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High-fat diet ingestion correlates with neuropathy in the duodenum myenteric plexus of obese mice with symptoms of type 2 diabetes

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Abstract

Obesity and type 2 diabetes are increasing in prevalence at an alarming rate in developed and developing nations, and over 50% of patients with prolonged stages of disease experience forms of autonomic neuropathy. These patients have symptoms indicating disrupted enteric nervous system function including gastric discomfort, gastroparesis and intestinal dysmotility. Previous assessments have examined enteric neuronal injury within either type 1 diabetic or transgenic type 2 diabetic contexts. This study aimed to assess damage to myenteric neurons within the duodenum of high fat diet ingesting mice experiencing symptoms of type 2 diabetes, as this disease context is most parallel to the human condition and disrupted duodenal motility underlies negative gastrointestinal symptoms. Mice fed a high fat diet developed symptoms of obesity and diabetes by 4 weeks. After 8 weeks, the total number of duodenal myenteric neurons and synaptophysin density index were reduced and transmission electron microscopy showed axonal swelling and loss of neurofilaments and microtubules, suggesting compromised neuronal health. High fat diet ingestion correlated with a loss of neurons expressing VIP and nNOS, but did not affect the expression of ChAT, substance P, calbindin and CGRP. These results correlate high fat diet ingestion, obesity and type 2 diabetes symptoms with a loss of duodenal neurons, biasing towards those with inhibitory nature. This pathology may underlie dysmotility and other negative GI symptoms experienced by human type 2 diabetic and obese patients.

Keywords

Myenteric plexus; nerve injury; type 2 diabetes; vasoactive intestinal peptide; nitric oxide synthase

Introduction

The autonomous enteric nervous system (ENS) runs the length of the gastrointestinal (GI) tract and consists of two main plexuses: the submucosal plexus and myenteric plexus. The ENS coordinates GI absorption, secretion and motility (Furness 2006). Neurons of the myenteric plexus regulate motility reflexes, using assemblies of intrinsic primary afferent neurons, descending and ascending interneurons, and excitatory and inhibitory motor neurons to the muscularis externa (Kunze and Furness 1999). Damage and plastic changes in myenteric neurons disrupts these neural circuits, causing symptoms of dysmotility that are

often experienced by patients with type 2 diabetes and obesity. GI neuronal cell injury in prediabetic and type 2 diabetic obese patients has not been fully characterized.

Increasing in prevalence at an alarming rate, type 2 diabetes (T2D) and obesity commonly develop in a mutual fashion, and are considered ‘twin epidemics’ (Smyth and Heron 2006). Frank T2D is marked by prolonged hyperglycemia and insulin resistance while pre-diabetes presents as elevated fasting glucose or impaired glucose tolerance (Schmidt et al. 2005). Both prediabetic and T2D patients experience forms of neuropathy (Dyck et al. 1993; Papanas and Ziegler 2012). This neuropathy is thought to be due to hyperglycemia or inflammation-induced oxidative stress, dyslipidemia, and growth factor deficiencies (Vincent et al. 2009; Yamagishi and Imaizumi 2005; Zochodne 2007). Nerve cells of the ENS are susceptible to the damage these factors cause, and this injury is believed to underlie symptoms of gastroparesis, dyspepsia, nausea and GI pain experienced by diabetics (Aring et al. 2005; Enck and Frierling 1997; Mearin et al. 1995). In support of this, reduced gastric emptying and GI transit times correlate with high BMI and poor regulation of blood glucose levels (Madsen 1992). Importantly, a change in GI nutrient flow of this nature is likely to exacerbate the existing dysfunction of whole body metabolism and glucose regulation in patients (Bytzer et al. 2001; Enck and Frierling 1997; Koch 1999).

To tie negative GI symptoms to an underlying pathology, previous studies have characterized changes to enteric neurons in several GI segments using type 1 and ob/ob or db/db transgenic T2D disease models (El-Salhy and Spangeus 1998; Furlan et al. 1999; 2002; He et al. 2001; Pereira et al. 2011; Spangeus and El-Salhy 2001; Surendan and Kondapaka 2005; Voukali et al. 2011). Importantly, the high-fat diet-ingesting mouse has been used in recent years as a model of western diet-induced prediabetes and T2D most parallel to the human condition (Surwit 1988; Winzell and Ahren 2004). The aim of the present study was to characterize change to neuron health and neuron phenotypes in the duodenum myenteric plexus of the high fat (HF) diet-induced diabetic mouse, as motility through the duodenum is important for proper gastric emptying, glucose and hormonal regulation (Chaikomen et al. 2007; Duchman et al. 1997; Heddle et al. 1988; Heddle et al. 1989; le Roux et al. 2011).

Enteric neuron phenotypes were assessed using immunohistochemistry and previously defined chemical codes (Furness et al. 1995; Lin et al. 2008; Phillips et al. 2004; Qu et al. 2008; Sang and Young 1998), while overall nerve health was assessed with scanning transmission electron microscopy (TEM) and the presence of synaptophysin. After acquisition of early-stage diabetic symptoms, HF diet mice had a reduction in the total number of myenteric neurons. Loss of neurons was biased towards nitric oxide synthase and vasoactive intestinal peptide containing cells. Synaptophysin density index was reduced and TEM analysis showed nerve injury, indicating that the overall health of remaining nerves became compromised. These results show that a HF diet causes neuropathy of enteric nerve tissue in the duodenum of obese and T2D mice, and may explain the GI symptoms experienced by obese and T2D patients.

Materials and Methods

Mice

7 week-old C57Bl/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME), kept on a 12:12 hour light cycle and housed in metal cages. Mice were acclimatized for 1 week and then fed either a standard chow (SC) diet composed of 6.2% fat (n = 6; Teklad Global 2018, Teklad Diets, Madison WI) or a HF diet containing 72% fat (n = 6, modified DIO 70% kcal fat diet with 2% additional corn oil, TestDiet, Richmond, IN) ad libitum for 8

weeks. Mice were exsanguinated under deep isoflurane anesthetic according to procedures approved by the University of Idaho Animal Care and Use Committee.

Obesity and T2D assessment

Mouse weights were recorded weekly throughout the study period. Prior to euthanasia, glucose intolerance was assessed by fasting glucose challenge. Mice were fasted for 6 hours during the onset of light cycle. Blood was drawn from a submandibular vein and blood glucose (BG) values were obtained using an Abbott AlphaTrak glucometer (Abbott Park, IL). An intra-peritoneal injection of glucose solution (1 g/kg^{-1}) was given and BG values were gathered both 30 and 60 minutes after. Homeostatic model (HOMA) values were generated to assess insulin resistance in test mice. On the day of euthanasia, mice were subjected to a 4 hour fast during the light cycle and blood was gathered from a submandibular vein. Serum was collected and stored at -80°C and whole blood was used to obtain BG values. Serum insulin values were obtained using a Millipore ELISA kit (Billerica, MA). HOMA was calculated as previously described (Matthews et al. 1985).

Duodenal tissue sampling

Duodenum segments extending from the distal end of the pyloric sphincter to the aboral end of the ligament of Treitz (at the duodenal-jejunal flexure) were immediately removed from euthanized animals. They were placed in prepared fixatives described below for TEM and cryostat sections, or in ice-chilled HEPES buffer (mmol L^{-1} : NaCl 134; KCl 6; CaCl_2 2; MgCl_2 1; HEPES 10; Glucose 10) for longitudinal muscle myenteric plexus (LMMP) whole-mount preparations. For each duodenum sample, the oral segment was used for TEM, a segment aboral to it was used for cryostat sections, and the distal segment was used for wholemount preparations. Unless otherwise specified, all reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

TEM tissue preparations

0.5 cm duodenal segments were cut along the mesenteric border, pinned flat and fixed in 2.5% glutaraldehyde with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 24 hours at 4°C . Tissues were washed three times in 0.1 M PBS, dehydrated in graded ethanol and stained with OsO_4 and uranyl acetate. After embedding in Durcupan Fluka resin and polymerization for 48 hours at 56°C , blocks were trimmed and cut into ultrathin 55 nm sections on an ultra-microtome. Sections were incubated in lead citrate after collection on Formvar-coated single-slot grids.

Cryostat Sections

1.0 cm of duodenum was placed in 4% paraformaldehyde with 0.2% picric acid and kept overnight at 4°C , to ensure adequate tissue penetration of fixative. Tissues were washed three separate times using 0.1 M sodium phosphate buffer (PBS; 0.1 M PO_4 , pH 7.4) before being placed in 30% w/v sucrose solution at 4°C on a rotator for 24 hours. Tissues were then placed in a 1:1 30% sucrose solution with O.C.T (Sakura FineTek, Torrance, CA) for 24 hours before being embedded in O.C.T medium and stored at -80°C . $10 \mu\text{m}$ transverse sections were cut using a microtome.

LMMP preparations

1.0 cm duodenal segments were cut along the mesenteric border, stretched mucosa side-up and pinned flat in sylgard (Dow Corning, Midland, MI) lined glass dishes. Tissues were fixed overnight, to ensure adequate penetration, in 4% paraformaldehyde with 0.2% picric acid at 4°C before being washed three times for 30 minutes each in 0.1 M PBS. Tissues

were dissected under a stereomicroscope using fine forceps to expose LMMP. Preparations were stored at 4 °C in 0.1 M PBS containing 0.01 M NaN₃.

Immunohistochemistry (IHC) staining of cryostat sections and LMMP preparations

Enteric neuron phenotypes can be determined based on cell morphology and projections to target tissues, electrophysiology, or the presence of specific proteins and transmitters (chemical code) (Sang and Young 1996). Chemical coding was utilized in the present study to determine changes within specific, neurochemically defined subpopulations (phenotypes) of duodenal neurons in test mice. Three classes of neurons in the duodenum LMMP were investigated using two primary antibodies (makers) for each, respectively: sensory (calbindin, calcitonin gene-related peptide [CGRP]), excitatory (substance P, choline acetyltransferase [ChAT]) and inhibitory (nitric oxide synthase [nNOS], vasoactive intestinal peptide [VIP])(Table 1). It should be noted that interneurons contain many chemical markers used herein, and were likely stained during IHC for each neural class listed in Table 1, and accounted for in analysis. Total neuron population analysis was completed with an antibody for HuC/HuD, apoptosis was studied using an antibody for active caspase 3 and neuronal function was assessed indirectly using an antibody for synaptophysin. HuC/HuD, calbindin, substance P, ChAT and nNOS were analyzed in LMMP preparations while synaptophysin, calbindin, CGRP, and VIP were analyzed in muscularis layers of cryostat sections. The rationale for specific preparation analysis can be found in Table 1. Manufacturer and dilution information for primary antibodies, secondary antibodies, and dyes can be found in Table 2.

All tissue washing and incubation steps were done at room temperature on a multi-purpose rotator. LMMP tissue preparations were washed three times for 20 minutes each in 0.1 M PBS containing 0.5% Triton X-100 (washing buffer) before being incubated for one hour in washing buffer containing 5% normal donkey serum (NDS; Jackson ImmunoResearch, West Grove, PA; used for active caspase 3, ChAT and HuC/HuD) or 5% normal goat serum (NGS; Jackson Immunoresearch, West Grove, PA; used for synaptophysin, calbindin, CGRP, substance P, nNOS and VIP). Primary antibodies were prepared in either NDS or NGS, respectively, and incubated for 18–24 hours. Tissues were rinsed three times in washing buffer, and incubated in secondary antibodies diluted in NGS or NDS for 3 hours. Tissues were washed and mounted on slides (HuC/D and ChAT) using VectaShield Mounting Medium (Vector Labs, Burlingame, CA) or incubated in dye diluted in NGS (see Table 2) for one hour before a final wash and mounting. Cryostat sections were stained using the same procedure, but washing steps were 5 minutes in duration.

To ensure nucleated cell localization, colocalization of DAPI was used for LMMP preparations. Primary and secondary antibody negative control staining was performed for all antibodies used.

Quantification of number of neurons and density indices in LMMP

Tissues were photographed using a Nikon Eclipse Fluorescent microscope with a 20x objective lens (Nikon, Melville, NY) and analyzed using Metamorph2 software (Molecular Devices, Sunnyvale, CA). At least 10 randomly selected ganglia were photographed per tissue, per marker, using either a TRITC or FITC filter. Ganglionic areas were measured and recorded by tracing around stained cell somas (in the case of HuC/HuD), or by tracing around stained cells and/or nerve strands (calbindin, ChAT, substance P, nNOS), delineating ganglionic boundaries as the area where a strand becomes smaller than the width of two stained neurons. Within these ganglia, neurons were counted (HuC/HuD, calbindin, nNOS), or the image was manually thresholded in a blinded fashion to allow software to detect only stained neurons and nerve fibers within the ganglia, and this area was gathered (ChAT,

substance P). This thresholding was used in lieu of counting in cases where accurate quantification of individual neurons was considered highly subjective (see Table 1). Cell density (Number of neurons / ganglionic area; packing density), or density index (stained area / ganglionic area) were computed for each LMMP marker.

To determine changes in ganglionic size of mice ingesting a SC or HF diet, large numbers of images (n = 50 per animal) were randomly taken of LMMP preparations labeled with a HuC/HuD pan-neuronal marker (Lin et al. 2008). Areas of ganglia within images were measured (>100 ganglia per mouse). Means ganglionic sizes were computed.

Images to demonstrate HuC/HuD, active caspase 3, calbindin, ChAT, substance P and nNOS staining in LMMP were acquired using an Olympus Fluoview confocal multiphoton microscope with a 60x oil objective and FluoView ASW acquisition software (Olympus, Center Valley, PA).

Quantitative analysis of nerve cells and nerve fibers in cryostat sections

Four full-thickness images per animal were taken using the 20x objective. A tracing tool was used to define the muscularis externa in order to estimate tissue area. Stained nerve cells and nerve fibers in the muscularis externa were then manually thresholded in a blinded fashion to allow software to estimate stained areas within muscularis externa. Density index calculations (stained area / area of muscularis externa) were generated.

Nerve health analysis with TEM

Ten TEM nerve images of interganglionic fiber strands (n = 3, each diet group) were analyzed in a blinded fashion. Normal plasma membrane, membranous organelles, neurofilaments, microtubules, and lack of axonal swelling were determined as a 'healthy' state. If a majority of nerves within an image looked healthy, a score of '1' was given. A score of '0.5' was given if only half of the nerves were healthy, and a '0' was given if most or all nerves were compromised. Mean nerve health scores were generated.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc, La Jolla, CA). To compare means of SC and HF mice, an unpaired Student's t-test was used. In cases of multiple comparisons, a repeated measures ANOVA with Bonferroni post-hoc test was utilized. Significance was determined as $P < 0.05$, and values within figures are expressed as mean $\pm$ standard error of the mean.

Results

Obesity and T2D symptoms in 72% high-fat diet fed mice

We evaluated disease progression in test mice as previous (Ding et al. 2010) to determine the proper early stage disease time-point at which to assess changes in neurons of the duodenum.

To monitor obesity by weight gain, SC and HF diet-fed mice were weighed weekly. At each weekly point, the amount of weight gained was computed (Fig. 1a), and weight gained was also assessed bi-weekly as a percentage of initial weight in an effort to account for effects of original mouse size (Fig. 1b). When means were compared by t-test the amount of weight gained by HF diet mice was higher compared to SC after 4 weeks (Fig. 1a; $P < 0.03$, n = 3). The proportion of initial weight gain [initial weight (g) / total weight (g)] was statistically different between SC and HF mice at 4 and 8 weeks (Fig. 1b; Fig. 4 weeks, $P < 0.0027$, n = 16; 8 wks, $P < 0.001$, n = 16), showing that the onset of obesity/overweight occurred in HF

diet mice after 4 weeks and was not affected by initial body weight. To assess insulin resistance in HF diet mice, HOMA values were computed biweekly and compared to those of SC using an unpaired t-test. HF diet mice become insulin resistant compared to SC after 4 and 8 weeks (Fig. 1c, Fig. 4 wks, $P < 0.03$, $n = 5$; 8 wks, $P < 0.03$, $n = 6-8$). Meanwhile, glucose intolerance was assessed by comparing area-under-curve (AUC) values of SC and HF diet mice, generated after performance of fasting glucose challenge. Through this assessment, HF diet mice are glucose intolerant after 4 and 8 weeks of diet ingestion compared to SC (Fig. 1d, 4wks, $P < 0.01$, $n = 4$, 8wks, $P < 0.01$, $n = 9-13$).

Using these results as a basis, 8 weeks of diet ingestion was chosen as an early stage disease time-point at which to assess neurons of the small intestine.

Ingestion of a HF diet reduced the total number of neurons and correlated with poor nerve health in mouse duodenum

72% HF diet ingestion correlated with a loss of myenteric neurons and nerve damage in mouse duodenum (Fig 2a–n). It is generally accepted that the size of enteric ganglia is not affected by stretch (Karaosmanoglu et al. 1996), however, size and shape of component cells can vary with contraction and distension (Gabella and Trigg 1984), and it is plausible that neuropathy affects ganglionic size. Mean ganglionic areas were no different between SC and HF diet mice after 8 weeks (Fig. 2k; $n = 150-185$ per group). Meanwhile, the number of neurons per ganglionic area was reduced in these mice compared to SC (Fig. 2l; $n = 5$, $P < 0.05$). An analysis of the density index of synaptophysin in the myenteric plexus showed a reduction in HF mice compared to SC, indicating impaired nerve function (Fig. 2m; $n = 4$, $P < 0.05$). Qualitative assessment of active caspase 3 in the myenteric plexus demonstrated a range in staining intensity of caspase-3 immunoreactive neurons, and an increase in the number of active caspase 3 positive neurons in the myenteric plexus of mice fed a HF diet compared to standard chow (Fig. 2c–e). Also, a blinded analysis of nerve health performed using TEM images revealed injuries in myenteric nerve fibers of HF diet mice. Of cellular components analyzed, these damages included axonal swelling and a loss of neurofilaments and microtubules, suggesting an overall disruption of cytoskeletal networks. Mean nerve health scores of HF diet mice were statistically lower than those of SC (Fig. 2n; $n = 3$; $P < 0.05$) indicating this injury. This shows that although the size of ganglia in mice fed a HF diet for 8 weeks did not change, the total number of neurons within ganglia was reduced, and the health of remaining neurons became compromised.

High fat diet ingestion correlates with a reduction in nNOS neurons and VIP density indices in the myenteric plexus of mouse duodenum

nNOS is necessary for production of nitric oxide, a main inhibitory neurotransmitter to the muscle (Qu et al. 2008; Shuttleworth et al. 1991) and VIP is also an inhibitory neurotransmitter (Furness et al. 1995; Llewellyn-smith et al. 1988). Presence of these molecules is a proxy for demonstrating an ability of the small intestine to relax, which may be affected during gastroparesis (Koch 1999). Localization of both of these proteins was used to assess the effect of HF diet on inhibitory neuron bodies and processes in duodenum (Fig. 3 a–d). The number of nNOS stained neurons per ganglionic area was reduced in HF diet mice at 8 weeks (Fig. 3e, $P < 0.05$, $n = 4$). Similarly, VIP density indices of duodenal muscularis were reduced compared to SC (Fig. 3f, $P < 0.05$, $n = 4$). Average tissue area of muscularis externa did not differ between SC and HF mice (data not shown).

Consuming a HF diet for 8 wks does not alter density indices of ChAT and substance P in the myenteric ganglia of mouse duodenum

Excitatory motor neurons have been defined as containing choline acetyltransferase, calretinin and tachykinins (Qu et al. 2008), and a vast majority of cholinergic neurons

colocalize with substance P (Sang and Young 1998). The present study used antibodies for both ChAT and the tachykinin substance P to assess changes in excitatory neuron phenotype in the myenteric ganglia of mouse duodenum during HF diet ingestion (Fig. 4a–d). After 8 weeks of HF diet ingestion and subsequent symptoms of early diabetes in mice, the density index (stained area / ganglionic area) of ChAT labeled neurons and nerves fibers in the myenteric ganglia was similar to SC control (Fig. 5e, $n = 4$). The same was seen for substance P, as density indices were not statistically different in HF diet compared to SC control mice (Fig. 5f, $n = 4$). Overall, consumption of a HF diet for 8 weeks did not affect the density indices of ChAT and substance P in mouse duodenum LMMP.

Consumption of a HF diet does not alter the number of calbindin-immunoreactive neurons in LMMP, or density indices of calbindin and CGRP in the muscularis of mouse duodenum

Sensory neurons can be defined by their morphology, but also by the presence of calbindin and CGRP (Furness 2006; Kunze and Furness 1999; Sang and Young 1996). We aimed to characterize the effect of a HF diet on intrinsic sensory neurons in the myenteric plexus by staining LMMP preparations with calbindin. Meanwhile, extrinsic and intrinsic sensory nerves were studied in full thickness sections using both CGRP and calbindin antibodies, respectively (Fig. 5a–f). The density indices of calbindin in muscularis of SC and HF diet mice did not differ significantly (Fig. 3g, $n = 4$). The number of calbindin-immunoreactive neurons per ganglionic area in LMMP was also no different between diet treatments (Fig. 3h, $n = 4$). Likewise, CGRP density indices in muscularis of HF mice were not significantly different from that of SC (Fig. 3i, $n = 4$). Conclusively, consumption of a HF diet for 8 weeks did not affect the expression of calbindin and CGRP sensory markers in mouse duodenum.

Discussion

The goal of this study was to perform an in-depth phenotypic assessment of myenteric neurons in the duodenum of adult mice (16 weeks of age) to determine the effects of HF dietary intervention. The main findings are that mice ingesting a 72% HF diet have diabetic symptoms at 4 weeks, evidenced by glucose intolerance and insulin resistance, which has been seen previously (Cani et al. 2007). Compared to lean control, mice fed a HF diet are also considered obese by 4 weeks. Given the quick onset of these symptoms, and likely presence of circulating components previously shown to damage peripheral nerves (Yamagishi and Imaizumi 2005; Zochodne 2007), 8 weeks of ingestion was determined as an early diabetic stage for which to assess injury to nerve cells in the duodenum myenteric plexus. The total number of myenteric neurons/ganglia and synaptophysin density index were reduced while active caspase 3 was qualitatively increased in HF mice, suggesting neuronal cell death and disrupted function. A phenotypic assessment showed that cell death is selective toward inhibitory motor neurons. This was accompanied by axonal swelling and a disrupted cytoskeletal network of microtubules and neurofilaments. Whether axonal injuries seen through TEM occur in a specific class of neurons remains to be determined.

A reduced number of total myenteric neurons in the small intestine, large intestine and stomach have been seen previously during type 1 diabetes (Du et al. 2009; Pereira et al. 2011; Zannoni et al. 1997), and HuC/HuD is currently regarded as a robust marker for quantification of total enteric neuron populations (Lin et al. 2008; Phillips et al. 2004). In contrast to our results though, a previous study of HuC/HuD in the small intestine of mice on a HF diet actually showed a correlation with a higher number of neurons (Baudry et al. 2012). However, this was in 5 week old mice, and dietary intervention was occurring during development. Given that ganglionic sizes and the thickness of muscularis did not differ in the present study of adult mice, we are confident in the observed reduction in packing density, likely due to neuron death. An observed decline in the number of nNOS neurons,

VIP density indices, and TEM ultra-structural nerve injury supports these HuC/HuD results. Importantly, nerve injuries of this nature have previously been seen through similar TEM analysis of the colon of streptozotocin-induced diabetic mice (Anitha et al. 2006) and the submucosal plexus of diabetic humans (Schmidt et al. 1984). The qualitative increase of active caspase 3 stained myenteric neurons observed in our HF diet mice suggests that the loss of neurons seen in this study is due to apoptotic cell death. Further, our results of a decline in synaptophysin expression indicates that this death is likely associated with impaired nerve cell function. Previously, reduced synaptophysin expression has been reported in the retina of diabetic rats (D'Cruz et al. 2012) while an increase has been observed in the bypassed duodenum and gastro-jejunal segments of C57Bl6/J mice with diet-induced diabetes (Woods et al. 2011), supporting a notion that bypass surgery may reverse the intestinal nerve injuries reported here during diabetes. Our observations warrant studies to determine whether the synaptophysin reduction, caspase 3 activation, and axonal swelling of HF diet mice occurs in a selected class of neurons, and whether or not an alteration in neurotransmission is a consequence.

The decline in the number of nNOS LMMP neurons and muscularis VIP density indices implies that resultant injury and loss of cells is occurring within a subset of inhibitory motor neurons. A reduction in the number of NO containing neurons, or in the expression of nNOS has been seen during type 1 diabetes (Anitha et al. 2006; Du et al., 2009; Furlan et al. 2002; He et al. 2001; Spangeus et al. 2000), and a loss of nNOS neurons has also been noted in ob/ob and db/db obese mice (Spangeus and El-Salhy 2001; Surendran and Kondapaka 2005). These results may underlie the development of diabetic gastropathy, as impairment of the nNOS protein or nNOS containing inhibitory motor neurons can result in enhanced intestinal peristalsis (Yoneda et al. 2001) and delayed gastric emptying (Huang et al. 1993). In support of our observations, a transgenic T2D mouse study showed the number of VIP containing neurons reduced in the duodenum of test animals (Spangeus and El-Salhy 2001). This is in light of previous type 1 diabetic studies showing conflicting results with up or down regulation of the peptide (El-Salhy and Spangeus 1998; He et al. 2001; Spangeus et al. 2000). Segmental variation in diabetes-induced changes of VIP, and implications in GI motility remain to be determined, however. It is important to note that the analysis of VIP in the present study may be taking into account projections of secretomotor or vasodilator neurons not originating in the LMMP, which could also be affected in this disease context (Ellis and Mawe 2003).

In the intestine of the mouse, ChAT and substance P are produced by excitatory motor neurons, as acetylcholine is the main excitatory neurotransmitter to the muscle and substance P is associated with stimulation of peristalsis (Donnerer et al. 1984; Qu et al. 2008). Injury to cholinergic (ChAT) and tachykininergic (substance P) neurons could contribute to the altered patterns of intestinal motility seen in obese and type 2 diabetics, but our results show these duodenal neurons to be unaffected in mice with a parallel disease. Previously, a study of ChAT in streptozotocin-induced diabetes showed reductions in proximal and distal areas of rat colon (Du et al. 2009), while an increase was seen in the ileum, antrum and duodenum of guinea pig (LePard 2005; Spangeus et al. 2000). Substance P has also shown variable results in diabetic animal models. In streptozotocin-induced rats, the number of duodenal substance P reactive nerve fibers increased after 4 weeks (Feher et al. 2006), while a study of longer duration (10 weeks) showed a reduction of substance P total content in stomach and small intestine (Ballman and Conlon 1985). Interestingly, a study looking at the small intestine and stomach during prolonged (12–18 month) streptozotocin-induced diabetes showed no change in substance P innervation between diabetic and control rats (Schmidt et al. 1988). Although tachykinins are shown to have excitatory nature (Qu et al. 2008), they are also shown to co-localize with markers of sensory neurons, including calbindin and CGRP (Chandrasekharan and Srinivasan 2007;

Furness et al. 1995; Gibbins et al. 1985; Sang and Young 1998), which may confound the results of all studies. Regardless, the ENS contents of ChAT and substance P during both type 1 and T2D seems to vary based on the type and duration of disease, segment of digestive tract studied, and methods of quantification used.

Knowing changes in sensory neurons within a diabetic context may be useful, as intrinsic primary afferent neurons responding to chemicals in the GI lumen or tension in the smooth muscle may be altered during symptoms of GI dysmotility (Furness 2006). The number of calbindin immunoreactive neurons and the muscularis density indices of both calbindin and CGRP were not affected during HF diet ingestion in our mice, suggesting that these duodenal intrinsic and extrinsic sensory nerve molecules are unaffected during early stages of disease. This is in contrast to findings in streptozotocin rat ileum, which showed a reduction in CGRP immunoreactivity and electrically stimulated release of the peptide (Belai et al. 1991; Shotton et al. 2004). In addition, our findings contrast an observation of reduced calbindin expression in hippocampus neurons of Zucker fatty rats (Nam et al. 2012), and up-regulated calbindin expression in retinal nerve cells during type 1 diabetes (Ng et al. 2004). Although calbindin has not been previously studied in the ENS of diabetic animals or humans directly, expression of the protein in cultured myenteric neurons was unaltered by chemicals that mimic a T2D environment (Voukali et al. 2011), supporting our results. Importantly, it remains possible that nerve injuries shown by synaptophysin reduction and TEM analysis occurred in sensory and excitatory motor nerve cells of our HF diet mice regardless of IHC results, as structural and functional changes may occur without an effect on expression of marker proteins.

The exact cause of neurodegeneration in the duodenum myenteric plexus of HF diet mice remains unknown, although microbial factors, hyperglycemia, dyslipidemia, oxidative stress from generation of advanced glycation end products (AGEs), and inflammatory molecules are likely causes (Anitha et al. 2006; Barbier de la Serre et al. 2010; Cani et al. 2007; Pereira et al. 2011; Vincent et al. 2009). It is also not clear why HF diet-induced cell death affects only a subset of nNOS and VIP expressing cells. Addressing the cellular mechanisms behind enteric nerve damage was not the goal of the present study, but it has been previously proposed that the presence of NO exacerbates oxidative stress caused by AGE formation, inducing neuronal apoptosis in inhibitory neuron populations (Cellek, et al. 2004). Importantly, oxidative stress of this nature has also been shown to cause GI motor dysfunction (Chandrasekharan et al. 2011).

Taken together, these results reveal the nature of HF diet-induced neuropathy in the small intestine myenteric plexus, which consists of a decline in nNOS neuronal populations and VIP density indices, with observed axonal swelling, damage to cytoskeletal structures and reductions in the presence of synaptophysin. The neuropathy shown here has broader implications for disrupted neuromuscular control in the duodenum, and a resulting alteration in gut-brain signaling crucial for glucose homeostasis, appetite regulation and proper gastric emptying (Aring et al. 2005; Enck and Frieling, 1997; Heddl et al. 1988; 1989). Our results expose a need for further investigation into the mechanisms behind diet-induced enteric neuropathy, and the functional changes that transcend them.

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Fig. 1. C57Bl/6 mice ingesting a 72% HF diet develop symptoms of obesity and T2D. After 4 weeks of ingestion, HF diet mice have greater total weight gain than those ingesting SC (a; $n = 3$, $*P < 0.05$, unpaired t-test). This weight gain was independent of mouse initial body weight, as proportion of initial body weight gain was still higher (b) at 4 ($n = 16$, $**P < 0.01$, unpaired t-test) and 8 ($n = 16$, $***P < 0.001$, unpaired t-test) weeks. HOMA values for insulin resistance were higher in mice on a HF diet (c) starting at 4 ($n = 5$, $**P < 0.01$, unpaired t-test) and continuing through 8 ($n = 6-8$, $**P < 0.01$, unpaired t-test) weeks. Meanwhile, a curve generated by plotted values of blood glucose before and after 1g/kg-1 IP glucose injection (d) showed higher area-under-curve (AUC) values in HF diet mice at 4 ($n = 4$, $*P < 0.05$, unpaired t-test) and 8 ($n = 9-13$, $*P < 0.05$, unpaired t-test) weeks, indicating glucose intolerance

Fig. 2.

Mice ingesting a 72% HF diet have a loss of myenteric neurons and nerve damage in the duodenum. Images of LMMP were taken of SC (a) and HF (b) preparations after staining with a HuC/HuD antibody to label all neurons and quantify the number of neurons per ganglionic area (neuronal packing density). Images of active caspase 3 stained LMMP suggest that compared to SC (c), more immunoreactive neurons are present in the myenteric ganglia of HF mice (d, e arrows), suggesting an increase in apoptotic signaling. Arrow heads show background staining in myenteric neurons. The normal range of caspase 3 immunoreactive neurons per ganglion was 0–1. However, ganglia containing more than one caspase 3 immunoreactive neurons were occasionally observed (d). A synaptophysin

antibody was used to stain full-thickness cryostat sections (f, g) and quantify densities of this vesicle protein in the myenteric plexus of both diet groups (arrows; myenteric presence of synaptophysin protein). TEM images of SC (h) and HF (i and insert, j) mice demonstrate an ultrastructural HF diet-induced nerve injury. This includes axonal swelling and loss of neurofilament proteins and microtubules (i and j; asterisks). Basal laminae showed no signs of thickening (j; arrowheads) and healthy neurites were seen (j; arrow). Scale bar for TEM is 2.5 μm in h–i, and 0.5 μm in j, respectively. Summary data (k–n) shows that the average ganglionic area was not affected by HF diet (k, $n = 4$), but the number of HuC/HuD labeled neurons per ganglionic area was reduced (l; $n = 4$, $*P < 0.05$, unpaired t-test) and synaptophysin density index was reduced as well (m; $n = 4$, $*P < 0.05$, unpaired t-test). Meanwhile, TEM average nerve health score in the muscularis of HF diet mice was significantly lower than that of SC ($n; n = 3$, $*P < 0.05$; unpaired t-test).

Fig. 3. Inhibitory motor neurons are negatively impacted after 8 weeks of HF diet ingestion. Duodenal LMMP preparations of SC and HF diet mice were stained with nNOS (a, b), and full thickness cryostat sections were stained with VIP (c, d). Scale bar for full thickness = $25\mu\text{m}$. Number of stained nNOS neurons per ganglionic area was reduced in HF diet mice (e; $n = 4$, $*P < 0.05$, unpaired t-test). Density index of VIP in HF diet mouse muscularis was also reduced compared to SC (f; $n = 4$, $*P < 0.05$, unpaired t-test)

Fig. 4. Excitatory motor neurons were not affected by HF diet ingestion. Duodenal LMMP preparations of SC and HF diet mice were stained with choline acetyltransferase (ChAT; a, b) or substance P (c, d) antibodies. Density indices (stained area / total ganglionic area) of ChAT were no different between SC and HF diet mice (e; $n = 4$). Substance P density indices were also no different between diet treatment groups (f; $n = 4$)

Fig. 5.

Sensory neurons were not affected by HF diet ingestion. A calbindin antibody was used to stain full thickness duodenum cryostat sections and LMMP preparations of SC (a, c) and HF diet mice (b, d). Full thickness duodenum cryostat preparations of SC and HF diet mice were also stained with calcitonin gene-related peptide (CGRP; e, f). Scale bar for full thickness = 25 μm . The density indices (stained area / total muscularis area) of calbindin in muscularis externa of SC and HF diet mice were not significantly different (g, $n = 4$). Likewise, the number of calbindin immunoreactive neurons per ganglionic area was also not significantly different between diet treatment groups (h, $n = 4$). Concurrently, CGRP density index in muscularis was not affected by HF diet (i, $n = 4$).

Table 1**Antibody Markers for Neuron Phenotypes Assessed by Immunohistochemistry**

Neuron class/phenotype	Primary antibody (marker) used	Tissue type(s) analyzed	Reasons for using WM or FT tissue preparations
All neurons	HuC/HuD	WM	Total number of myenteric neurons are best studied in WM
Inhibitory motor	Neuronal nitric oxide synthase (nNOS)	WM	High efficacy of staining in WM only
	Vasoactive intestinal peptide (VIP)	FT	High efficacy when staining FT & WM; However, quantification of neurons in WM would require colchicine for block of VIP axonal transport, complicating assessment of HF diet effects
Excitatory motor	Choline acetyltransferase (ChAT)	WM	High efficacy of staining in WM only
	Substance P (SP)	WM	Compared with FT, high efficacy of staining was observed in WM preparations
Sensory	Calbindin	WM, FT	High efficacy of staining in both FT and WM preparations; WM used for neuron quantification
	Calcitonin- gene-related peptide (CGRP)	FT	High efficacy when staining FT preparations
Neuronal synaptic vesicles	Synaptophysin	FT	Density indices in FT preparations is most thorough, as it provides quantification not only in ganglionic cells but nerve cell axon and dendrite projections
Neuronal cell death	Active Caspase 3	WT	High efficacy of staining in WM preparations, able to detect the number of myenteric neurons undergoing cell death in WM only

WM: longitudinal muscle myenteric plexus whole mount, FT: 10 µm full thickness cryostat sections.

Table 2**Antibody and Manufacturer Information**

Primary Antibody, Dilution	Secondary Antibody, Dilution	Dye, Dilution
All Neurons/Projections		
Ms monoclonal HuC/HuD, 1:200 <i>Molecular Probes, Eugene, OR A21271</i>	Dk anti Ms IgG Dylight 488, 1:300 <i>Jackson ImmunoResearch, West Grove, PA 715-485-150</i>	N/A
Rb polyclonal Synaptophysin, 1:100 <i>Aviva Systems Biology, San Diego, CA ARP45435_P050</i>	Biotinylated Gt anti Rb IgG, 1:300 <i>Vector Labs, Burlingame, CA BA1000</i>	Streptavidin Cy3 conjugate, 1:600 <i>Life Technologies, Grand Island, NY SA1010</i>
Rb polyclonal Caspase 3, 1:150 <i>Abcam, Cambridge, MA, USA ab2302</i>	Dk anti Ms IgG Dylight 488, 1:300 <i>Jackson ImmunoResearch, West Grove PA 715-485-150</i>	
Inhibitory Motor Neurons		
Rb polyclonal VIP, 1:1000 <i>Abcam, Cambridge, MA Ab43841</i>	Biotinylated Gt anti Rb IgG, 1:300 <i>Vector Labs, Burlingame, CA BA1000</i>	Streptavidin Cy3 conjugate, 1:600 <i>Life Technologies, Grand Island, NY SA1010</i>
Rb polyclonal nNos, 1:500 <i>Invitrogen, Camarillo, CA #61-7000</i>	Biotinylated Gt anti Rb IgG, 1:300 <i>Vector Labs, Burlingame, CA BA1000</i>	Streptavidin Cy3 conjugate, 1:600 <i>Life Technologies, Grand Island, NY SA1010</i>
Excitatory Motor Neurons		
Gt polyclonal ChAT, 1:200 <i>Millipore, Billerica, MA AB144P</i>	Dk anti Gt IgG Dylight 594, 1:300 <i>Jackson ImmunoResearch, West Grove, PA 705-515-003</i>	N/A
Rt monoclonal Substance P, 1:150 <i>Millipore, Billerica, MA MAB356</i>	Biotinylated Gt anti Rt, 1:300 <i>Invitrogen, Camarillo, CA #62-9540</i>	Streptavidin Cy3 conjugate, 1:600 <i>Life Technologies, Grand Island, NY SA1010</i>
Sensory Neurons		
Rb polyclonal Calbindin, 1:1250 <i>Santa Cruz Biotechnology, Santa Cruz, CA Sc28285</i>	Biotinylated Gt anti Rb IgG, 1:300 <i>Vector Labs, Burlingame, CA BA1000</i>	Streptavidin Alexa Fluor 488 conjugate, 1:600 <i>Jackson ImmunoResearch, West Grove, PA 016-540-084</i>
Rb polyclonal CGRP, 1:200 <i>Sigma Aldrich, St. Louis, MO #c8198</i>	Biotinylated Gt anti Rb IgG, 1:300 <i>Vector Labs, Burlingame, CA BA1000</i>	Streptavidin Cy3 conjugate, 1:600 <i>Life Technologies, Grand Island, NY SA1010</i>

Ms: Mouse, Dk: Donkey, Rb: Rabbit, Gt: Goat, Rt: Rat