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Diet-Induced Obesity Blunts Sensitivity of Intestinal Enteric Neurons: FIRST Evidence of Modulation of Activity of Enteric Neurons by Luminal Nutrients

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ABSTRACT

The enteric nervous system (ENS) has well-established roles in gut motility, epithelial secretion, and blood flow. However, its role in luminal nutrient sensing remains elusive. Given that the nerve endings of enteric neurons terminate at the basolateral surface of epithelial cells and do not contact the luminal milieu, the involvement of enteric neurons in luminal sensing is thought to be indirect and secondary to epithelial nutrient absorption. Our study demonstrates that intestinal enteric neurons are activated by dietary glucose and oleic acid, in the *absence* of mucosal enterocytes and enteroendocrine cells (EECs). Using primary enteric neuronal cultures generated from the intestinal submucosa, after exclusion of the mucosa, muscle layers, glial, and smooth muscle cells, we studied neuronal activation using intracellular Ca^{2+} transients as a surrogate. We show that diet-induced obesity (DIO) blunts the sensitivity of enteric neurons, as evidenced by lower (42%–52%), delayed (22–34 s), and sustained peak fluorescence (1.5–3.7-fold), and prolonged decay time (1158–1432 s). These findings significantly advance the field of enteric neuronal circuitry by revealing an unexplored, critical physiological function with potential therapeutic roles in the amelioration of obesity and associated comorbidities, including type 2 diabetes.

1 | Introduction

Recent evidence shows that enteric neurons express nutrient sensing transporters and receptors [1, 2], including the sodium glucose transporter (SGLT1) [3], monocarboxylate transporters (MCT2) [4], the short chain fatty acid receptor GPR41 [5], the dipeptide transporter Pept2 [6], and amino acid receptors activated by glutamate, glycine, or GABA [7, 8], suggesting plausible direct sensing machinery. We report for the first time that enteric neurons isolated from the submucosal plexuses (SPs) of proximal intestines of mice are activated by dietary glucose and oleic acid. Additionally, activation profiles of SP neurons of diet-induced obese (DIO) mice are distinct from those of their lean, healthy counterparts.

2 | Methods

2.1 | Mice

Six- to eight-week-old, male C57BL/6J mice (JAX #000664) were fed high-fat diet (45% calories, Research Diets Inc) or a 10% low-fat control diet (10% calories, Research Diets Inc) for 12 weeks.

2.2 | Microdissection of Intestinal Layers

The proximal small intestines (~16-cm distal to the pyloric sphincter) were flushed clean with ice-cold PBS, and the longitudinal muscle myenteric plexus (LMMP) was gently peeled off, following which the submucosal plexus (SP) was microdissected. Care was taken to minimize shredding of the fragile muscle layers.

2.3 | Primary Neuronal Cultures

SPs were digested (30 min, 37°C) to separate the neurons from the surrounding smooth muscle cells using liberase (3.75 mg/mL) and DNase (0.10 μg/mL). Digestion was stopped by adding cold Neurobasal A media and centrifuged (350*g*, 8 min, 4°C). Pellets were resuspended in complete neuronal growth media (Neurobasal A containing 10 ng/mL human recombinant glial cell line-derived neurotropic factor, 1% fetal bovine serum, B27 supplement, 1 mM pyruvate, 2 mM glutamine), filtered (100 μm), and plated on glass bottom dishes coated with laminin (5 μg/mL, 100 μL/cm^2) and poly-L-lysine (0.01%, 150 μL/cm^2).

2.4 | Measurement of Intracellular Ca^{2+} Transients

Please refer to Supporting Information Methods.

2.5 | Live Cell Recording and Quantitation

Please refer to Supporting Information Methods.

2.6 | Statistical Analyses

Data were analyzed by linear mixed effects modeling with likelihood ratio tests using R (v4.1.1) running in R Studio. Please refer to the figure legends for specific tests used.

3 | Results

3.1 | Primary Cultures Are Enriched in Neurons

As expected, mice fed a high-fat diet had significantly higher total body weights than low-fat diet fed controls (Figure 1A). After 24 h in culture, enteric neurons attached and developed short processes that became longer and branched over the next 1–3 days (Figure S1A–C). No differences in morphology and growth dynamics were observed in lean and DIO-derived submucosal neurons. Neuronal surface areas (Figure S1D) and numbers (Figure S1E) were comparable in cultures from both groups. Neurons were found to robustly express pan-neuronal marker Pgp9.5 and β-III tubulin that specifically marks ENS neurons (Figure 1B), thereby confirming neuronal phenotype. Cultures showed minimal glial cells, as determined by the absence of glial markers, glial fibrillary acidic protein (GFAP), and Sox 10 (Figure 1B), and were negative for α-smooth muscle actin (Figure S1F), demonstrating minimal smooth muscle contamination. About 76%–79% and 22%–23% of neurons in lean and DIO-derived cultures expressed sensory markers, calretinin and calcitonin gene-related peptide (CGRP), respectively (Figure 1C). About 40%–42% co-expressed calretinin and CGRP, and 9.5%–12.6% were negative for both (Figure 1D). About 50%, 30%–33%, and 10%–11% of lean and DIO-derived neurons expressed vasoactive intestinal peptide (VIP), choline acetyltransferase (ChAT), and neuronal nitric oxide synthase, respectively (Figure 1C,E), known to be expressed by secretomotor and interneurons. Additionally, about 22%–23% and 16%–19% of neurons in lean and DIO-derived cultures expressed sensory markers, Neuromedin U (Nmu) and Somatostatin (Sst, Figure 1F). Thus, high-fat feeding did not alter the neurochemical coding of neurons in culture.

Neuronal viability was similar in cultures from both groups of mice. Based on data from the MTT assay and the neuronal viability assay, the viability of cultures harvested from lean and DIO mice was virtually indistinguishable (Figure S2A–F). Additionally, neurite outgrowth, a widely accepted index of neuronal viability, was comparable in both groups (Figure S2E,G). Furthermore, lean and DIO-derived neurons in culture demonstrated equivalent maturity, as evidenced by equivalent surface areas and cell body densities (Figure S2H), total neurite lengths (Figure S2I), total number of neurites per neuron (Figure S2J), higher order branching of neurites (Figure S2K), and synaptophysin levels (Figure S2L).

3.2 | SP Enteric Neurons Are Activated in Response to Nutrients: DIO Blunts Sensitivity of SP Enteric Neurons

Stimulation of cultures (from lean mice) with 25 mM glucose or 100 μM oleic acid led to a robust increase in intracellular Ca^{2+} transients (Figure 2A,B, respectively). A ~5-fold increase in peak fluorescence was observed within 100 s, which decayed within 10 min (Figure 2A,B). However, in DIO-derived cultures, peak fluorescence was reduced by 42%–52% (Figure 2C) and persisted longer (Figure 2C). A significant delay in peak fluorescence was also observed upon stimulation with glucose (33.7 s) and oleic acid (22.2 s) (Figure 2C). Additionally, decay time (T_{50}) was prolonged by 1432 s and 1158 s with glucose and oleic acid stimulation, respectively (Figure 2C). Taken together, diet-induced obesity blunts the sensitivity of enteric neurons in the submucosal layer, as evidenced by lower peak fluorescence, longer peak duration, delay to peak, and prolonged decay time.

3.3 | DIO Shifts Glucose and Oleic Acid Dose–Response Curves

As shown in Figure 2D, compared to isotonic and iso-osmotic controls, 25 mM glucose stimulation led to a robust increase in fluorescence in lean-derived cultures. However, no response was observed in DIO-derived cultures at that concentration; a minimum of 50 mM glucose was needed to detect a response (Figure 2D). Notably, DIO-derived cultures showed no significant difference in fluorescence when stimulated with the positive control, ATP, a purinergic receptor agonist, implying that high-fat feeding does not impact functionality per se. A similar

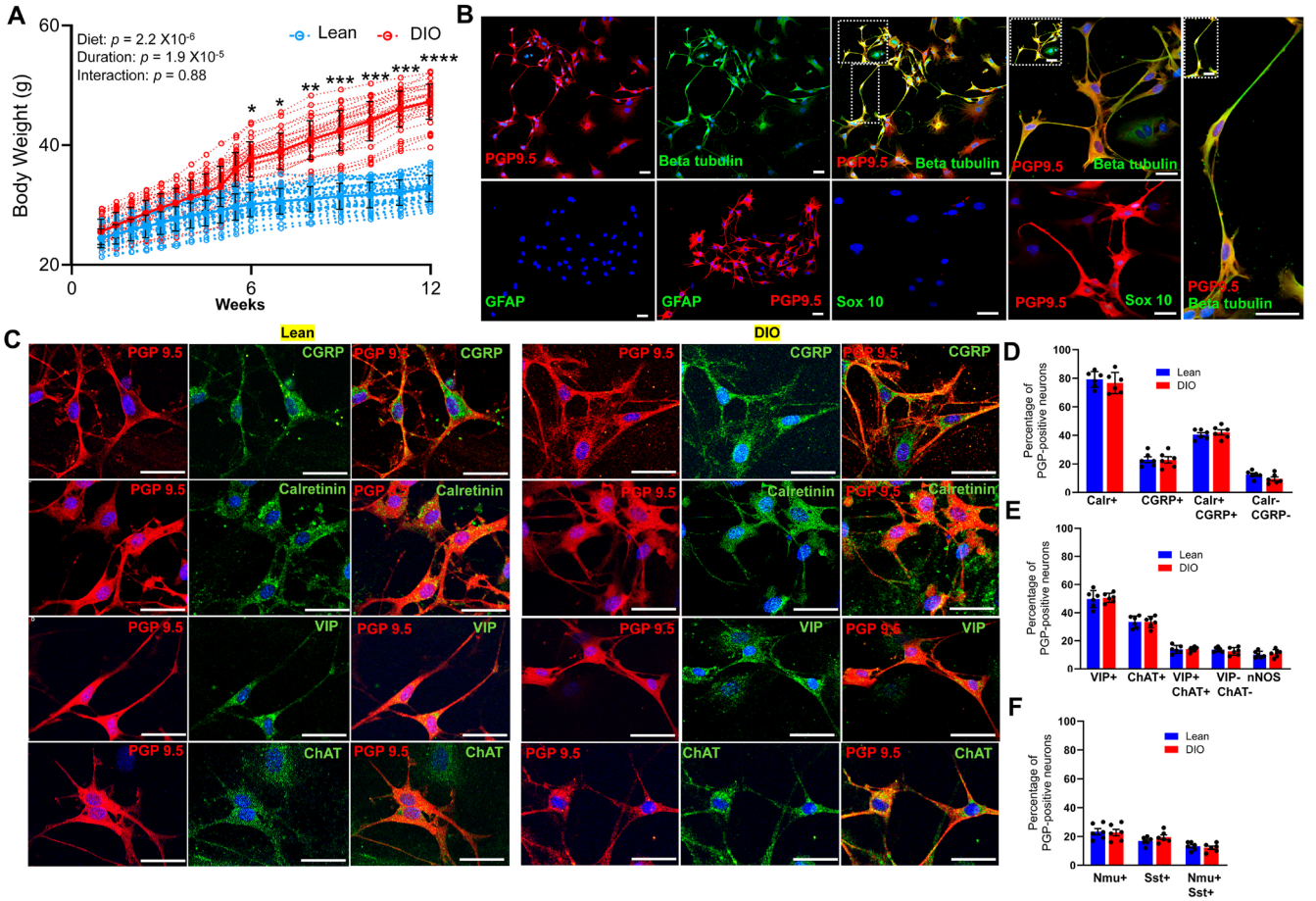

FIGURE 1 | Submucosal enteric neurons are activated by glucose and oleic acid. (A) Longitudinal effects of high-fat feeding for 12 weeks on total body weights as determined by linear mixed effects model with likelihood ratio tests. Data were compared by multiple Mann–Whitney test with Bonferroni correction (Lean: $n = 20$; DIO: $n = 22$, see figure panels for p values, asterisks denote significance between lean and DIO groups at a given time point). (B) Immunofluorescent (IF) staining of primary neuronal cultures generated from proximal intestinal submucosal plexuses (SP) and stained for pan neuronal markers, PGP9.5, βeta tubulin-III, and glial markers, Glial fibrillary acidic protein (GFAP) and Sox 10. (C) IF staining of three-day-old primary neuronal cultures generated from proximal intestinal submucosal plexuses (SP) from lean and DIO mice, stained for CGRP, Calretinin, VIP, and ChAT, Nmu, and Sst along with pan neuronal marker, PGP9.5. Quantitation of neuronal cultures showing proportion of PGP9.5-positive neurons expressing calretinin, CGRP (D), VIP, ChAT, nNOS (E), and Nmu and Sst (F). For quantitation in (D–F), data from 65 to 75 neuronal cell bodies across 5–7 fields of view, per replicate per mouse (n = triplicates from 6 mice per diet) are presented. Each data point represents the average proportion (relative to all PGP 9.5-positive neurons) of CGRP, Calretinin, ChAT, and VIP positive neurons in the proximal intestinal submucosal plexuses. Original Scale bars: (*B*) – 50 μm, insets in B-20 μm, (C)-10 μm.

pattern was observed with oleic acid stimulation (Figure 2E). The percentage of responders ($\Delta F/F_0 \geq 2$) also increased dose-dependently in cultures from lean mice up to 50 mM, after which no further increase was observed (Figure 2F). Significantly fewer responders were observed when cultures from DIO mice were stimulated with 10–50 mM glucose (Figure 2F). A similar pattern was observed when cultures were stimulated with oleic acid (Figure 2F).

4 | Discussion

Collectively, our study demonstrates that submucosal enteric neurons in mice are activated in response to luminal nutrients, glucose, and oleic acid. This finding significantly advances the ENS field by providing evidence that enteric neurons can directly sense luminal nutrients, independent of upstream signaling mediators and/or non-neuronal cells. Additionally, the observed loss of sensitivity with DIO offers insights into the reported dysregulated nutrient sensing associated with chronic nutrient excess and the body's maladaptive response to nutrient excess as observed during obesity.

Neuronal characterization reveals close similarities between neurochemical signatures on primary cultures and neurons in vivo. Submucosal neurons in the small and large intestines of guinea pigs and mice [9–13] have been reported to be ChAT+ and VIP+, and nNOS+ in the duodenums [14] of guinea pigs [12] and mice [11]. This pattern was found to be mimicked by neurons in culture. Calretinin is expressed by about 90% of submucosal neurons in the adult mouse duodenum [14], also mimicked by cultures in our study. Similarly, CGRP, the next abundant sensory subtype in culture, mimicked the distribution in the adult mouse and guinea pig intestines [10–12, 14]. The retention

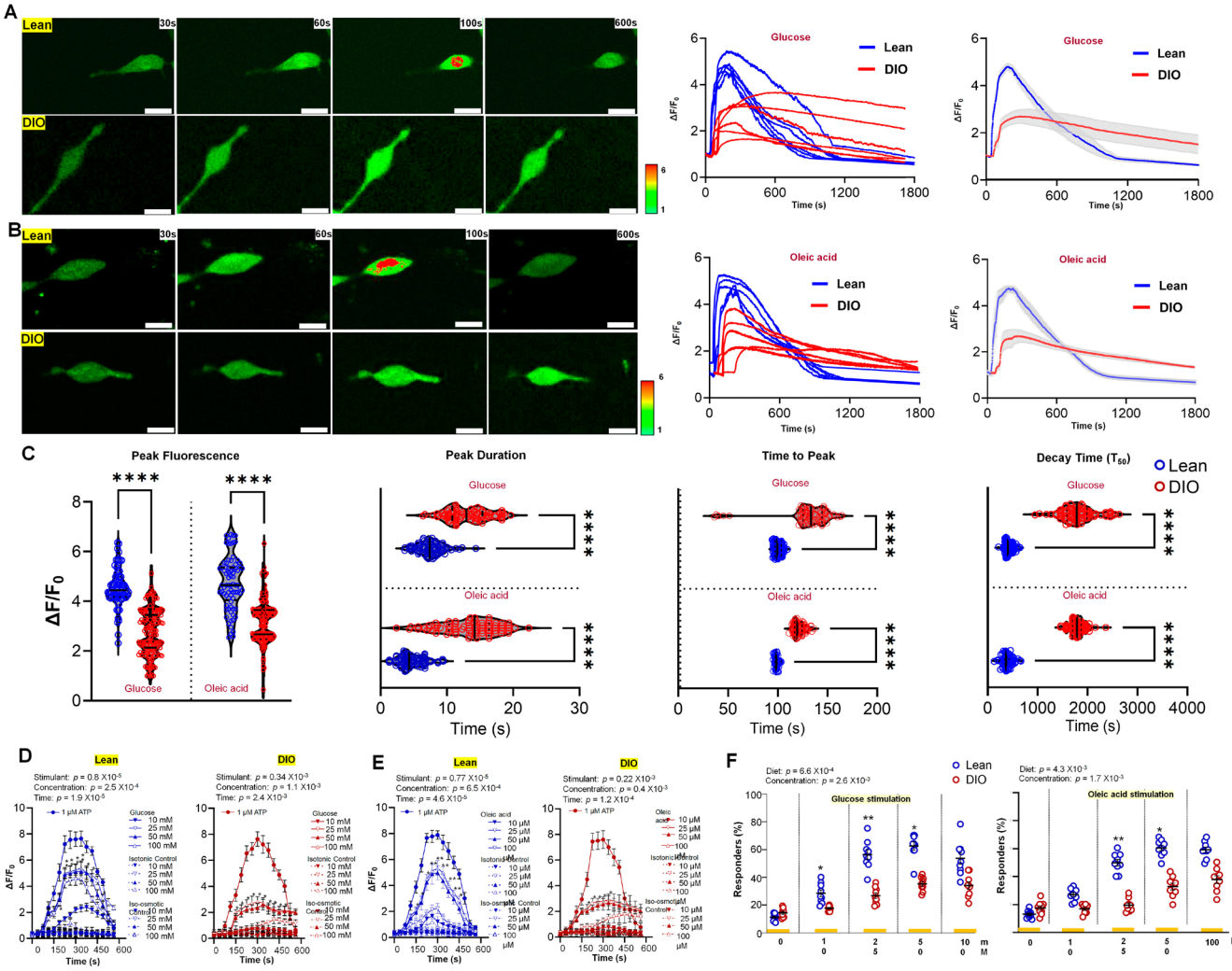

FIGURE 2 | DIO blunts sensitivity of SP enteric neurons to ex vivo stimulation with glucose and oleic acid. (A, B) Snapshots (*left panel*) and quantitation of intracellular Ca^{2+} transients (*right panel*) in primary neurons from SPs of lean and DIO mice stimulated with glucose (A) or oleic acid (B), recorded using time-lapse fluorescence microscopy. Graphs show individual and integrated fluorescence tracings of 75–80 regions of interest (ROIs) per mouse, normalized to baseline after subtraction of background fluorescence ($\Delta F/F_0$, $n = 5$–7 mice/diet/stimulant). (C) Peak fluorescence, peak duration, time to peak, and decay time (T_{50}) of SP neurons stimulated with glucose and oleic acid, calculated using pClamp 11 software ($n = 75$–80 ROIs from 5 to 7 mice/diet/stimulation, asterisks denote significance between lean and DIO groups for each stimulant). Data in (C) were analyzed using two-way ANOVA and post hoc Scheirer–Ray–Hare test with continuity correction. (D, E) Dose-dependent response to stimulation of primary neuronal cultures from SPs of lean and DIO mice with 10–100 mM glucose (D), 10–100 μM oleic acid (E) and 1 μM ATP, measured by intracellular Ca^{2+} transients recorded using time-lapse fluorescence microscopy. Fluorescence intensities of ROIs were normalized to baseline after subtraction of background fluorescence ($\Delta F/F_0$). Data in (D, E) were analyzed using repeated two-way ANOVA and post hoc Bonferroni test ($n = 5$–8 ROIs/stimulant concentration/mouse, 6–8 mice/diet). (F) The percentage of primary neuronal culture responders in response to 10, 25, 50, and 100 mM glucose and 10, 25, 50, and 100 μM oleic acid stimulation. $\Delta F/F_0 \geq 2$ was used to detect responders. Data in (F) were analyzed by multiple Mann–Whitney tests with Bonferroni correction ($n = 7$–10 ROIs/stimulant concentration/mouse, 8–9 mice/diet, asterisks denote significance between lean and DIO groups at a given concentration). All data are Mean ± SEM; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. Original magnification: Scale bars on panels A, B: 20 μm.

of neurochemical signatures ex vivo is promising and suggests that the primary culture model can be used to reliably investigate functional readouts. Our studies report for the first time that submucosal enteric neurons in the proximal small intestines of mice are activated by luminal nutrients. High-fat feeding blunts the sensitivity of these neurons, despite no changes in health, viability, and maturity.

One of the limitations of this study is the limited translatability of primary cultures. Future investigations directed toward activity measurements in vivo are warranted. Furthermore, although live calcium imaging is an established surrogate for electrophysiological measurements and allows simultaneous analyses of neuronal activity in multiple cells, consideration of differences in calcium handling mechanisms by the two distinct electrophysiological classes of neurons in the ENS, after-hyperpolarizing (AH)- and synaptic (S)-neurons is less robust. A handful of studies have shown that in the guinea pig, a calcium transient will accompany a single action potential in AH-neurons, but multiple action potentials are required to generate

a calcium transient in S-neurons. Hence, the conclusion that an increase in Ca^{2+}- driven fluorescence translates into generation of neuronal action potential may not be accurate for all neuronal populations across species. Activity measurements in targeted neuronal populations using chemogenetic manipulation and other streamlined approaches are warranted to decipher differences in electrophysiological profiles of distinct neuronal subpopulations.

Author Contributions

Sinju Sundaresan: conceptualization and experimental design, funding acquisition, project administration and supervision. **Ava Grandberry, Naomi Rajesh, Robert Murphy:** data collection. **Sinju Sundaresan, Ava Grandberry:** data analyses. **Sinju Sundaresan, Ava Grandberry:** original draft and editing. All authors have read the manuscript and approved the final version.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available in the Materials and Methods, Results, and/or Supporting Information of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.