A Western diet increases serotonin availability in rat small intestine

Abbreviated title: Serotonin and obesity

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Abbreviations: 5-HT, 5-hydroxytryptamine or serotonin; EC cells, enterochromaffin cells; SERT, serotonin reuptake transporter.

Précis: A Western diet is associated with increased 5-HT availability in the rat ileum which may lead to altered intestinal motility or sensation.

Abstract

Diet-induced obesity is associated with changes in gastrointestinal function and induction of a mild inflammatory state. Serotonin (5-HT) containing enterochromaffin (EC) cells within the intestine respond to nutrients and are altered by inflammation. Thus, our aim was to characterize the uptake and release of 5-HT from EC cells of the rat ileum in a physiologically relevant model of diet-induced obesity. In chow-fed (CF) and Western diet-fed (WD) rats electrochemical methods were used to measure compression evoked (peak) and steady state (SS) 5-HT levels with fluoxetine used to block the serotonin reuptake transporter (SERT). The levels of mRNA for tryptophan hydroxylase 1 (TPH1) and SERT were determined by quantitative PCR while EC cell numbers were determined immunohistochemically. In WD rats, the levels of 5-HT were significantly increased (SS: 19.2±3.7 μM; peak: 73.5±14.1 μM) compared to CF rats (SS: 12.3±1.8 μM; peak: 32.2±7.2 μM) while SERT-dependent uptake of 5-HT was reduced (peak WD: 108% of control versus peak CF: 212% control). In WD rats, there was a significant increase in TPH1 mRNA, a decrease in SERT mRNA and protein, and an increase in EC cells. In conclusion, our data show that foods typical of a Western diet are associated with an increased 5-HT availability in the rat ileum. Increased 5-HT availability is driven by the up-regulation of 5-HT synthesis genes, decreased re-uptake of 5-HT, and increased numbers and/or 5-HT content of EC cells which are likely to cause altered intestinal motility and sensation *in vivo*.

Introduction

Enterochromaffin (EC) cells function as sensory transduction elements in the gastrointestinal (GI) mucosa, responding to chemical and mechanical stimuli by releasing serotonin (5-HT) and other potential mediators onto afferent nerve terminals to initiate GI reflexes and modulate visceral perception (1-3). As such, these cells are ideally placed to contribute to GI dysfunction and the symptoms of bowel disease. This has led to a great deal of interest in how EC cells release 5-HT and how 5-HT availability is affected during disease (e.g., 4, 5).

The EC cells can respond directly to ingested nutrients by a variety of mechanisms, including the use of taste transduction machinery (reviewed in 6). EC cells contain the taste G-protein α-gustducin (7, 8) as well as taste receptors (T1R and T2R), second messenger systems (PLCβ2) and channels (TRPM5) that are normally associated with the tongue (7, 9-13). In addition, Nozawa *et al* (14) have shown that TRPA1, the receptor for pungent compounds such as mustard, is also expressed by EC cells. Together, these studies suggest that EC cells can respond directly to ingested nutrients. As such, we predict that an altered diet may influence EC cell behavior.

Diet-induced obesity is an increasing health care burden in many Western countries and is due to environmental, lifestyle and genetic influences (15). Obesity has been associated with decreases in GI motility such as constipation and an increased incidence of colorectal cancer (16, 17). The levels of intestinal hormones secreted from enteroendocrine cells include factors controlling satiety and blood glucose (e.g., ghrelin, orexin and GLP-1) and these are also changed in obesity (e.g., 18, 19). In fact, surgical treatments for obesity such as Roux-en-Y gastric bypass or gastric banding owe much of their effectiveness to altered GI hormone production (20, 21). Animal studies show that changes in EC cell

numbers occur in obese leptin deficient *ob/ob* mice (22), as well as leptin receptor deficient *db/db* mice (23), and in a model of acute hyperglycemia (dexamethasone-treated rats) (24). One mechanism by which obesity could alter EC cell numbers or function is through the induction of a mild inflammation. Obesity has been shown to be a mild inflammatory disease (25-28) and, as inflammation is known to alter 5-HT availability (reviewed in 29), we predict that EC cells would respond to the obesity associated inflammatory milieu with altered cell numbers or release characteristics to change gut function.

Recent electrochemical studies in healthy GI mucosa have shown that it is possible to gain more precise information about 5-HT release (30, 31) and 5-HT uptake via SERT (32) by monitoring 5-HT levels in real-time close to the mucosal surface. Recently, our group has shown that 5-HT availability is increased during dextran sulfate sodium induced colitis (6), but it is unknown whether similar changes in regulation occur during obesity. Therefore, the aim of this study was to characterize the release and uptake of 5-HT in the ileum of a rat model of diet-induced obesity.

Materials and Methods

Diet and sample collection

Male Sprague-Daly rats weighing 211±2 g (6-7 weeks) were obtained from Animal Resources Centre (Perth, Western Australia, Australia). Experimental protocols were approved by the Animal Experimentation Ethics Committee of the University of New South Wales. Animals were randomly divided into two groups. For 16 - 20 weeks the first group was fed a standard rat chow (11 kJ/g, 14% fat, Gordon's Specialty Stockfeeds, Yanderra, NSW; chow-fed; CF) and the second group was fed a highly palatable cafeteria-style diet which consisted of high fat foods of a known caloric content (average 15.33 kJ/g, Western diet; WD) (33, 34). The WD consisted of foods such as meat pies, cakes and deep-fried potatoes in addition to standard chow. The WD was approx 60% carbohydrate and 32% fat, and the CF diet was 65% carbohydrate and 13.7% fat (35). During this time, veterinary staff monitored animals for signs of disease. Body weight was measured weekly and 24 hour food intake (kJ) was measured every 2-3 weeks. At the end of the diet period rats were anesthetized with sodium thiopentone (100 mg/kg i.p., Abbott, Kurnell, NSW) and blood samples were taken by cardiac puncture with blood glucose measured immediately (Accu-Chek Advantage, Roche Diagnostics Australia, Castle Hill, NSW). Rats were then sacrificed by guillotine with left retroperitoneal (Rp) and testicular fat removed and weighed, and segments of ileum removed for assay. The distal ileum was chosen as this region normally does not see a high nutrient load. We hypothesised that during establishment of diet-induced obesity, this region would change from a low to high nutrient load and, thus, may show a greater change than more proximal regions.

Electrochemistry

Tissue preparation and data gathering

Segments of ileum 5-10 cm from the ileo-cecal junction were removed from CF and WD rats and placed in physiological saline (Krebs; in mM: 126 NaCl, 2.5 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 5 KCl, 25 $NaHCO_3$, and 11 glucose) that was gassed with 95% O_2/5% CO_2. The ileum was flushed and opened along the mesenteric border and a small segment (~10 mm wide by ~10 mm in length) placed in a small volume (3 mL) recording chamber lined with a silicone elastomer (Sylgard 184, Dow Corning, MI, USA) and superfused with warmed (35^{o}C) Krebs containing, when needed to reduce muscle contractions, papaverine (100 μM; phosphodiesterase inhibitor (31)) at a flow rate of 6 mL/min. Preparations were pinned out mucosa up with 150 μm pins and visualized at 20x magnification using an upright dissecting microscope.

Electrochemical recordings were made as described in detail previously by ourselves (5, 30-32, 36) and others (37, 38). The carbon fiber electrodes (7 μm diameter, 200-400 μm exposed) were calibrated with freshly made 5-HT (10-20 μM; Sigma-Aldrich, Castle Hill, NSW, Australia) before each set of mucosal 5-HT measurements (i.e., control and in fluoxetine). Carbon fiber electrodes were voltage-clamped at +400 mV versus a Ag/AgCl ground and were placed above or touching the mucosa (see Figure 1A) using a mechanical micromanipulator (MP-1, Narishige Scientific Instruments, Tokyo, Japan). Recordings of oxidation currents were made using a VA-10 amplifier (NPI Electronics, Tamm, Germany) with a Ag/AgCl ground, digitized at 10 kHz (Digidata 1440), and recorded on a personal computer. For analysis and graphing, the signals were filtered at 50 Hz (notch) and reduced by 5x using PClamp9 (MDS Analytical Technologies, Sunnyvale, CA, USA) and then analyzed with PClamp9 and graphed with Origin7.5 (MicroCal, Northampton, MA, USA). Evoked release of 5-HT

was accomplished by compression of the epithelium with 1 - 2 mg of force using the carbon fiber electrode as a von Frey hair (e.g., 10 mg breaking force is the minimum used to activate extrinsic colonic afferents, 39). The oxidation current was recorded at 3 - 6 locations per preparation and converted to the peak (direct compression evoked) and steady state concentrations of 5-HT (Figure 1B). Total 5-HT measured is a combination of apical and basal release (Figure 1B). Levels of 5-HT were recorded under control conditions and during superfusion of fluoxetine (1 μM, specific serotonin reuptake inhibitor; 20 min equilibration; Sigma-Aldrich, Castle Hill, NSW, Australia).

RT-PCR, western blot and immunohistochemical analyses

Real-time quantitative PCR analysis of mRNA expression

Scrapings of ileal mucosa (taken using a scalpel blade; mucosa-only; 50 mg) had total RNA extracted from CF or WD rats using the TRizol method (Invitrogen, Mulgrave, Vic, Australia) followed by a DNase treatment (3U at 37°C for 20 min) to remove residual DNA. Single strand cDNA (sscDNA) was reverse transcribed from 2 μg of total RNA using a SuperScript III First-Strand Synthesis System (Invitrogen) and random hexamers (50 ng/μL) at 1 cycle of 25˚C for 10 min, 50˚C for 50 min and 85˚C for 5 min.

Real-time quantitative PCR was carried out to determine mRNA expression of SERT, TPH1 (tryptophan hydroxylase 1 enzyme), villin (intestinal epithelium brush border protein), GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and β-actin using RealMasterMix SYBR ROX (5 PRIME, Quantum-Scientific, Murarrie, Qld, Australia). Each PCR reaction was performed in a volume of 25 μL containing 500 ng of sscDNA, 0.05 U HotMaster Taq polymerase, 4 mM magnesium acetate, 0.4 mM dNTP with dUTP, SYBR Green I dye, and a pair of gene specific primers for which the

optimal concentrations were predetermined. GAPDH, β-actin and villin were used as housekeeping genes (HKGs), and in each real-time PCR assay, a designated calibrator RNA (from one control rat ileum) was used to allow inter-run comparisons. The PCR amplification conditions were 1 cycle at 95˚C for 2 min, followed by 40 cycles of 95˚C for 15 s, 60˚C for 15 s and 68˚C for 20 s. In the final step, the melting curve analysis was carried out during gradual temperature elevation from 60˚C to 95˚C. Oligonuclear primers used for real-time PCR were as follows: SERT-forward: 5'-atggagaccacacccttga-3' and reverse: 5'-gtggggacacccttctgta-3'; TPH1-forward: 5'-caaggagaacaaagaccattc-3' and reverse: 5'-cgcagtccacaaaaatctca-3'; Villin-forward: 5'-tcaaaggctctctcaacaccac-3' and reverse: 5'-agcagtcaccatcgaagaagc-3'; GAPDH-forward: 5'-gtcggtgtgaacggatttg-3' and reverse: 5'-tggaagatggtgatgggttt-3'; β-actin-forward: 5'-gcgcaagtactctgtgtgga-3' and reverse: 5'-acatctgctggaaggtggac-3'.

The mRNA level for each gene was expressed as fold change, in which each target gene was normalized to GAPDH, β-actin or villin, and expressed relative to the calibrator using the formula: Fold change=$2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct=[Ct_{(target)} - Ct_{(HKG)}]_{sample} - [Ct_{(target)} - Ct_{(HKG)}]_{calibrator}$ (40). The HKGs GAPDH, β-actin or villin were not found to change in the mucosa-only samples during WD (Supplemental Figure 1) nor was villin found to change when compared directly to GAPDH or β-actin (Supplemental Figure 2).

Western blotting for SERT

Approximately 50 mg of ileal mucosa scrapings (n=4 from each group) were frozen in liquid nitrogen and if needed, stored at -80°C. Scrapings were ground in liquid nitrogen using a pestle and mortar, re-suspended in phosphate buffered saline (PBS) pH 7.4 containing complete protease inhibitor cocktail

(Roche, Castle Hill, NSW, Australia) and 0.1% Triton x100, and centrifuged (3000 x g, 4˚C, 5 min). The supernatant was removed and placed on ice, aliquoted, snap frozen in liquid nitrogen and stored at -80^{o}C. Protein concentration of the samples was determined using the Bradford protein assay (BioRad, Gladesville, NSW, Australia).

Aliquots of protein extracts (20 µg protein) were dissolved in lithium dodecyl sulphate (LDS) sample buffer (0.5% LDS, 62.5 mM Tris-HCl, 2.5% glycerol, 0.125 mM EDTA, pH 8.5) for 10 minutes at 70^{o}C. The samples were separated by electrophoresis in bis-Tris polyacrylamide gels using MOPS SDS running buffer and electroblotted onto PVDF membranes overnight at 4˚C according to the recommendations of the manufacturer (Invitrogen). Following transfer, membranes were thoroughly washed, blocked and probed with anti-SERT primary antibody (ImmunoStar Inc., Hudson, WI, USA) overnight at 4C. Specific binding was visualized using alkaline phosphatase conjugated secondary antibody and chemiluminescence according to the instructions of the manufacturer (Invitrogen). Membranes were then stripped and re-probed with anti-actin antibody (Sigma-Aldrich) for 2 hours at room temperature and specific binding was visualized as before. The intensity of the band corresponding to SERT or actin protein expression was determined using Photoshop CS3 extended (Adobe Systems Incorporated, San Jose, CA, USA) software, normalized against sample specific Coomassie stained protein bands and statistically analyzed using Prism5 software (GraphPad Software, Inc., La Jolla, CA, USA).

Immunohistochemistry

Immunohistochemical analyses were made as described in detail previously (5). Briefly, 1 cm segments of ileum were removed from rats and immersed in fixative (4% paraformaldehyde in PBS,

pH 7.4) for 4h at 4ºC. Tissue was rinsed with PBS 3 x 5 min at room temperature, immersed in cold 20% sucrose overnight at 4ºC, and embedded in Tissue-Tek OCT Compound (Sakura Finetek, ProSciTek, Thuringowa, Qld, Australia). Sections (14 µm) were cut on a cryostat, thaw mounted onto 0.3% gelatin coated glass slides (Fisherbrand Superfrost plus) and stored at -80ºC if required. Prior to immunolabeling slides were thawed and washed for 3 x 10m in 0.1 M PBS with 0.5% Triton X-100 (Sigma-Aldrich). Tissue sections were blocked with 1% normal donkey serum (NDS) in 0.1 M PBS with 0.1% Triton X-100 for 1h at room temperature in a humid chamber, briefly washed, and subsequently incubated with rabbit anti-5-HT primary antiserum, 1:5000 (Immunostar, Inc., Hudson, WI, USA) in 1% NDS in 0.1M PBS with 0.1% Triton X-100 overnight at 4˚C. The following day slides were washed and incubated with donkey anti-rabbit IgG Cy3 secondary antibody, 1:200 (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) for 2h at room temperature in a humid chamber, then washed. Tissue sections were then incubated with goat anti c-Kit primary antibody, 1:400 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in 1% NDS in 0.1M PBS with 0.1% Triton X-100 overnight at 4˚C to detect mast cells, washed and incubated with donkey anti-goat fluorescein isothiocyanate secondary antibody, 1:200 (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) for 2h at room temperature. They were then washed, covered with buffered glycerol and coverslipped.

Immunoreactivity was analyzed with an epifluorescence microscope (BH-2, Olympus, Tokyo, Japan). c-Kit is a cell surface cytokine receptor for stem cell factor which, in the gut, can be used as a marker for mast cells and interstitial cells of Cajal. In rodent, mast cells and EC cells both contain 5-HT, thus, the absence of c-Kit staining in a 5-HT positive cell suggests it is an EC cell. Thus, only cells that were within the epithelial border and were c-Kit negative and 5-HT positive were counted as EC cells. The number of 5-HT immunoreactive EC cells in each of four non-adjacent fields of view at x40

magnification was quantified per sample, as well as the number of crypts in each, and from that the average number of EC cells per crypt was calculated.

Solutions

All reagents were purchased from Sigma-Aldrich unless otherwise noted. Fluoxetine was made up as a 10 mM stock solution in EtOH, stored at room temperature and diluted into physiological saline on the day of the experiment. 5-HT was made up fresh from powder on the day of the experiment or frozen as a 10 mM solution in dH_2O.

Statistics

Population data are presented as mean ± standard error (SE) with the range and/or median given where appropriate. In all cases the 'n' value refers to the number of animals used while the number of repetitions is given when more than one response from a single animal was studied. A student's t test was used to make single comparisons while a one way ANOVA was used to make multiple comparisons of data. Tests were paired or unpaired as noted; a Tukey-Kramer post hoc test was used with the ANOVA. A Wilcoxon signed-rank test was used for non-parametric data (such as percentages). A value of $P \leq 0.05$ was taken as the cut-off for statistical significance.

Results

Effect of a Western diet on rats

During the diet period the average daily energy intake of animals maintained on the WD (675±23 kJ/rat/day; n=16; Supplemental Figure 3) was significantly increased compared to CF (308±12 kJ/rat/day; n=14; $P<0.001$, Supplemental Table 1). At the end of the diet period the obese animals on the WD had increased body weight and fat mass (retroperitoneal and testicular fat; $P<0.0001$, Supplemental Table 1). Blood glucose was also increased from 8.7±0.3 mM in CF to 10.2±0.4 mM in WD rats ($P<0.01$, Supplemental Table 1).

Characterization of 5-HT release from rat ileum

EC cells release 5-HT at rest and this has become an important measure of serotonin availability. We assessed the ongoing release of 5-HT from the EC cells (termed steady state (SS) levels) using electrochemical amperometry techniques. The electrode was held at a constant potential of +400 mV, just above the oxidation potential for 5-HT (30). When the electrode was brought into gentle contact with the mucosa, an increase in oxidation current was detected (as illustrated in Figure 1A). The steady state levels of 5-HT were measured from multiple sites on the mucosal surface; 3-6 sites were found to be consistent within a single preparation and between preparations. Using calibration data specific to each electrode allowed us to calculate that the oxidation current generated was equivalent to a level of 12.3±1.8 μM 5-HT at steady state (n=8; Figure 1B; Figure 2B, right panel).

EC cells are known to release 5-HT during mechanical stimulation (e.g., 41). We determined the peak amount of compression-evoked 5-HT release by using the recording electrode to compress the mucosal

epithelium while simultaneously measuring 5-HT oxidation current. This evoked release of 5-HT was calculated to be 32.2±7.2 μM (n=12; Figure 1B; Figure 2B left panel). The compression-evoked 5-HT oxidation current peaked shortly after the electrode contacted the mucosa (0.7±0.2 s; n=8) and decayed back to steady state levels within ~15 s. Overall, the average compression-evoked peak level of 5-HT was 271±48% of the steady state levels (n=12). In order to rule out any contribution of movement artifacts to the oxidation current, we confirmed that when the electrode potential was held at 0 mV, no oxidation current was detected during compression of the mucosa (data not shown).

WD rats exhibited a significant increase in the 5-HT levels detected with the carbon fiber electrode from the mucosal epithelium (Figure 2A). Steady state levels were significantly increased from 12.3±1.8 μM in CF rats to 19.2±3.7 μM in WD rats (156% of CF rats; n=12,11; *P*=0.044; unpaired t-test; Figure 2B). Peak compression-evoked 5-HT release was also significantly increased from 32.2±7.2 μM in CF rats to 73.5±14.1 μM in WD rats (228% of CF rats; n=12, 11; *P*=0.005; unpaired t-test; Figure 2B).

Changes to the uptake of 5-HT during obesity

The uptake of 5-HT by SERT-dependent mechanisms is a key factor in controlling 5-HT availability in the GI tract. The real-time SERT-dependent uptake of 5-HT can be inferred using single carbon fiber electrochemical techniques (42, 43). We have recently extended this to monitor SERT function in the colon of the mouse (5) while others have examined guinea pig (37). These later studies have shown that activity of the epithelial SERT helps to reduce steady state levels of 5-HT and can reduce peak compression evoked levels. The difference between 5-HT levels before and during SERT blockade with 1 μM fluoxetine can be taken as an estimate of SERT function. We measured the 5-HT oxidation

current during compression of the mucosa (Figure 3A) and found that both peak and steady state levels of 5-HT were increased during SERT blockade. The average peak levels of 5-HT were significantly increased from 39.1±11.6 μM in control to 84.8±27.2 μM in fluoxetine (217%; n=7; *P*=0.015; paired t-test; Figure 3A). To reduce inter-experiment variability, fluoxetine data were also expressed as a percent of control for individual experiments and these percentages were averaged. Again, fluoxetine was again seen to cause a robust doubling of peak levels of 5-HT (212±23% of control; Figure 3A; *P*=0.008, Wilcoxon signed-rank test). During steady state the average 5-HT levels were also significantly increased from 11.4±1.8 μM in control to 18.6±3.2 μM in fluoxetine (163%; n=7; *P*=0.034; paired t-test). When the average percent of control data were analyzed, there was also a large increase (180±34% of control, *P*=0.039, Wilcoxon signed rank test) (32).

The capacity for SERT-dependent uptake of 5-HT was significantly reduced in WD rats. Peak compression evoked release was unchanged from 61.9±10.9 μM before fluoxetine to 60.2±7.6 μM during SERT blockade (97%; n=7; *P*=0.433; Figure 3B). When the average percent of control data were analyzed, this was not significantly different at 108±15% of control (data not illustrated). Similarly, blockade of SERT had no effect on steady state release from 12.8±2.4 μM before fluoxetine to 11.8±2.7 μM in the presence of fluoxetine (n=9; *P*=0.275; Figure 3B). When the average percent of control data were analyzed, this was unchanged at 120±17% of control (data not illustrated). In order to determine whether a high fat diet had an effect on the magnitude of SERT-dependent uptake of 5-HT, the effect of fluoxetine was compared between WD rats and CF rats. From the preceding data, the contribution of SERT to peak compression-evoked 5-HT levels was clearly reduced (CF: 228% versus WD: 108%; data not illustrated).

Properties of 5-HT release during obesity

We examined whether the properties of 5-HT release from the EC cells were altered during obesity. We found that the rundown of the response during compression-evoked release was similar in both CF and WD rats with peak release reduced by ~50% during the second evoked response. In CF rats, the second peak was 53±7% of control (interval: 43±15s; n=6; data not illustrated) while in WD rats the second peak was 62±9% of the first peak (interval: 33±14; n=7; data not illustrated).

The pressure exerted on the mucosa by the electrode is similar to that from a von Frey hair and when advanced onto the surface bends at around 2 mg of force. However, a lighter touch can be used and this was exploited to examine whether the previous release of 5-HT had any effect on the measured steady state values. The steady state values in CF rats for a light touch were 6.4 ±1.7 μM (n=7) while heavy touch at the same spot showed a significantly higher steady state level of 8.5±2.2 μM (P=0.01; data not illustrated). Similarly, in WD rats the steady state values recorded after a light touch (12.9±3.7 μM; n=6) were significantly higher after a heavy touch (17.7±3.7 μM; P=0.02; data not illustrated). Similar to our overall data, the steady state values in WD rats were significantly higher than in the corresponding CF rats (i.e., WD: 12.9 versus CF: 6.4 μM for light touch; WD: 17.7 versus CF: 8.5 μM for heavy touch; data not illustrated).

Relationship between blood glucose, weight and 5-HT availability

Diet-induced obesity is associated with increased levels of 5-HT at the mucosal surface, but whether increased body weight and/or blood glucose were directly related to the magnitude of 5-HT present is unknown. We tested this idea by plotting the relationship between 5-HT levels, blood glucose and weight in CF and WD rats (Figure 4). When data on 5-HT levels and blood glucose were plotted for

individual rats, there was no significant relationship for peak compression evoked 5-HT release (R=0.27; Figure 4A) or for steady state 5-HT levels (R=0.58; Figure 4B; *P*=0.124). As expected, when the peak compression evoked release of 5-HT and the steady state levels of 5-HT were plotted, there was a significant positive correlation (*P*=0.001; Figure 4C). In a similar manner, a strong positive correlation between blood glucose and final weight was also found (*P*=0.006; Figure 4D).

Effect of diet length and composition

In order to determine whether 16-20 weeks of WD was necessary to see this increase in 5-HT availability, we examined animals that were on the WD for only 10 weeks. Age-matched control rats fed a standard chow diet were used as a comparison. As with the longer diet, rats fed a WD for 10 weeks were significantly heavier (CF rats: 363±22g and WD rats: 448±19g; *P*=0.006; n=7,5). Similarly, WD rats exhibited a significant increase in 5-HT levels detected at the mucosal epithelium. Steady state levels of 5-HT were significantly increased from 11.7±2.4 μM in CF rats to 31.6±11.9 μM in WD rats (270% of CF rats; n=7, 5; *P*=0.028; data not illustrated). Peak compression-evoked 5-HT release was also significantly increased from 25.2±3.2 μM in CF rats to 53.3±15.2 μM in WD rats (219% of CF rats; n=7, 5; *P*=0.014; unpaired t-test; data not illustrated).

Based on these results, we wondered whether changes to 5-HT availability were taking place within the first few weeks of the WD. Ingested nutrients are in direct contact with the EC cells which have a 16 day turnover time (44), so significant changes to EC cell function might occur. To investigate, animals were placed on a 10 day WD and 5-HT levels measured. Rats on a 10 day WD were significantly heavier than control rats (CF: 266±17g; WD rats: 308±7g; *P*=0.014; n=3,6). Steady state levels of 5-HT were unchanged (6.2±0.7 μM in CF rats; 6.5±0.5 μM in WD rats; 105% of CF rats; n=3, 6; *P*=0.36;

data not illustrated). In contrast, peak compression-evoked 5-HT release was significantly increased from 26.2±4.9 μM in CF rats to 34.9±2.0 μM in WD rats (133% of CF rats; n=3, 6; *P*=0.042; unpaired t-test; data not illustrated).

We were also interested in whether the fat content of the WD was the most important contributor to the increase in 5-HT availability. To test this, we examined rats fed a medium fat pellet diet (22% fat chow; Specialty Feeds, WA; as compared to 32% fat in WD) for 16-20 weeks and compared these with age-matched control rats fed a standard chow diet. Control diet rats were 642±16g and medium-fat diet rats were 723±30g which was a significant increase (P=0.037; n=4,3). In contrast to WD rats, those fed a medium fat pellet diet did not show a change in 5-HT availability. Steady state levels were not changed with 2.7±0.3 μM in CF rats and 3.3±1.2 μM in medium fat pellet fed rats (122% of CF rats; n=4, 3; *P*=0.310; data not illustrated). Peak compression-evoked 5-HT release was also not changed with 18.4±2.6 μM in CF rats and 15.6±2.1 μM in medium fat pellet fed rats (85% of CF rats; n=4, 3; *P*=0.218; unpaired t-test; data not illustrated). Together, these data suggest that the effects of the Western diet on 5-HT availability start within 10 weeks and that the fat content of the Western diet, although higher than the chow diet, may not be the most important component driving changes in 5-HT.

TPH-1 and SERT mRNA

Our electrochemical studies showed that 5-HT levels were increased and that the fluoxetine sensitive uptake of 5-HT was reduced in WD rats. To examine the mechanisms responsible for the reduction in SERT activity, we used quantitative real time RT-PCR to determine the levels of SERT mRNA expression in mucosa-only samples of rat ileum (Figure 5, left column). In line with our functional

assays of SERT activity, the mucosal levels of SERT mRNA, normalized to GAPDH mRNA, were reduced in WD rat ileum (n=14) compared to CF rats (n=9; *P*=0.037, unpaired t-test; Figure 5A). However, when compared to β-actin (Figure 5B) or the brush border protein villin (Figure 5C) there was no difference in mucosal SERT mRNA expression compared to CF rats.

Increased 5-HT availability could also come about by an increase in 5-HT production by the EC cells. To investigate this, we used quantitative real time RT-PCR to determine the levels of TPH-1 mRNA expression in mucosa-only samples (Figure 5, right column). TPH-1 is the rate limiting enzyme in the 5-HT synthesis pathway and increased levels could reflect either more TPH-1 per EC cell, or more EC cells. We detected a significant increase in the levels of TPH-1 mRNA when normalized to villin mRNA, in WD rat ileum compared to CF rats (*P*=0.021; n=9,12; unpaired t-test; Figure 5C); however, there was no change when TPH-1 was compared to GAPDH or β-actin (Figure 5A,B).

SERT protein

Western blots quantified the amount of SERT protein in relation to Coomassie staining which was used as a loading control. In WD rat ileum the ratio of SERT to Coomassie was 1.76±0.10 which was significantly decreased compared to 5.59±0.39 in CF rats (n=4 for each condition; *P*=0.001, unpaired t-test) (Figure 6). For the same samples, actin was compared to Coomassie and found to be decreased during diet-induced obesity from 7.71±0.77 in CF rats versus 2.56±1.19 in WD rats (n=4 for each condition; *P*=0.006, unpaired t-test).

EC cell numbers

To further examine the cause of the increased levels of 5-HT detected electrochemically in WD rats, we double labeled ileum from both groups against 5-HT and c-Kit using immunohistochemical techniques (Figure 7). Counts were made of 5-HT immunoreactive EC cells which were not c-Kit positive (differentiating them from mast cells) and quantified per mucosal crypt. WD rats had a significant increase in the number of EC cells/crypt-villus axis from 1.24±0.29 in CF to 1.90±0.17 in WD (n=4; P=0.032; unpaired t-test).

Discussion

The main finding of this study was that mucosal 5-HT availability was increased in the ileum of a rat model of diet-induced obesity. Real-time measurements of 5-HT levels showed that during obesity the release of 5-HT was enhanced; findings that are supported by an increase in the numbers of EC cells and an increase in TPH-1 mRNA. Our electrochemical data also showed in the WD rat ileum a decrease in SERT-dependent uptake of 5-HT that was mirrored by decreases in SERT mRNA and protein levels. This study is the first to demonstrate an increase in 5-HT availability in obese tissue and the first to characterize the molecular changes in serotonin signaling associated with a detrimental Western diet.

In the present study, carbon fiber electrodes were used to record 5-HT levels near the mucosa in the quiescent preparation and during compression of the epithelium with the electrode. We found that the levels of 5-HT released in CF rat ileum were approximately 32 μM at the peak of the response to compression and approximately 12 μM at steady state. Interestingly, these levels are higher than that found in the rat ileum in our previous study (compression evoked ~14 μM, steady state ~6 μM) (32). However, it is worth noting that the rats used in our previous study were younger than the rats used here (32). Along these lines we have also found evidence that in aged mouse ileum 5-HT levels are increased compared to young mice (45).

Diet-induced obesity increases 5-HT levels

In the present study, measurements from the ileum of rats fed a Western diet revealed a clear increase in 5-HT availability. Our electrochemical data showed that both compression evoked release and

steady state 5-HT levels are higher at the mucosal surface of WD rats. Additionally, the electrochemical data showed that 5-HT uptake is reduced in WD rats, which suggests a reduced SERT function. We tested the mechanisms by which this increased availability might have occurred. First, we examined WD rats at earlier time points and found that the effects of Western style foods were associated with an increase in mechanically evoked 5-HT release at 10 days and with an increase in both mechanical release and steady state levels at 10 weeks. Second, we examined rats fed a medium fat pellet diet for 16 weeks and found that these rats did not appear to have an increased availability of 5-HT; though these data are from a small sample size. Together, these data suggest that important changes are established within the first few weeks of WD and that the fat content of the diet, although higher than the chow diet, may not have been the most important component driving changes to 5-HT availability.

Obesity and a high fat diet have been associated with changes in GI function and in the levels of intestinal hormones secreted (16, 17, 21). Animal models of obesity have also shown changes in intestinal motility (46, 47) although there are conflicting reports as to whether there is an increase or decrease in transit or mixing (reviewed in 16). A high 5-HT availability could well bring about such pathophysiological changes to motility or secretion during obesity. For example, recently Hyland *et al*. (48) showed that diet induced obesity in rats is associated with increased intestinal secretion.

EC cell numbers increase and SERT function decreases during diet-induced obesity

The levels of 5-HT at the mucosal surface are controlled by several factors including the active release of 5-HT from EC cells, which is itself controlled by the numbers of EC cells and the amount of 5-HT contained within them. Following release, the diffusion of 5-HT into the surrounding solution and its re-uptake by SERT contribute to overall 5-HT availability.

The numbers of EC cells have been assessed in a variety of genetic models of obesity and diabetes and during dietary manipulations. In the leptin deficient *ob/ob* rat (22) and the leptin receptor deficient *db/db* mouse (23) a low density of ileal EC cells has been found. However, in an acute hyperglycemia model (dexamethasone-treated rats) (24) there was an increase in ileal EC cells. Diet has also been shown to have an effect on the general proliferation of enterocytes (49) and presumably EC cells. However, a different study using a diet-induced obesity model found that not all enteroendocrine cell numbers are increased as there was no change in the GLP/PYY containing L cells (50). We have recently confirmed this by showing a lack of change for PYY mRNA in our WD rats (51). In the present study, we show that EC cell numbers increase in the ileum of WD rats. This provides one explanation for the increase in 5-HT availability which we measured electrochemically. That the number of EC cells increased suggests the total amount of 5-HT increased, though future studies would need to tested this by measuring 5-HT stored within the tissue.

SERT is localized to the plasmalemma of GI epithelial cells (52, 53) and a novel splice variant has been identified that is specific for the intestinal epithelium (54). In our electrochemical experiments, the

function of SERT was reduced in WD rats suggesting that SERT expression on the epithelial cells may be down-regulated during obesity. To investigate this further we looked at the levels of SERT mRNA and protein. Quantitative RT-PCR showed that SERT mRNA was reduced in obese rat ileum. Quantitative Western blot data extended these observations, showing that the WD rat ileum had a reduction in SERT protein. SERT has been shown to be up-regulated by increases in the levels of 5-HT (55). Thus, the increase in 5-HT levels we saw in WD rats are at odds with the decrease in SERT function and expression we found, suggesting that obesity induces a significant down-regulation of SERT which over-rides the effect of local increased levels of 5-HT.

How does diet-induced obesity increase 5-HT availability?

A high fat diet has been associated with changes in the intestinal microflora (56, 57) which in turn has been linked to inflammation (57, 58) and obesity (59). While diet may alter the proliferation of EC cells (49), it is inflammation which has been associated with robust changes in EC cell numbers and, as mentioned, obesity is a mild inflammatory disease (25-28). In animal models of inflammation, EC cells undergo hyperplasia (60) and it is clear that many animal models of intestinal inflammation are associated with an increase in the number of EC cells (e. g., 5, 60, 61, 62). Similarly, in patients with ulcerative colitis, EC cell numbers have been increased (e. g., 63). SERT expression has also been shown to be decreased during inflammation. In mouse trinitrobenzene sulfonic acid induced colitis 5-HT availability was increased due to a decrease in SERT but with no change in EC cell numbers (64). Previous studies of guinea-pig colitis and ileitis have found that there were decreased levels of SERT mRNA expression coupled with increased EC cell numbers (61, 65), mirroring the findings of the present study. Of note, one mechanism by which inflammation reduces SERT was shown by Foley *et al*. (66) who reported that the inflammatory mediators tumor necrosis factor alpha and interferon

gamma both decrease SERT mRNA and protein in the Caco2 human intestinal epithelial cell line. In another recent study, De La Serre *et al*. (57) confirmed that a high fat diet is associated with inflammation in the rat ileum. Inflammation, however, is not the only mechanism which may alter 5-HT availability in the intestine, as there may be direct effects of diet on EC cells. The EC cells respond directly to ingested nutrients by a variety of mechanisms, including taste transduction for compounds such as sugars, bitter tastants and mustards (reviewed in 6). Thus, EC cells themselves may have responded to the Western diet used in the present study, which may have influenced their behavior. Further work will have to done to disentangle the effects of inflammation and a Western diet on EC cell function and subsequent availability of 5-HT in the intestine.

Conclusions

The findings of this study demonstrate that 5-HT availability is increased in the ileum of rats fed a Western diet. Our electrochemical data show that both compression induced release and steady state levels of 5-HT are increased during obesity. We have demonstrated that the physiological uptake of 5-HT by SERT is reduced during obesity, as is SERT mRNA and protein. Further, the number of EC cells and mRNA for the 5-HT synthesis enzyme TPH-1 was increased during diet-induced obesity. Taken together, these data suggest that obesity has differential effects on components of the 5-HT system in the small intestine which may lead to altered motility or sensation. Implications of the present data for human dietary obesity and its treatment confer an urgency for further study.

Acknowledgements

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Figure Legends

Figure 1. Illustrations of how 5-HT release was measured. **A.** Side view of the rat ileum showing the structure of the villus and crypt, including enterochromaffin cells (EC cells). *1*, when the carbon fiber electrode was positioned >500 μm above the mucosa, no 5-HT was detected. *2*, when the electrode was lowered to touch the mucosa (dashed arrow) it had much better access to 5-HT released by EC cells. In addition, compression of the mucosal epithelium by the electrode stimulated the mechanosensitive release of 5-HT from EC cells (illustrated as a cloud with dots of 5-HT). This represents 'peak' release of 5-HT. Total 5-HT measured is a combination of 5-HT which is released apically (solid arrow) and 5-HT released basally which leaks into the luminal space (dotted arrow). *3*, Once released, 5-HT passively diffuses into the superfusing solution (straight arrows) and is actively removed via the actions of the serotonin reuptake transporter (SERT, curved arrows; EPI - epithelium). This represents the steady state (SS) levels of 5-HT at the mucosal surface. **B.** An individual trace showing 5-HT oxidation current due to the compression of the rat ileal mucosa with the carbon fiber electrode. The dotted lines indicate the levels at which measurements of the peak (compression-evoked) and steady state oxidation current were taken. The numbers (1, 2 and 3) correspond to the time of recordings illustrated in A.

Figure 2. Electrochemical measurements of 5-HT release in chow-fed and Western diet fed rats. **A.** Representative traces from chow-fed (CF) rat ileum (lower trace) and from Western diet (WD) fed rat ileum (upper trace). Each trace shows the peak and steady state (SS) 5-HT concentrations during mucosal compression (during the grey bar) with the carbon fiber electrode. Dotted lines show points at which peak and SS measurements were taken. **B.** Bar graphs showing the average peak and SS levels of 5-HT in CF and WD fed rat ileum. On average, rats fed a WD had a significantly increased peak and SS level of 5-HT compared to CF rats (asterisks, $P<0.05$; n=8 each).

Figure 3. Electrochemical measurements of SERT function in chow-fed and Western diet fed rats. A. Average data from chow-fed rats. Bar graphs show both average peak and SS levels of 5-HT before (open bars) and during fluoxetine (closed bars; 1 μM; n=7). Fluoxetine significantly increased both peak and SS levels in CF rats. **B.** Average data from Western diet fed (WD) rats. Bar graphs show both average peak and SS levels of 5-HT before (open bars) and during fluoxetine (closed bars; 1 μM; n=9). Fluoxetine did not have any significant effects on either peak or SS levels in WD rats.

Figure 4. The availability of 5-HT compared to blood glucose and weight. Scatter plots showing peak and steady state 5-HT levels from a combined group of both chow-fed and Western diet fed rats. **A.** Peak compression evoked 5-HT release was compared to blood glucose. An unconstrained linear regression found a very weak relationship. **B.** Steady state 5-HT levels were compared to blood glucose. Higher levels of 5-HT were positively correlated with a higher blood glucose measurement in CF and WD rats. **C.** As expected, there was a significant correlation between the peak compression evoked release of 5-HT and the steady state levels of 5-HT (* P=0.001). **D.** Blood glucose and final weight were also significantly correlated (* P=0.006).

Figure 5. SERT and TPH1 mRNA expression during diet-induced obesity. Scatter dot plots showing quantitative PCR data for SERT (right) and TPH1 (left) mRNA as compared to housekeeping genes GAPDH (A.), β-actin (B.) or the epithelial brush border protein 'villin' (C.). Individual points are data from mucosa-only samples from single animals, the bar indicates the mean data. *Left*. The expression of SERT mRNA was significantly reduced in WD rats only when compared with GAPDH (top panel; $P=0.037$). *Right*. TPH-1, the rate limiting enzyme in the 5-HT synthesis pathway, showed a significant increase in WD fed rats only when compared with villin as the housekeeping gene (bottom panel; $P=0.021$). CF - chow-fed rat, WD - Western diet fed rat.

Figure 6. Western blot for SERT protein in chow-fed and Western diet fed rats. A. Raw Western blots showing SERT and actin protein expression in ileum from chow-fed rats (CF; n=4) and Western diet fed rats (WD; n=4). Coomassie staining was used as a loading control. **B.** Histogram showing average densitometry data from the blots in A. There was a significant reduction in protein for SERT and actin in WD rats compared to age matched CF rats when compared to Coomassie staining.

Figure 7. Western high fat diet (WD) increases the number of 5-HT immunoreactive cells in rat ileum. Representative photomicrographs of 14 μm sections showing 5-HT immunoreactive cells from chow-fed (CF) rat ileum (A.) and WD rat ileum (B.). **Top** (A, B), low power view encompassing several villus-crypt units; scale bar is 100 μm. The dashed boxes indicate areas enlarged below**.** **Bottom** (A', B'), high power view showing individual EC cells; scale bar is 20 μm. Arrow heads show examples of 5-HT positive cells that were c-Kit negative indicating that they were EC cells (and not mast cells). Immunohistochemical analysis revealed a significant increase in the number of 5-HT immunoreactive EC cells in the WD rats compared to CF rats.

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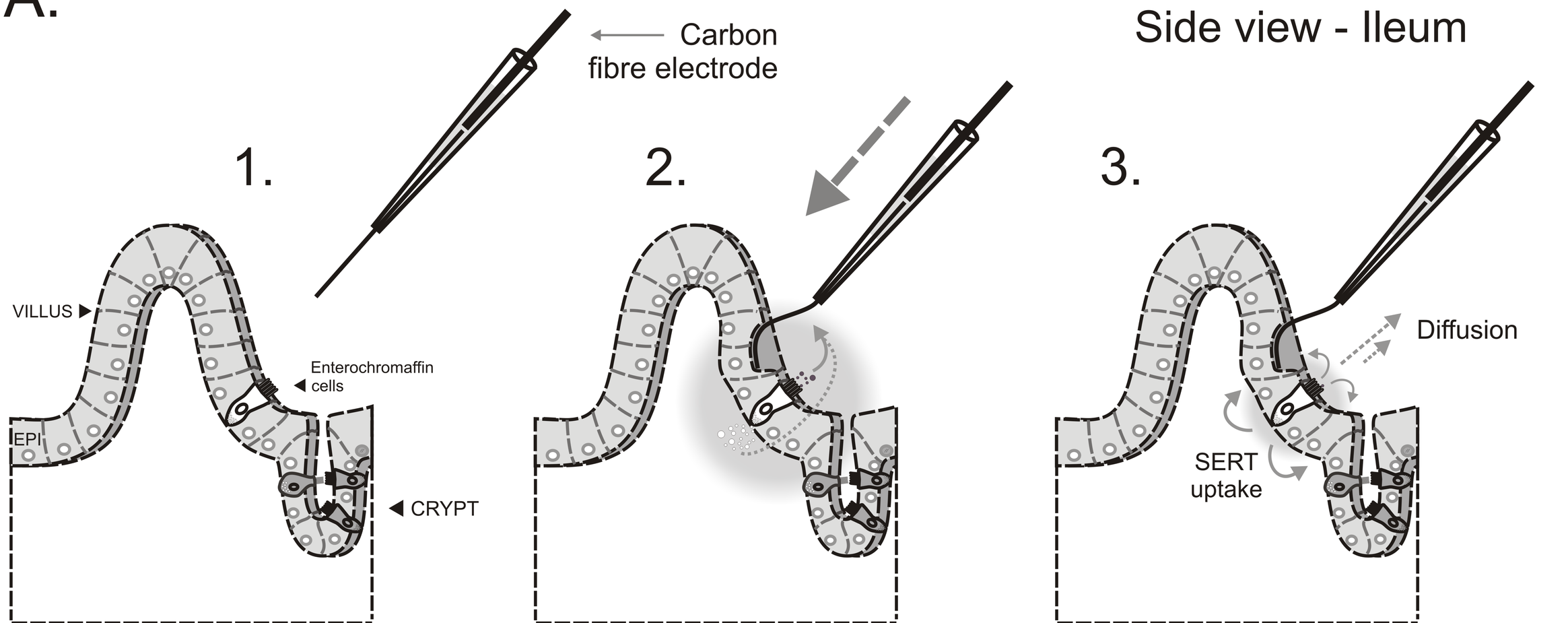

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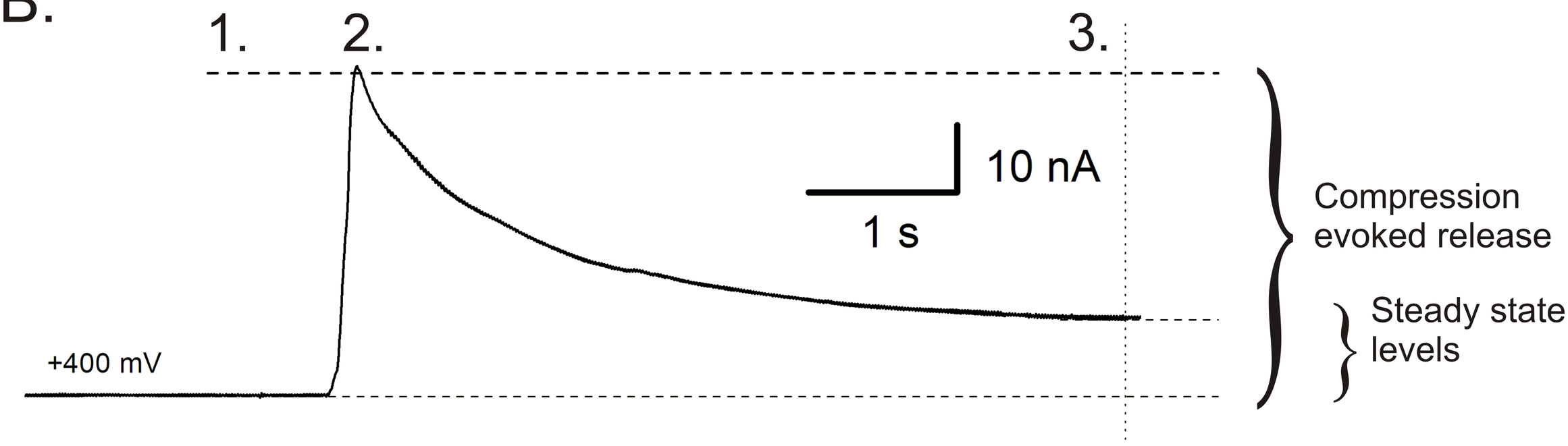

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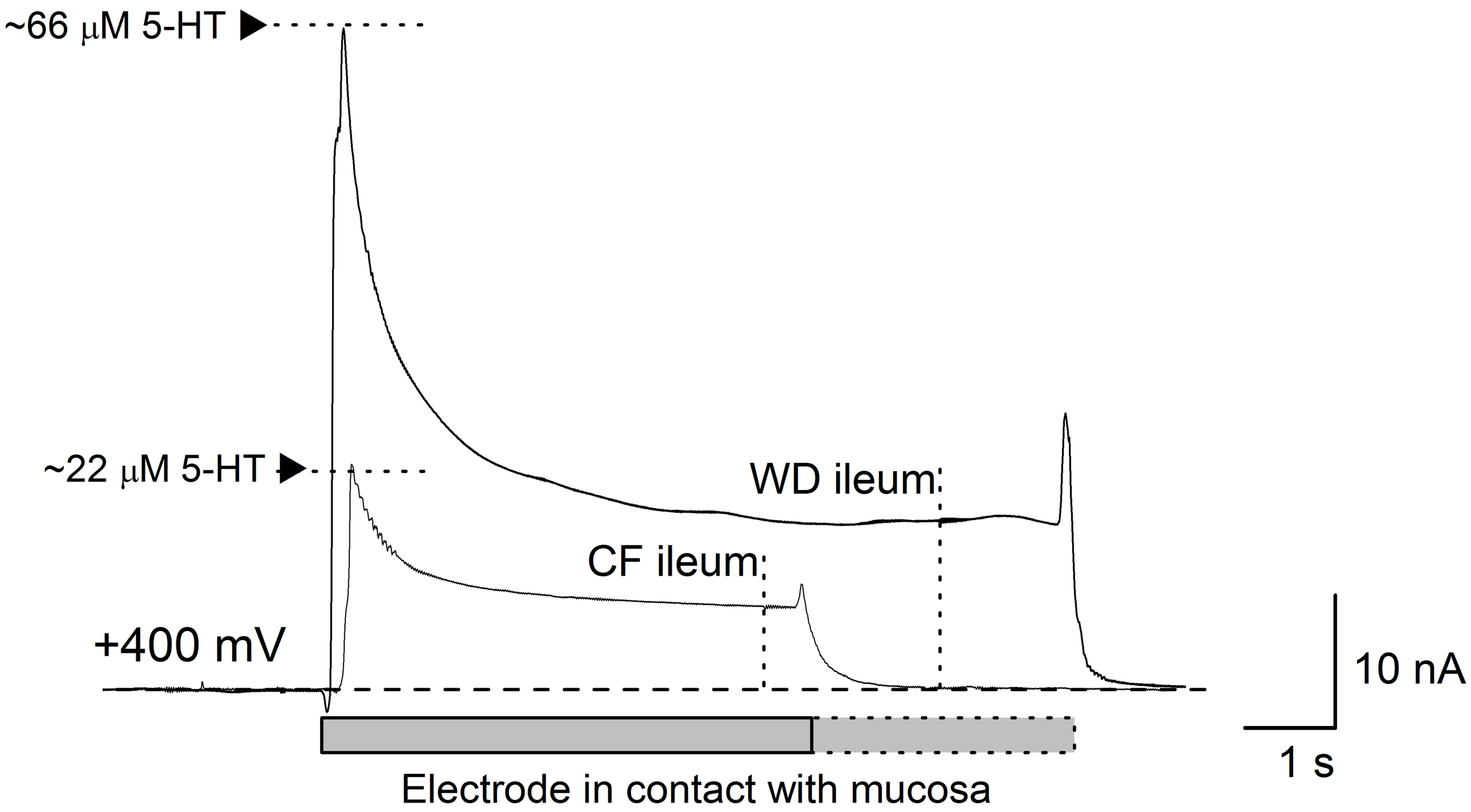
A. Compression-evoked 5-HT release



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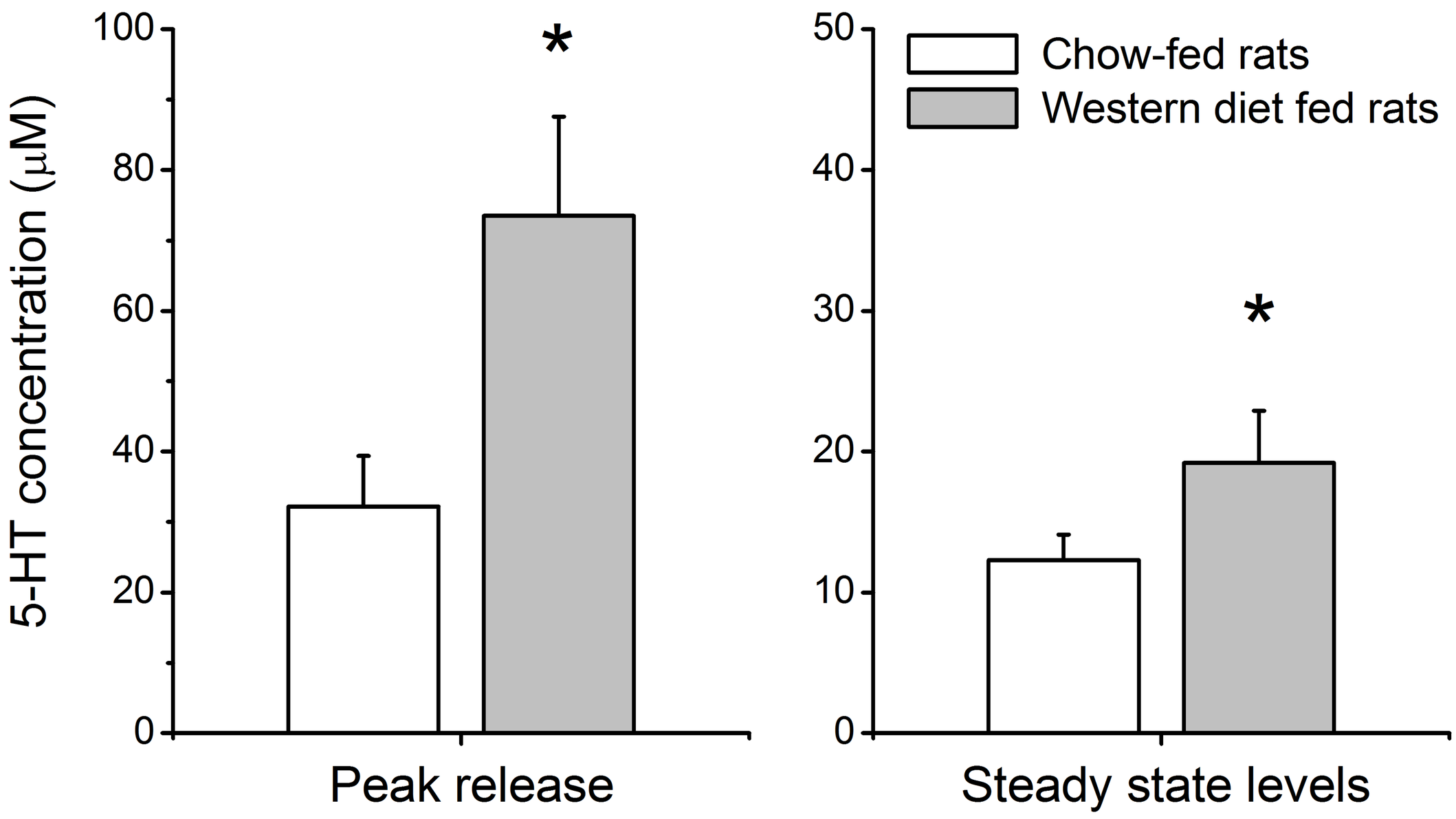

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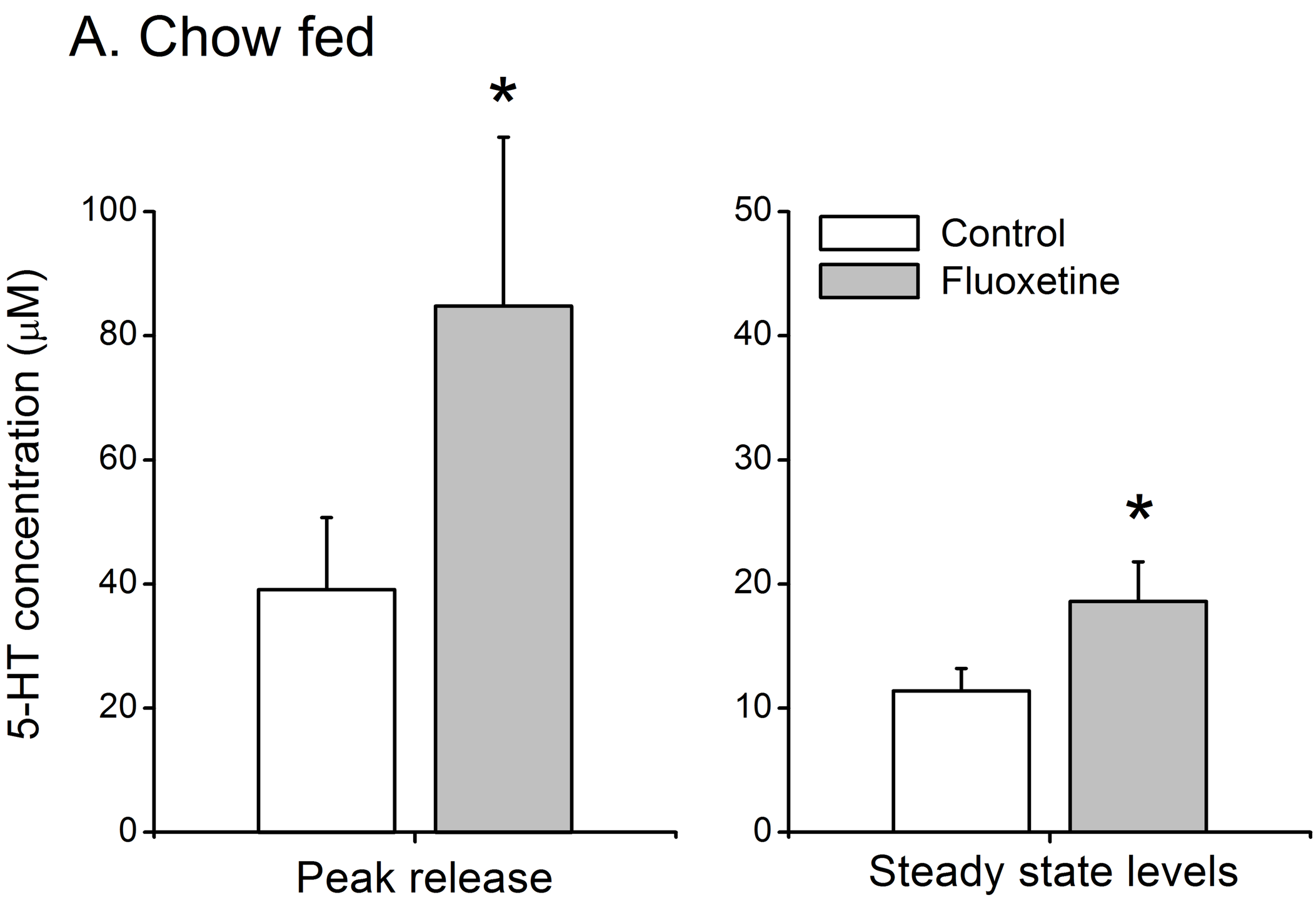
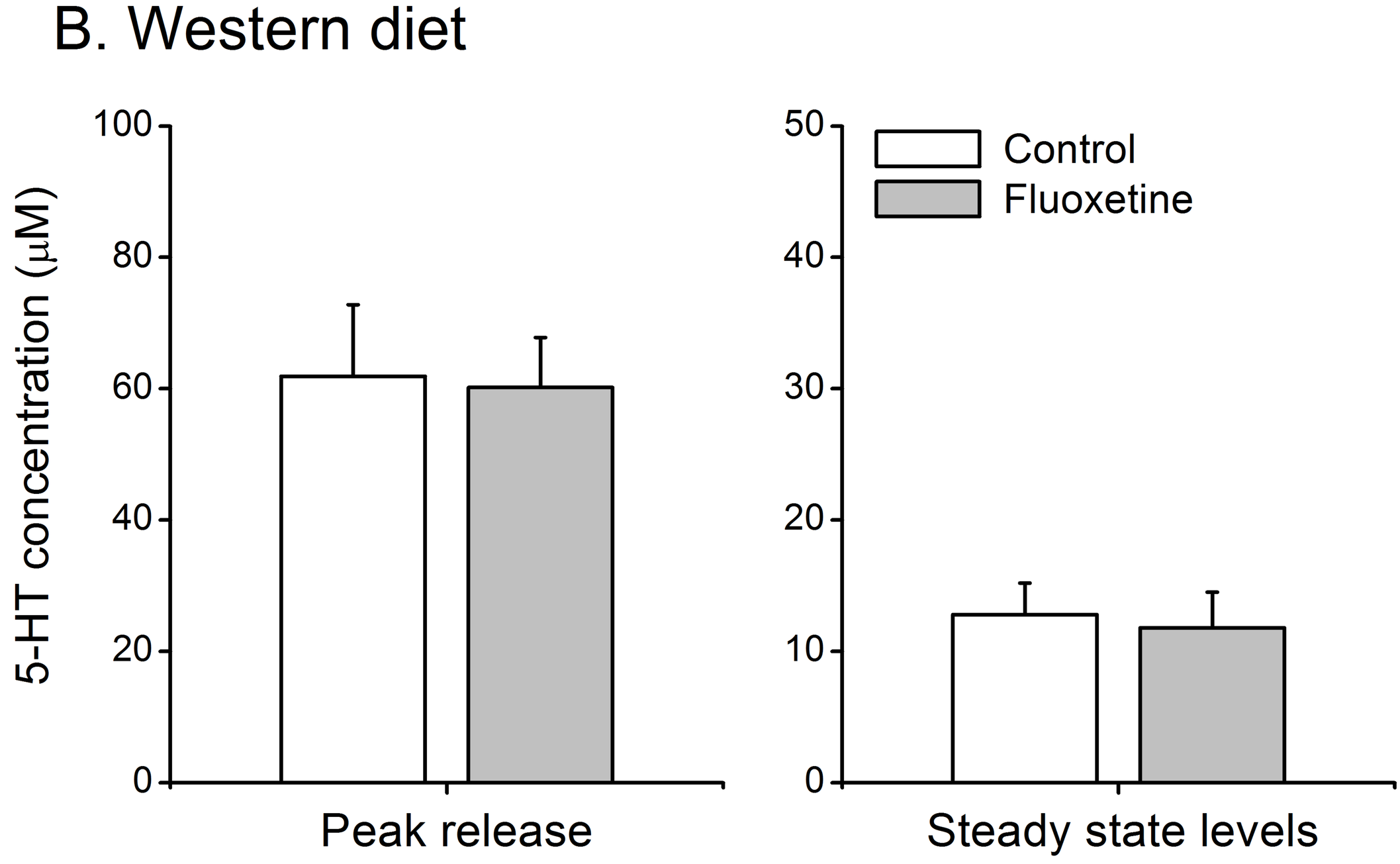
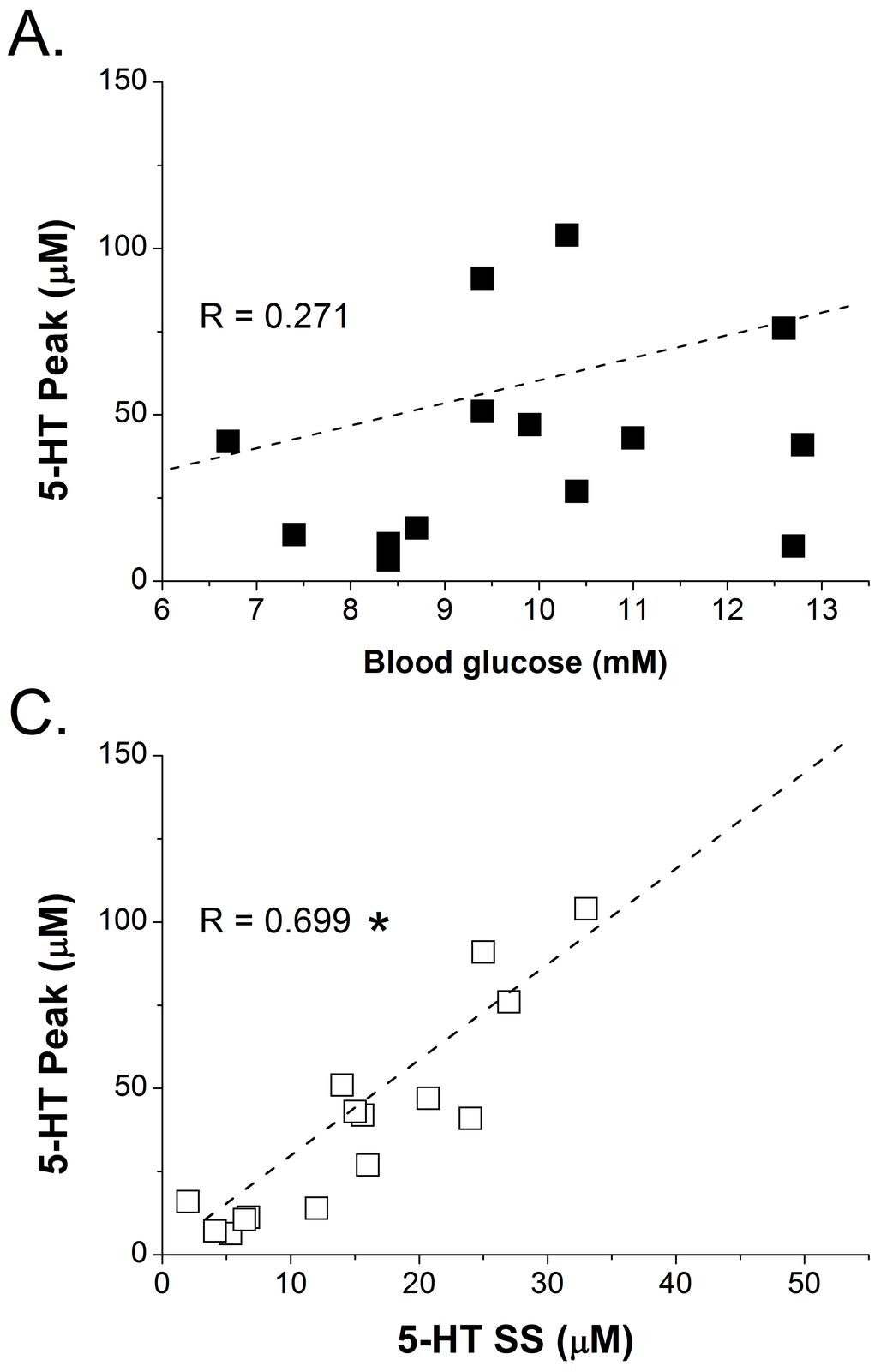
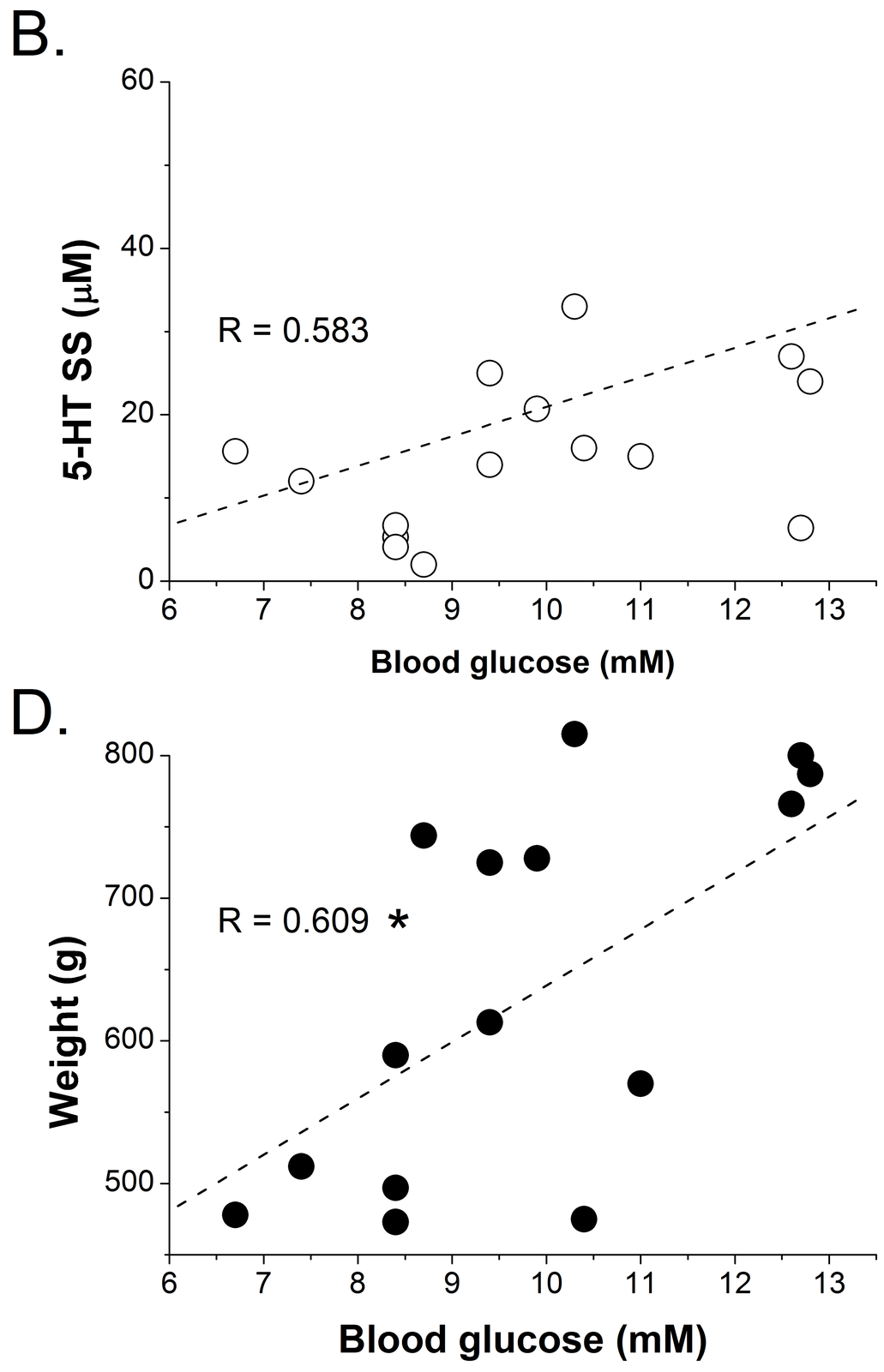




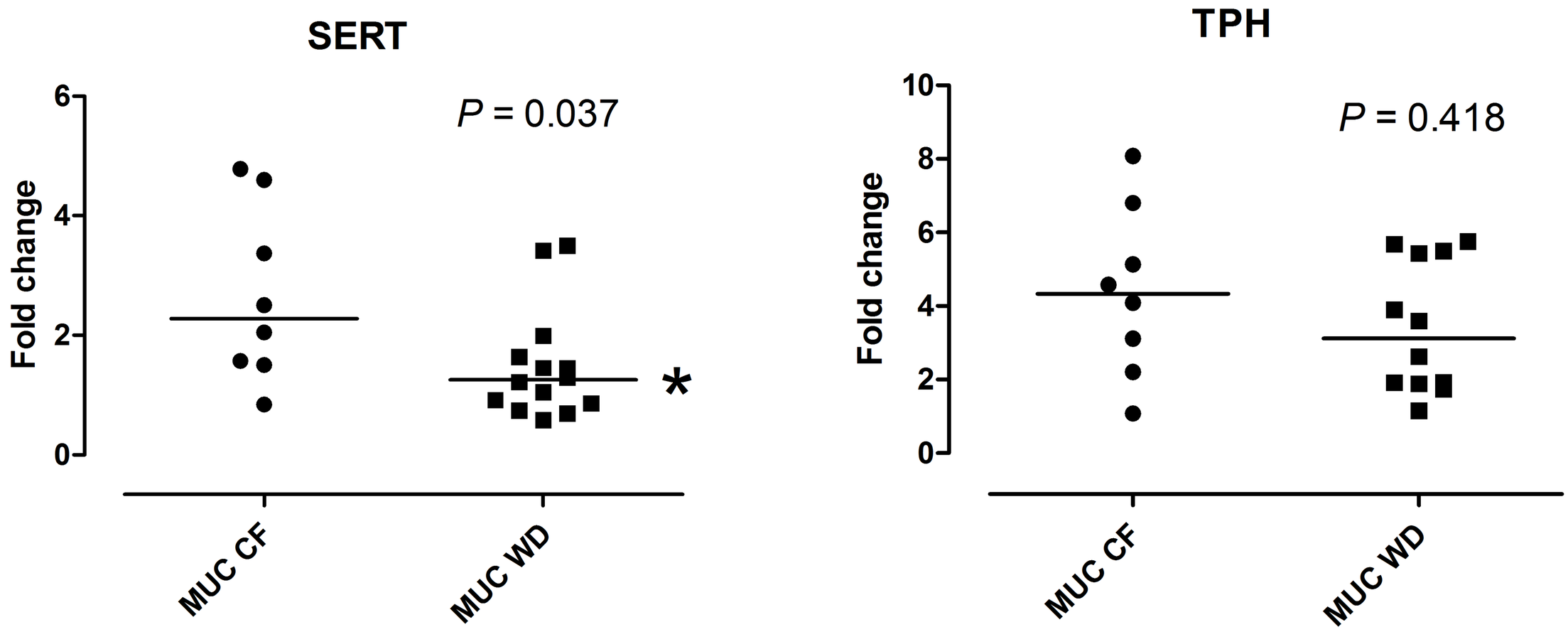
Bertrand, Figure 3



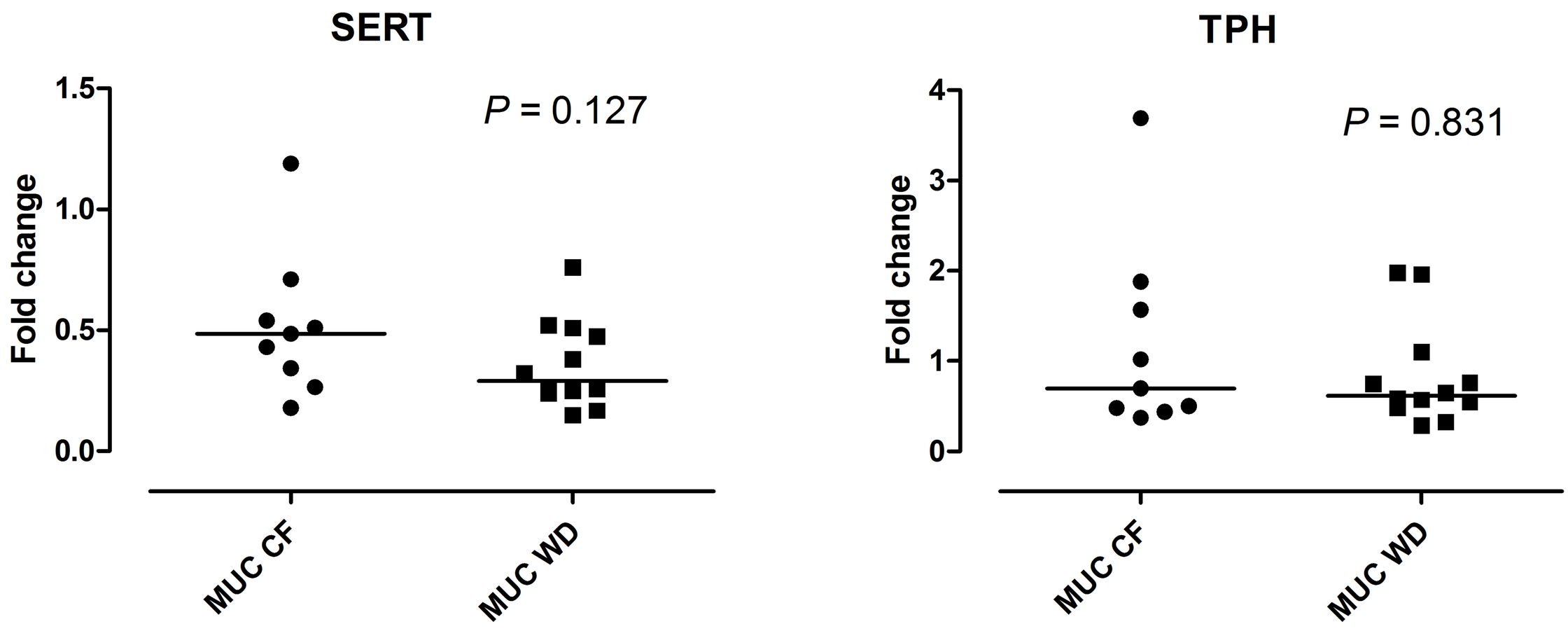


Bertrand, Figure 4

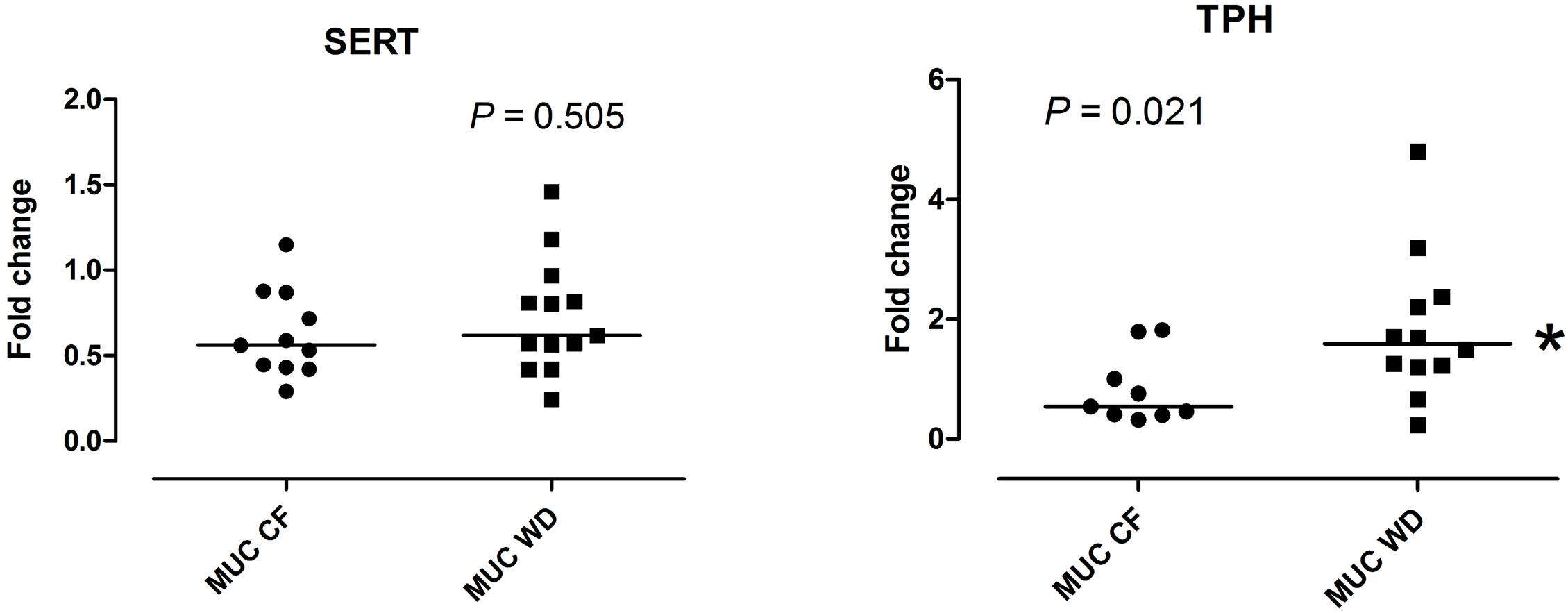
A. SERT and TPH versus GAPDH



B. SERT and TPH versus β-Actin

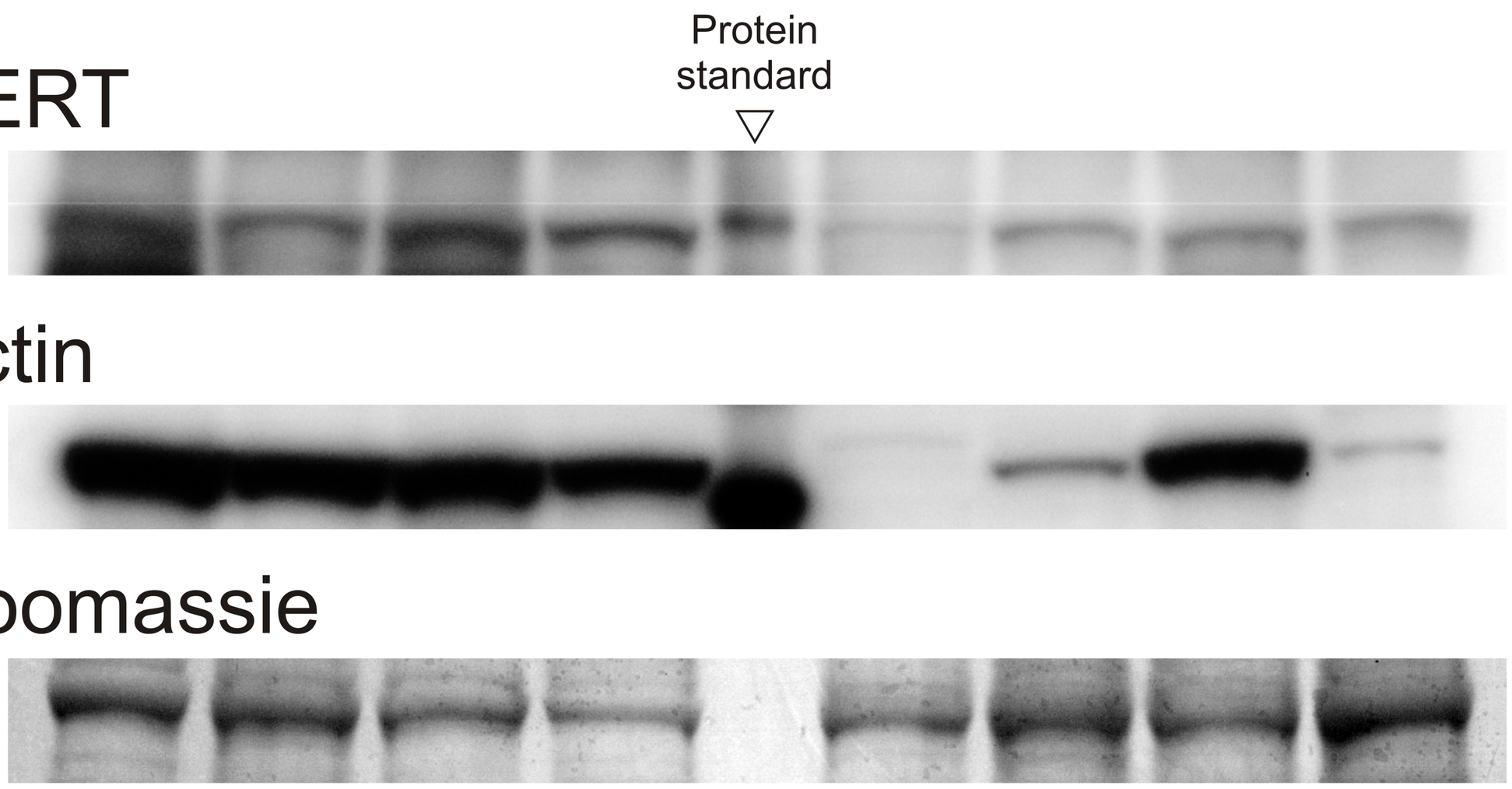


C. SERT and TPH versus villin

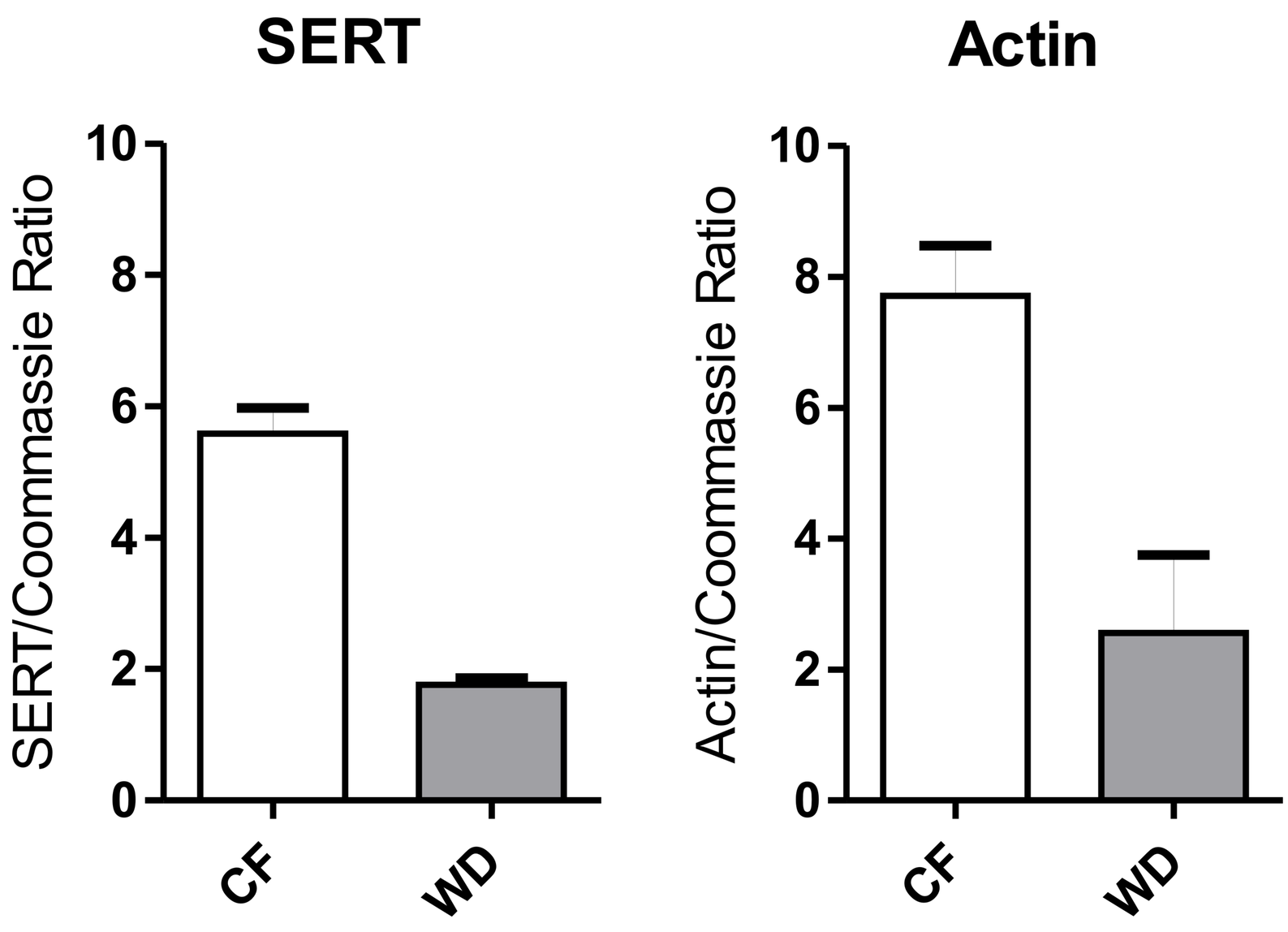


Bertrand, Figure 5

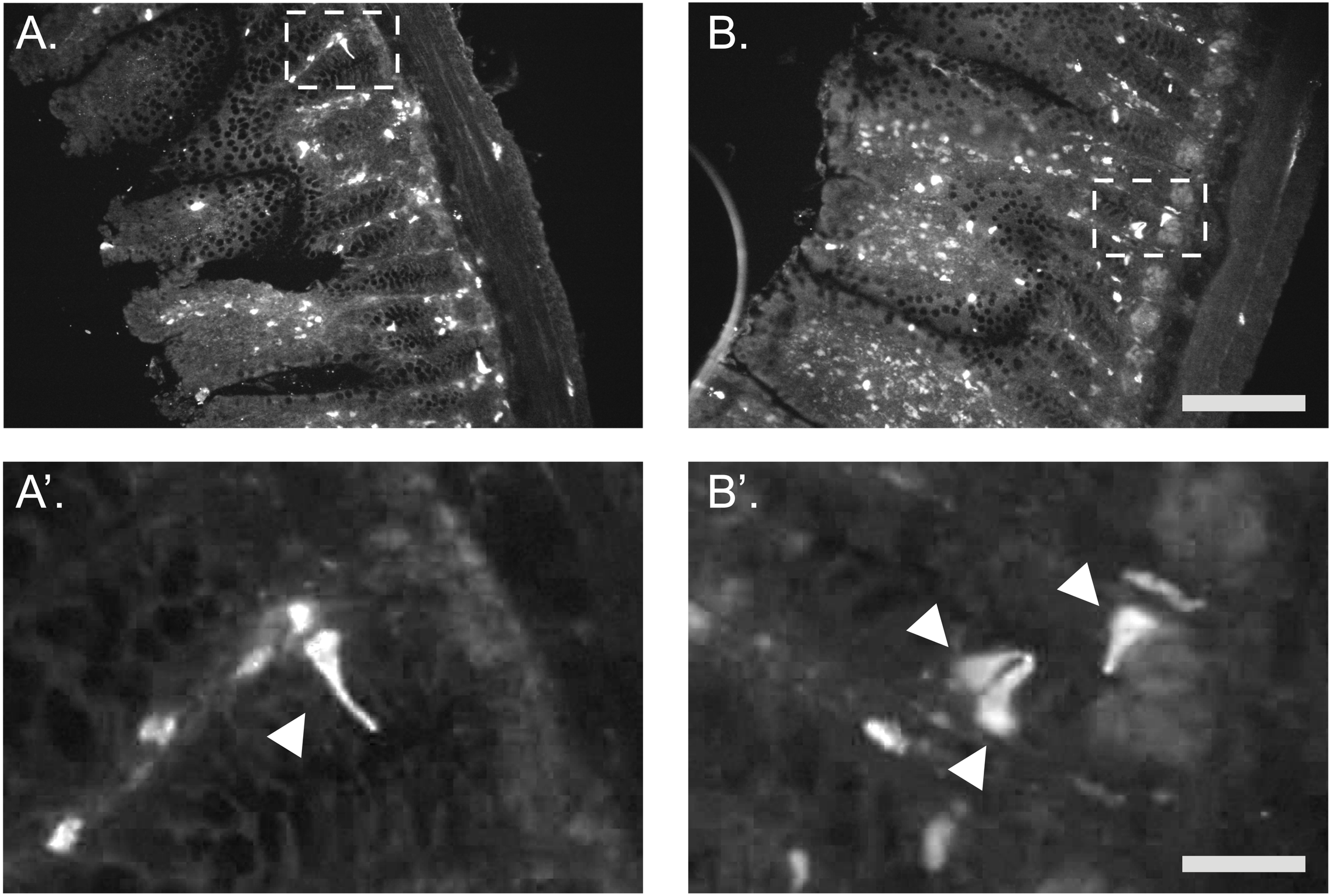
A. Western blot



B. Average data



Bertrand, Figure 6



Bertrand, Figure 7