 with little in other segments ($*P < 0.05$ vs HFD segment #4). All data are mean $\pm$ SEM.

Figure 3.

Alosetron, a 5-HT₃ receptor antagonist, changed gastric emptying and GC in CD-fed mice. Following 30 min after intraperitoneal (i.p.) administration of alosetron (0.1 mg kg⁻¹), mice were gavaged with FITC-dextran. Gastric emptying and the GC value were measured after an additional 30 min. (A) Alosetron treatment increased gastric emptying in CD-fed males compared to saline-treated mice (n=8, *P<0.05) but not in HFD-fed male mice (n=6) or in female mice fed CD (n=6) or HFD (n=8). (B) Alosetron did not alter the GC in CD or HFD fed male mice. The GC value was decreased in alosetron-treated CD-fed females compared to saline-treated controls (*P<0.05). Alosetron did not alter the distribution of FITC-dextran along the length of the small intestine in either CD-fed (C) or HFD-fed (D) males. (E) Alosetron-treated HFD-fed males share a similar pattern of FITC-dextran distribution along

the small intestine as saline-treated CD-fed males. Alosetron-treated CD-fed (F) and HFD-fed (G) female mice show a leftward shift of the fraction of fluorescence along the small intestine compared to saline-treated CD-fed and HFD-fed females, respectively. (H) The pattern of FITC-dextran distribution in alosetron-treated HFD-fed females overlies that of saline-treated CD-fed females. Data are mean $\pm$ SEM. (* $P < 0.05$, $n = 6 - 8$).

Figure 4.

Jejunal 5-HT₃ receptor function is similar in HFD-fed and CD-fed mice. 5-HT (0.01–10 $\mu\text{mol L}^{-1}$) generated longitudinal muscle contractions in jejunal segments (black circles). The 5-HT₃ receptor antagonist ondansetron (0.1 $\mu\text{mol L}^{-1}$) in the presence of methysergide (1 $\mu\text{mol L}^{-1}$) (black open circles) produced a similar inhibition of 5-HT-induced contractions at the greatest 5-HT doses in HFD-fed and CD-fed jejunal segments in both males (A) and females (B). Methysergide was used as a control to block non-target 5-HT receptors (grey open squares). Measurements are expressed as a percentage of maximum contraction by ACh (100 $\mu\text{mol L}^{-1}$). Data are mean $\pm$ SEM, n = 6–8.

Figure 5.

Continuous amperometry (CA) method for 5-HT uptake in jejunal preparations. (A) Schematic showing the flow bath housing the jejunal preparation and diamond microelectrode position at the mucosal surface. (B) Representative cyclic voltammogram of 5-HT ($10 \mu\text{mol L}^{-1}$) made in Krebs buffer (grey line) and in Krebs buffer alone (black line). The E_p^{ox} of 5-HT is $\sim +0.65 \text{ V}$ (arrow). Scan rate was 0.1 V s^{-1} . (C) Current approach curves obtained at electrode distances of 750–10 μm from the mucosal surface before (Krebs buffer; pH = 7.4), during and after (washout) application of fluoxetine ($1 \mu\text{mol L}^{-1}$; 5 min)., Fluoxetine produced a reversible increase in 5-HT oxidation currents. (D) Current vs. distance plots in which current is plotted against the tissue-electrode distance. (E) Representative natural log (ln) of the currents (pA) shown in (D) plotted against tissue-electrode distances without (black squares) and with fluoxetine (grey squares). The slope is a measure of 5-HT uptake and fluoxetine reduces the slope. Data are mean $\pm$ S.E.M.

Figure 6.

Jejunal SERT function and expression are altered in HFD-fed mice. (A) CA measurements in the presence of fluoxetine (1 $\mu\text{mol L}^{-1}$) reduced 5-HT uptake (slope) in CD-fed, but not HFD-fed male jejunum while fluoxetine reduced 5-HT uptake in CD- and HFD-fed female jejunum (* $P < 0.05$, $n = 8$). (B) Extracellular 5-HT was higher in preparations from CD vs. HFD-fed female mice and ($n = 10$ for each) and vs. CD- and HFD-fed male jejunum ($n = 15$). (C) 5-HIAA levels in jejunal mucosa were similar in HFD-fed compared to CD-fed males ($n = 15$). 5-HIAA levels in CD- and HFD female ($n = 10$) mucosa were higher than in CD and in HFD-fed male mice. (D) Representative Western blot showing SERT protein levels in jejunal mucosa from female and male CD-fed and HFD-fed mice. SERT protein was found at the apparent MW of approximately 100 kDa (closed arrow) and LDH at 35 kDa (open arrow). (E) SERT protein was unaffected by diet in male mice, but was increased in HFD-fed compared to CD-fed female mice ($n = 8$ for each). (F) SERT mRNA expression (normalized to GAPDH) was similar in CD- and HFD-fed female jejunum ($n = 8$). Data are mean $\pm$ SEM, * $P < 0.05$.

Figure 7.

Mucosal 5-HT levels in the jejunum are sex-dependent, but diet-independent. (A) Representative image of EC cells in the jejunal mucosa. The EC cells were identified (dark gray) by immunohistologic staining using anti-5-HT antibody in thin (4–5 μ m), paraffin-embedded sections. Scale bar = 100 μ m. (B) EC cells number was unaffected by diet, but was greater in CD-fed females compared to CD-fed males ($n = 10$). (C) 5-HT levels in jejunal mucosa measured using HPLC with electrochemical detection was greater in females ($n=10$) vs. males ($n=15$) but was unaffected by diet. Data are mean $\pm$ SEM, * $P < 0.05$.

Figure 8.

DAT is present in the jejunal mucosa and clears 5-HT in HFD-fed male jejunum. (A) In CA measurements, the DAT blocker, GBR 12909 ($0.1 \mu\text{mol L}^{-1}$) reduced 5-HT uptake compared to Krebs buffer in male HFD-fed but not CD-fed jejunum ($n = 5$). (B) DAT western blot identified two predominant bands at ~ 60 and ~ 100 kDa (black arrowheads) in jejunal mucosa (left: 1° and 2° antibody; right: 2° antibody alone). (C) Western blot comparing DAT protein levels between CD-fed and HFD-fed male jejunal mucosa. LDH is shown at ~ 35 kDa (open arrowhead). (D) The DAT/LDH ratio was increased in HFD-fed compared to CD-fed males for the 100 kDa band and when both the 60 and 100 kDa bands are combined but not for the 60 kDa band alone ($n = 8$ each). (E) DAT-ir in paraffin-embedded sections was observed in enterocytes (solid arrow) and in the lamina propria (dotted arrow). (G) DAT-ir is shown in the myenteric and submucosal plexus and absent in secondary only controls (F, H). Scale bar = $50 \mu\text{m}$. Data are mean $\pm$ SEM, $*P < 0.05$.

Figure 9.

HFD did not alter total plasma sex hormone levels in female or male mice. (A, B) Plasma estradiol and progesterone levels from un-staged CD-fed and HFD-fed females were similar ($n = 10$). (C) Plasma testosterone levels in CD-fed and HFD-fed males were similar ($n = 10$). Data are mean $\pm$ SEM.

Table 1

Summary of major sex and diet related changes in gut motility and monoamine signaling and transporter expression.

Measurement	Males	Females
Gastric emptying	↑ in HFD vs. CD	↑ in CD vs. CD-fed males
Small intestinal transit	↑ in HFD vs. CD	↑ in CD vs. CD-fed males
Alosetron-gastric emptying	↑ in CD vs. CD-saline	No change
Alosetron-small intestinal transit	No change	↓ GC in CD Oral shift in FITC-distribution in CD and HFD
5-HT ₃ receptor function	No change	No change
SERT function	↓ in HFD vs. CD	No change
SERT protein	No change	↑ in HFD vs. CD
Extracellular 5-HT	No change	↑ in CD vs. HFD ↑ in CD and HFD vs. males
Mucosal 5-HT	No change	↑ in CD and HFD vs. males
EC cell number	No change	↑ in CD vs. CD-fed males
DAT function/protein	↑ in HFD vs. CD	Not measured
Plasma sex hormone levels	No change	No change