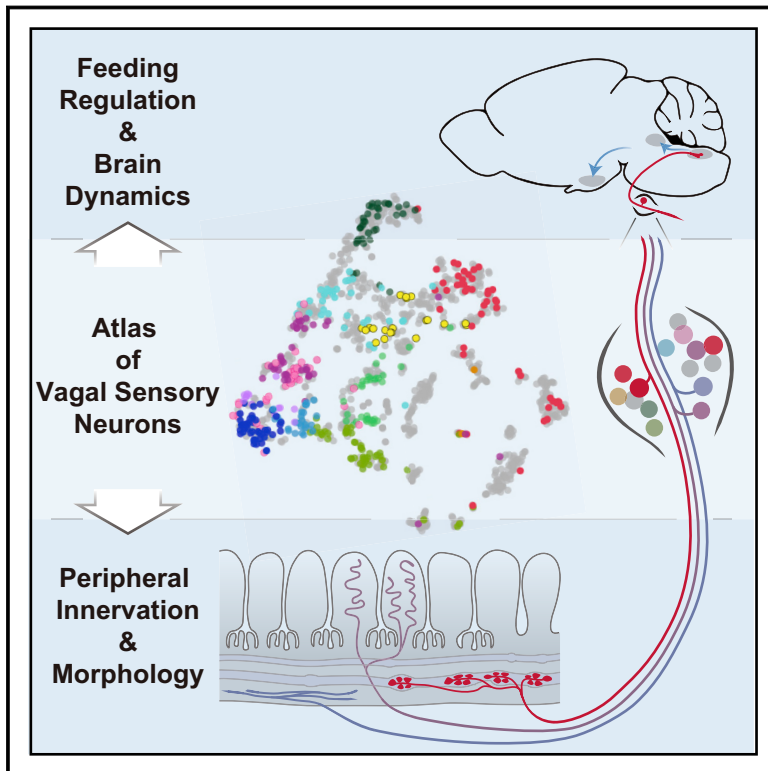


Genetic Identification of Vagal Sensory Neurons That Control Feeding

Graphical Abstract



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In Brief

A single-cell approach cataloging the sensory neurons in the vagus nerve identifies populations that suppress hunger through mechanosensation in the intestine.

Highlights

- A molecular map of vagal sensory neurons innervating the abdominal viscera
- Molecular identity correlates with innervation pattern and axon terminal morphology
- Stimulation of vagal IGLE mechanoreceptors, but not mucosal endings, inhibits feeding
- Intestine distension or mechanoreceptor activation inhibits hypothalamic AgRP neurons

Data Resources

GSE138651



Genetic Identification of Vagal Sensory Neurons That Control Feeding

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<https://doi.org/10.1016/j.cell.2019.10.031>

SUMMARY

Energy homeostasis requires precise measurement of the quantity and quality of ingested food. The vagus nerve innervates the gut and can detect diverse interoceptive cues, but the identity of the key sensory neurons and corresponding signals that regulate food intake remains unknown. Here, we use an approach for target-specific, single-cell RNA sequencing to generate a map of the vagal cell types that innervate the gastrointestinal tract. We show that unique molecular markers identify vagal neurons with distinct innervation patterns, sensory endings, and function. Surprisingly, we find that food intake is most sensitive to stimulation of mechanoreceptors in the intestine, whereas nutrient-activated mucosal afferents have no effect. Peripheral manipulations combined with central recordings reveal that intestinal mechanoreceptors, but not other cell types, potently and durably inhibit hunger-promoting AgRP neurons in the hypothalamus. These findings identify a key role for intestinal mechanoreceptors in the regulation of feeding.

INTRODUCTION

The size of each meal is tightly regulated by a physiological system that measures the quantity and quality of ingested food (Chambers et al., 2013; Cummings and Overduin, 2007; Grill and Hayes, 2012). This measurement happens primarily in the gastrointestinal (GI) tract, but the identity of the key cells, signals, and pathways remains poorly defined.

The vagus nerve contains the primary sensory neurons that monitor GI signals (Berthoud and Neuhuber, 2000; Dockray, 2013; Travagli and Anselmi, 2016). Vagal sensory neurons have cell bodies located in nodose ganglia and axons that bifurcate into two branches, one of which innervates visceral organs and

the other of which projects to the brainstem. Vagal afferents are anatomically heterogeneous, and their peripheral axons form characteristic sensory endings that are specialized for detection of chemical (mucosal endings) or mechanical (primarily intraganglionic laminar endings, or IGLEs) stimuli (Berthoud et al., 2004; Brookes et al., 2013). Within these broad classes, electrophysiological studies have revealed a diversity of response properties, including cells that respond to hormones, GI luminal nutrients, osmolytes, pH, GI distension, or luminal stroking (Berthoud and Neuhuber, 2000; Brookes et al., 2013; Dockray, 2013; Grundy and Scratcherd, 1989). Thus, vagal afferents represent a diverse class of sensory neurons that survey the gastrointestinal milieu and relay this information to brain.

Given their sensory capabilities, vagal afferents are uniquely positioned to regulate food intake. One way this is thought to occur is through hormones, such as cholecystokinin (CCK), peptide YY (PYY), and glucagon-like peptide-1 (GLP-1), which are secreted from enteroendocrine cells in the intestine in response to nutrient ingestion (Dockray, 2013). These hormones can directly modulate vagal afferent terminals within the intestinal mucosa that are in proximity to (or direct synaptic contact with) enteroendocrine cells (Berthoud and Patterson, 1996; Kaelberer et al., 2018). Food intake also results in gastric distension, which can stimulate vagal sensory neurons with mechanosensitive IGLEs innervating the muscular layer of the stomach (Williams et al., 2016; Zagorodnyuk et al., 2001). This gastric distension signal may, in combination with vagal signals of nutrients arising from the intestine, contribute to the emergence of satiation during a meal (Berthoud, 2008; Cummings and Overduin, 2007; Powley et al., 2005).

Nevertheless, it remains unclear whether these mechanisms can fully account for how satiation is naturally regulated (Woods et al., 2018). One fundamental obstacle has been the absence of techniques for manipulating the vagus with cell-type specificity in awake, behaving animals. Traditional approaches, such as surgical or chemical vagotomy (Berthoud, 2008) or bulk stimulation (Browning et al., 2017; Han et al., 2018), do not target individual pathways that carry specific types of information and consequently cannot be used to test their causal role in behavior.



We reasoned that if we could identify genetic markers for functionally distinct populations of vagal neurons and then manipulate their activity during behavior, this might provide new insight into the nature of the gastrointestinal signals that regulate food intake. A similar approach has been applied to study the role of the vagus in autonomic reflexes (Chang et al., 2015; Williams et al., 2016), but to date, the effect of manipulating vagal cell types on behavior has not been described. Here, we have used target-guided, single-cell sequencing to generate a molecular map of vagal sensory cell types that innervate the GI tract and then systematically characterized their innervation pattern, terminal morphology, and behavioral and autonomic functions. This has revealed an unexpected role for intestinal mechanoreceptors in the regulation of feeding.

RESULTS

Anatomical Characterization of Vagal Sensory Neurons That Innervate GI Tract

We set out to relate the molecular identity of vagal sensory neurons to their anatomy and function. As a first step, we cataloged the innervation of the gastrointestinal (GI) tract by vagal afferents with different terminal morphologies. We injected AAV9-DIO-tdTomato bilaterally into the nodose ganglia of *vGlut2^{Cre}* mice, which resulted in *tdTomato* expression in the majority of vagal sensory neurons but not vagal motor neurons in the brainstem (Figures 1A and S1A–S1C). We then quantified the innervation of different parts of the GI tract by whole-mount staining and imaging.

In the stomach, we observed three types of classical sensory endings with distinct patterns of innervation (Figures 1B and 1C) (Fox et al., 2000; Wang and Powley, 2000). Intraganglionic laminar endings (IGLEs), which are thought to be the mechanoreceptors detecting GI stretch (Berthoud and Neuhuber, 2000; Williams et al., 2016; Zagorodnyuk et al., 2001), densely innervated the antrum, fundus, and the greater curvature of corpus (Figures 1D and 1I). A second type of putative mechanoreceptor, intramuscular arrays (IMAs), showed highly restricted innervation near pyloric antrum and cardiac sphincter and were sparser across the rest of the stomach (Figure 1F). The mucosal endings of putative chemosensory vagal terminals were restricted to the antrum and corpus, with the highest density near the cardiac sphincter (Figures 1E, 1K, and S1D). Those mucosal afferents may also be responsive to gentle luminal stroking, but they are not activated by innocuous GI distension (Grundy and Scrat-herd, 1989). In the intestine, we observed the highest density of IGLEs and mucosal endings proximal to the pylorus, consistent with prior reports (Berthoud et al., 2004; Fox et al., 2000; Wang and Powley, 2000), but both endings were also distributed throughout the entire length of the intestine, including the large intestine (Figures 1D, 1E, and 1H–1L).

We also examined visceral organs other than the GI tract. Vagal innervation of the portal vein has been proposed to contribute to metabolic regulation by detecting absorbed nutrients en route from the intestine to the liver (Berthoud et al., 1992). Consistent with this possibility, we observed sparse vagal-derived *tdTomato*⁺ fibers running along the portal blood vessels and nearby medial lobe of liver (Figure 1G). We also

observed occasional vagal fibers in the gallbladder but not in the pancreas (data not shown). The sparsity of this innervation indicates that the vast majority of subdiaphragmatic vagal sensory neurons innervate either the stomach or intestine.

To test whether these regions receive innervation from distinct sets of vagal sensory neurons, we performed dual-color retrograde tracing by injecting fluorescent tracers into combinations of five targets and then quantifying overlap in nodose ganglion (Figures 1M–1O and S1H). Only a small percentage of vagal sensory neurons were double labeled by injections into any two different sites (ranging from 3% to 17%). In contrast, tracer injection into the same structure tended to label the same cells (ranging from 86% to 97%), and control experiments confirmed that in the periphery, fluorescent tracers were restricted to the injection site (Figures S1E–S1G). This indicates that different regions of the GI tract are innervated by different vagal sensory neurons.

Target-scSeq Identifies Vagal Cell Types Innervating Distinct Visceral Organs

To identify the vagal sensory cell types innervating different regions of the GI tract, we performed RNA sequencing of individual cells retrogradely labeled from different peripheral innervation targets (target-scSeq) (Y.L., A.D., and M.A.K., in preparation). We injected a retrograde tracer into different sites within the abdominal viscera and then manually picked 501 fluorescently labeled vagal sensory neurons from nodose ganglion of 34 mice (Figure 2A). After filtering out samples that produced low-quality sequencing data, we obtained 395 single-cell profiles for analysis, with an average of 1.72 million mapped reads per cells (Figures S2A–S2E).

We first performed unsupervised graph clustering based on the gene expression profiles and identified 12 sets of genetically distinct subdiaphragmatic vagal sensory neurons (Figures 2B and S2F–S2H). For most of the clusters, we were able to identify individual genes that function as unique markers, including t01 (*Oxtr*⁺), t02 (*Olfir78*⁺), t03 (*Npas1a*⁺), t04 (*Sst*⁺), t05 (*Calca*⁺), t06 (*Vip*⁺/*Uts2b*⁺), t07 (*Prom1*⁺), and t09 (*Edn3*⁺). Other clusters lacked a single unique marker but could be identified by the combined expression of two or more genes (Figures 2G and S2L).

Many of these purely gene-expression-based clusters were associated with a unique pattern of putative gastrointestinal innervation (Figures 2B–2E, S2I, and S2J). Seven clusters are mainly composed of neurons labeled by injection into one or two of the visceral organs: t04 and t05 were highly enriched in neurons labeled by stomach injection (59% and 77% respectively); t12, t9, and t8 were enriched in neurons labeled by injection into proximal intestine (79%), middle/distal intestine (73%), or large intestine (57%), respectively; and t01 and t03 were enriched in cells labeled by injection into stomach or large intestine (54% and 76%, respectively). Finally, three clusters, t06, t07, and t11, contained cells that were labeled by injection into many targets, suggesting that these clusters may identify vagal neurons with broad projection patterns.

The correspondence between the gene-based clustering and the retrograde tracing targets (Figures 2B–2E) suggests that these clusters identify cell types with organ-specific projection patterns. To confirm this, we injected retrograde tracers into

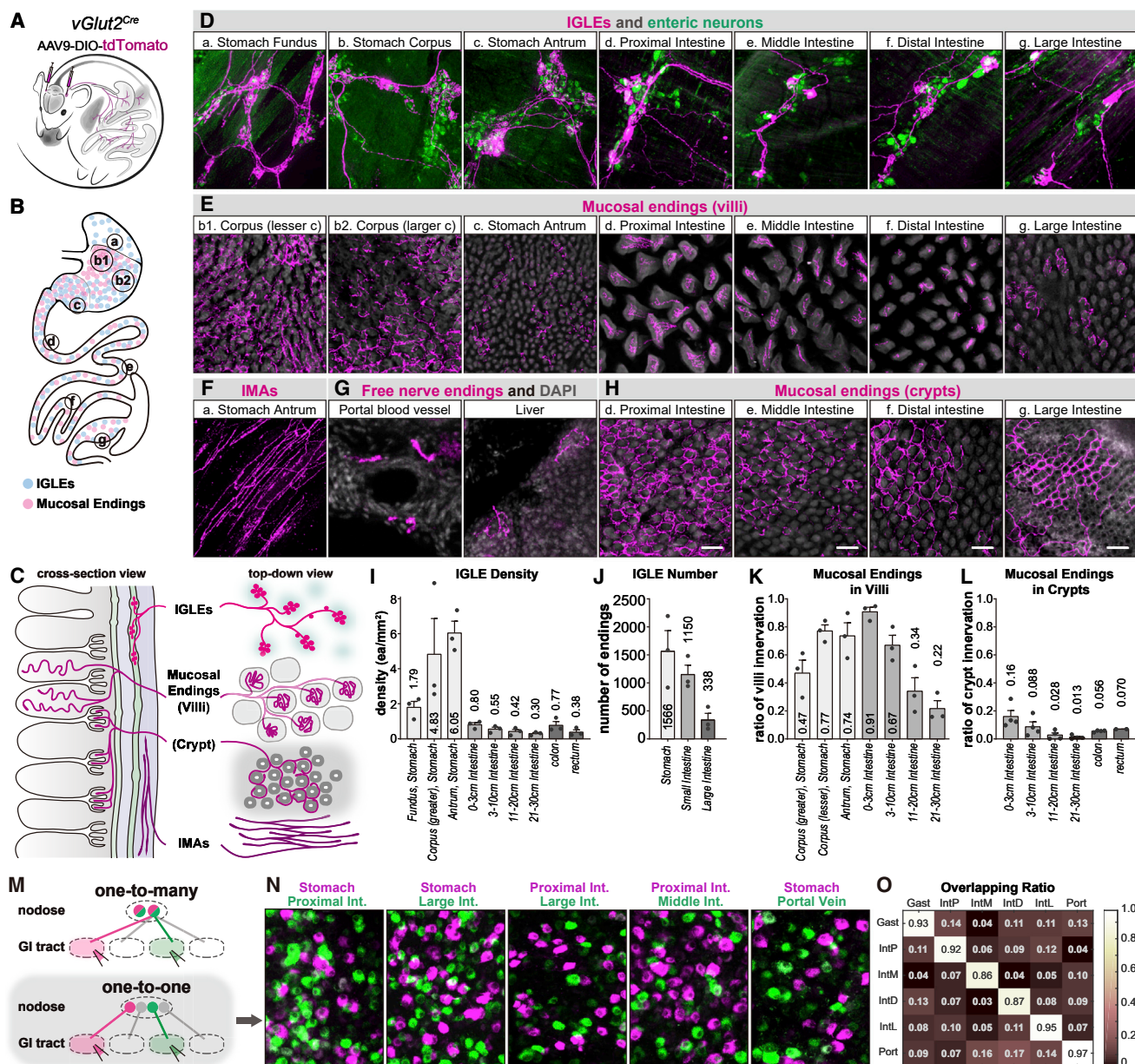


Figure 1. Anatomical Characterization of Vagal Sensory Neurons That Innervate the GI Tract

(A) Anterograde tracing strategy.

(B–C) Schematic for the distribution (B) or morphology (C) of vagal sensory terminals.

(D–H) Whole-mount (D–F and H, top-down view) and cross sections (G) showing the innervation of *tdTomato*⁺ vagal sensory terminals (magenta) in different tissues (annotated above each panel). Autofluorescence in the UV channel (gray in E and H) shows villi or crypts. FluoroGold (green) labels enteric neurons.

(I–L) Distribution of *tdTomato*⁺ vagal sensory terminals (mean \pm SEM).

(M) Schematic of the two ways vagal-GI innervation could be organized.

(N and O) Whole-mount nodose ganglion showing segregation of vagal sensory neurons that are retrogradely labeled from different GI targets (N) and quantification (O).

Scale bar, 100 μ m. See also Figure S1.

visceral organs and then quantified their overlap in nodose ganglion with marker genes identified by target-scSeq (Figures 2H and 2J). We identified *Sst* and *Calca* as markers for putative stomach-projecting neurons in clusters t04 and t05, and, consistent with this, we found that *Sst* and *Calca* each labeled approx-

imately 10% of stomach-projecting vagal neurons but less than 1% of neurons retrogradely labeled from intestine. Likewise, *Uts2b* was identified as a marker for cells in cluster t06 that broadly innervate the intestine, and this marker co-localized with tracer injected into the intestine but not the stomach. Our

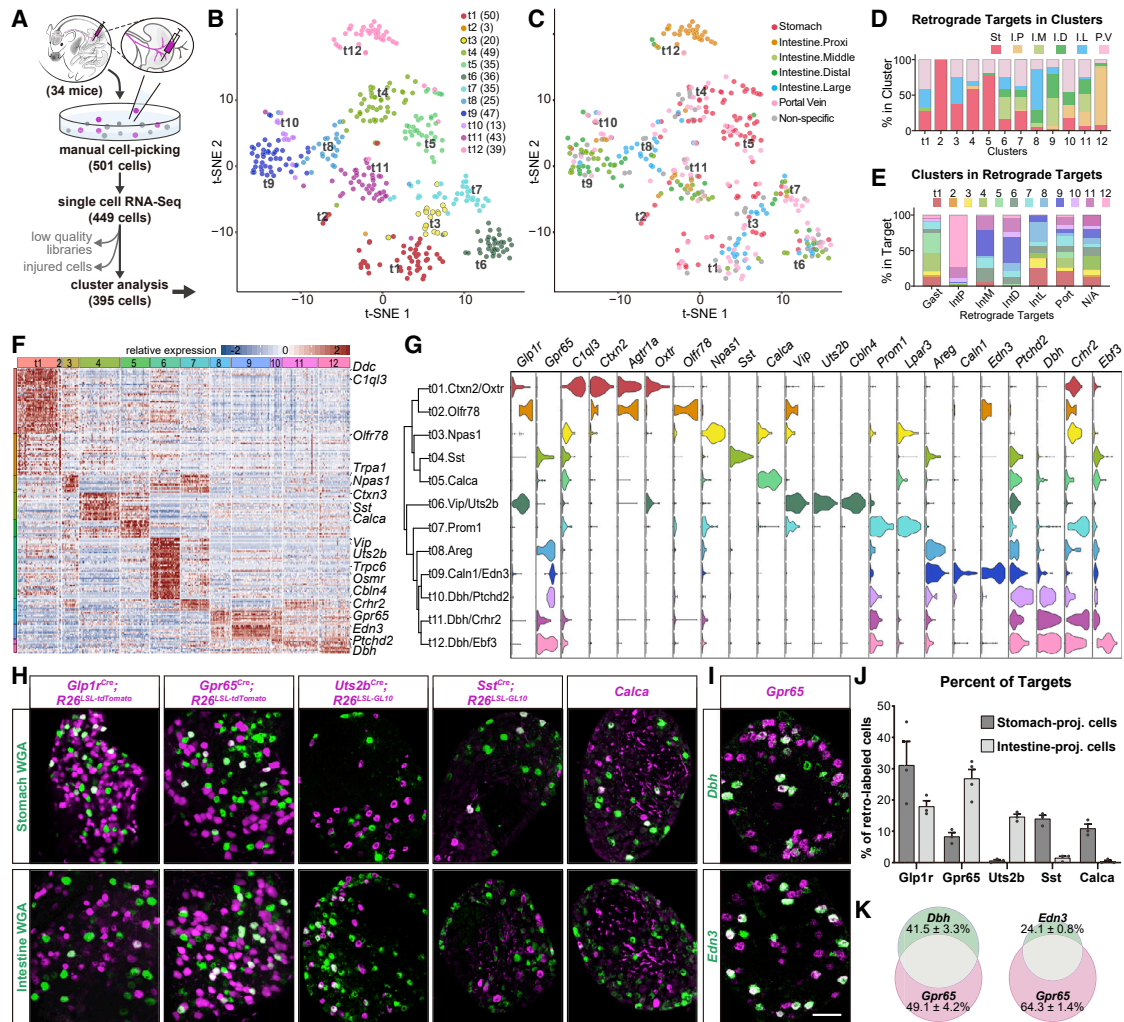


Figure 2. Target-scSeq Identifies Vagal Cell Types Innervating Distinct Visceral Organs

(A) Target-scSeq strategy.

(B and C) Spectral tSNE colored by gene-based clusters (B) or retrograde-tracing targets (C).

(D and E) Percentage of cells retrogradely labeled from different targets within individual clusters (D) or vice versa (E).

(F) The relative expression of subtype-enriched genes (rows) across cells sorted by cluster (column).

(G) Dendrogram showing relatedness of clusters, followed by violin plots showing the expression of cluster-specific marker genes.

(H) Immunostaining reveals the overlap between vagal cell-type markers (magenta) and retrograde tracer (green).

(I) RNAscope reveals that *Gpr65*⁺ cells are partially labeled by *Dbh* (t10-t12) and *Edn3* (t09).

(J) Quantification of (H), showing the percentage of stomach- or intestine-projecting cells that express each marker gene (mean \pm SEM).

(K) Quantification of (I); $n = 4$ mice.

Scale bar, 100 μ m. See also Figure S2.

data also revealed heterogeneity in the previously identified markers *Glp1r* and *Gpr65* (Williams et al., 2016), which marked neurons labeled by tracers injected into both stomach and intestine (Figure 2G–2K and S4K). This demonstrates that target-scSeq can robustly link the anatomy of sensory neurons to their molecular identity.

Comparison of Whole-Nodose and Target-scSeq Reveals the Organization of Vagal Sensory Subtypes

In addition to the abdominal viscera, vagal sensory neurons also innervate the heart, lung, and other supradiaphragmatic targets.

To place our target-scSeq data in this broader context, we next performed unbiased, droplet-based scSeq of the entire nodose ganglion. We sequenced 1,108 individual cells from the nodose ganglion of adult mice, removed low-quality cells and non-neuronal cells (Figures S3A–S3E) (Kupari et al., 2019), and obtained 956 vagal sensory neurons for subsequent analysis. Next, we merged this whole nodose-scSeq with the Gl target-scSeq datasets and performed integrated clustering analysis based on gene expression (Stuart et al., 2019). This revealed 27 clusters of vagal sensory cell types (n1–27; Figure 3A), each associated with the expression of unique marker genes

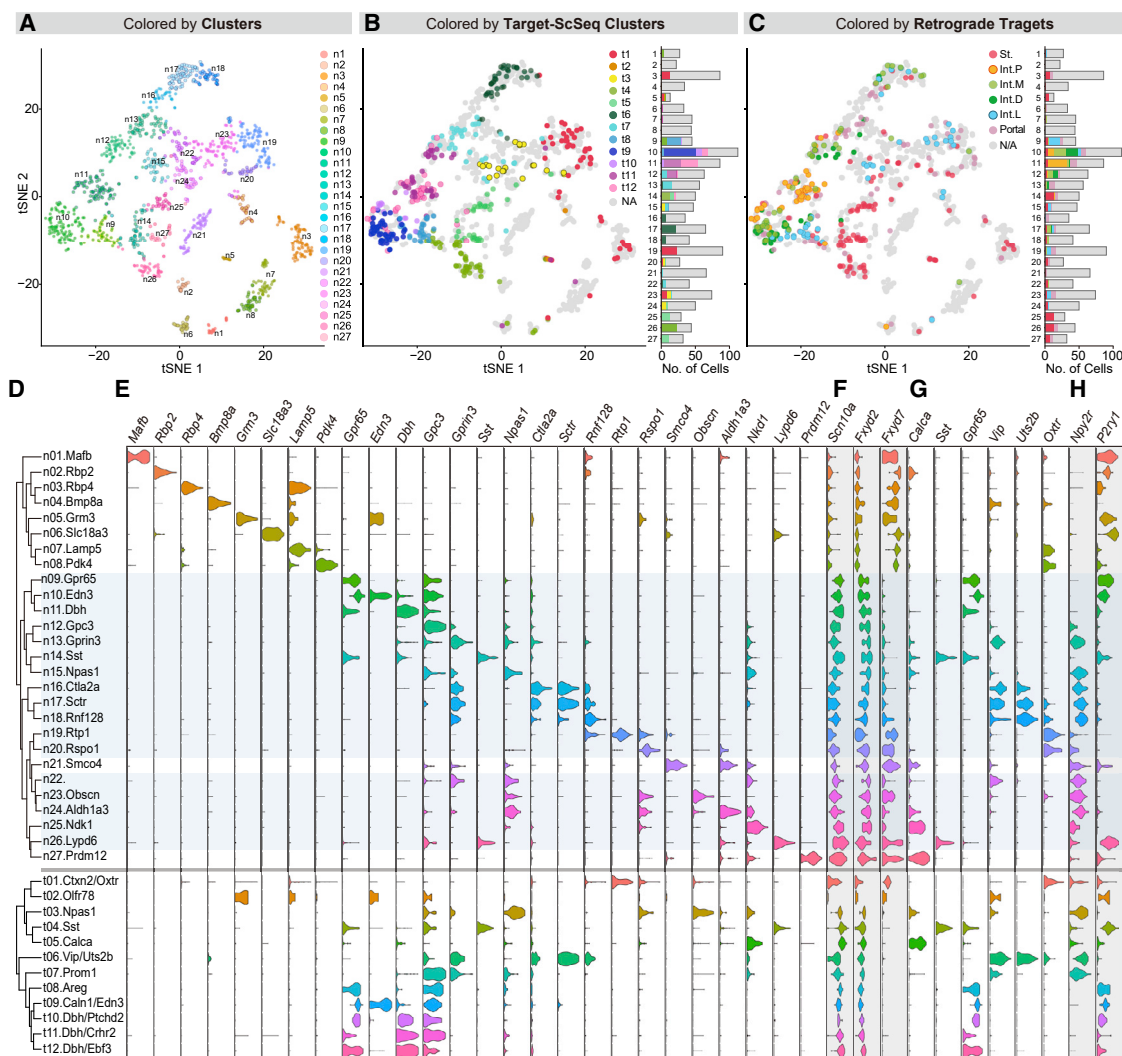


Figure 3. Comparison of Whole-Nodose and Target-scSeq Reveals the Global Organization of Vagal Sensory Subtypes

(A) Spectral tSNE plot shows gene-based clusters, combining GI target-scSeq and unbiased whole-nodose scSeq.

(B and C) tSNE plots (left) or stacked bar graphs (right) showing neurons in each cluster that originated from individual target-scSeq clusters (B) or were labeled from individual GI targets (C).

(D–H) Dendrogram showing relatedness of clusters (D) followed by violin plots showing gene expression across clusters of combined nodose scSeq (top, n1–n27) and GI target-scSeq (bottom, t01–t12).

(E–H). Genes included are cluster markers of combined scSeq (E), markers enriched or excluded in subdiaphragmatic clusters (F), markers used for later anatomical characterizations (G), and genes listed in prior literature (H). The blue shadow marks putative subdiaphragmatic clusters from the combined whole-nodose scSeq.

See also Figure S3.

(Figures 3D–3H and S3F). 17 of the clusters are putative subdiaphragmatic vagal sensory subtypes (n9–20 and 22–26), based on the fact that (1) more than 10% of cells in each of these subdiaphragmatic clusters are derived from the GI target-scSeq (Figure S3H) and (2) each cluster is defined by marker genes that are also expressed in one or two of the GI target-scSeq clusters (Figure 3E). On the other hand, 10 clusters lacked cells from the GI target-scSeq or were labeled by marker genes not expressed in the target-scSeq (n1–8, 21, and 27; Figures 3E and S3H), suggesting that they contain cells with supra-diaphragmatic innervation.

We also identified genes that are broadly enriched in, or excluded from, the vagal sensory subtypes that project to the abdominal viscera (Figure 3F). For example, *Scn10a* and *Fxyd2* were enriched in all 17 putative subdiaphragmatic clusters but were excluded from most non-subdiaphragmatic clusters. In contrast, *Fxyd7* was highly expressed in all non-subdiaphragmatic clusters but only weakly expressed in a small subset of abdominal clusters (n19, 20, 23, and 26). Thus, these marker genes identify vagal sensory neurons based on their pattern of innervation above or below the diaphragm and may provide useful genetic access to these broad subsets of cells.

Genetic Identification of Vagal Subtypes with Unique Morphologies and Innervation Patterns

We next used the genetic markers identified by ScSeq to determine the terminal morphology and innervation targets of a panel of vagal neuron subtypes. For this purpose, we characterized six Cre mouse lines predicted to label distinct subsets of vagal sensory neurons (*Uts2b*, *Oxtr*, *Vip*, *Sst*, *Calca*, and *Nav1.8*) as well as two lines that label previously characterized populations (*Gpr65* and *Glp1r*). We then visualized the anatomy of these cell types by bilateral injection of AAV9-DIO-tdTomato into nodose ganglia of the corresponding Cre driver mice, which captures the expression pattern of these marker genes in the adult. The visceral innervation pattern of these cells was then cataloged by quantitative histological analysis and benchmarked to the innervation of *Vglut2^{Cre}* mice, which labels all vagal sensory neurons (Figure 4G).

Sst and *Calca* are predicted to label two populations of neurons innervating the stomach based on our ScSeq data (t04 and t05, respectively; Figures 2B–2D and 2G). Consistent with this, we found that *Sst^{Cre}* and *Calca^{CreER}* both label vagal sensory neurons that are mainly restricted to the stomach. These two cell types form mucosal endings innervating gastric villi but do so with distinct topography (Figures 4A, 4B, and 4G). *Sst⁺* mucosal endings are enriched in the pyloric antrum and are very sparse in the stomach corpus. In contrast, *Calca⁺* neurons form extensive villi innervation near the lesser curvature of the corpus, with density dramatically decreased near the pyloric antrum and greater curvature of the corpus. A subset of *Calca⁺* neurons also forms IMAs near the gastric antrum and large intestine (Figure S4K). Staining of cell bodies in the nodose ganglion further confirmed that the *Sst⁺* and *Calca⁺* neurons are non-overlapping (Figures S4A and S4B). Thus, *Sst* and *Calca* define distinct subtypes of gastric mucosal-ending neurons with unique innervation patterns.

The intestine is also densely innervated by mucosal endings (Figures 1K and 1L) (Berthoud et al., 2004). We observed expression of the mucosal marker *Gpr65* (Williams et al., 2016) in five clusters with broad intestinal projections (t08–12) as well as in the *Sst⁺* gastric mucosal-ending cluster (t04) (Figures 2D, 2G, 2I, 2K, and S2K). Consistently, dense *Gpr65⁺* mucosal endings were found in the stomach with a distribution that resembles that of the *Sst⁺* cells (Figures 4A, 4B, and 4G). We also found that *Gpr65⁺* mucosal endings innervate not only proximal intestine but also middle and distal intestine, accounting for approximately a third of total mucosal endings in the latter two regions (Figures 4C, 4D, and 4G). Thus, *Gpr65* labels multiple subtypes of mucosal-ending vagal afferents distributed across the entire GI tract.

The marker genes *Vip* and *Uts2b* identify a fourth type of mucosal ending neurons in our target-scSeq data. Visualization of these cells using *Vip^{Cre}* mice revealed that they exclusively innervate the intestinal villi and were evenly distributed across proximal, middle, and distal intestine (Figures 4C, 4D, and 4G). We confirmed this by generating a *Uts2b^{Cre}* mouse (Figures S2M–S2S), which showed an identical pattern of labeling (Figures 4D and 4G). *Vip/Uts2b* expression showed no overlap with the other intestinal mucosal ending marker, *Gpr65* (Figures S4A and S4E–S4F), indicating that the t06 *Vip/Uts2b⁺* cluster de-

fines a unique population of sensory neurons that innervates small intestine villi.

In addition to the diversity of mucosal ending neurons, we observed multiple cell types forming mechanosensitive IGLEs. Beyond the previously identified IGLE marker gene *Glp1r*, we found that *Oxtr^{Cre}* exclusively labels neurons that form IGLEs (Figure 4F). Both *Oxtr* and *Glp1r* are expressed in the cluster t01 (Figure 2G), but they are mutually excluded from each other (Figures S4A and S4I), suggesting that they may label a distinct subtype of cells. Indeed, quantitative examination of their anatomy revealed that *Oxtr⁺* IGLEs are largely restricted to the intestine, with the highest density in the proximal intestine, whereas *Glp1r⁺* IGLEs extensively innervate the stomach but not the intestine (Figures 4E–4G). Thus, *Oxtr* and *Glp1r* identify two distinct subsets of IGLE mechanoreceptors with different innervation patterns in the abdominal viscera.

Activation of Gastrointestinal Mechanoreceptors Potently Inhibits Food Intake

The identity of the vagal cell types that regulate feeding is unknown. To investigate this, we selected two sets of mucosal-ending neurons (*Vip^{Cre}* and *Gpr65^{Cre}*) and two sets of IGLE mechanoreceptors (*Oxtr^{Cre}* and *Glp1r^{Cre}*) for functional analysis (Figure 5B). Three of these cell types (*Vip⁺*, *Oxtr⁺*, *Glp1r⁺*) express *Cckar*, and all four express *Htr3a*, indicating that they are poised to receive nutritional signals from the gut (Figures 5A and S5A). We then targeted the excitatory opsin ChR2 to these cells and installed an optical fiber above the nucleus of the solitary tract (NTS) for selective photostimulation of their central terminals (Figures 5C–5E).

We first measured food intake in overnight-fasted mice. The *Vip⁺* t06 cluster showed the highest expression of *Cckar* in our sequencing data (Figures 5A and S5A), and *Gpr65⁺* neurons are activated by nutrients in the intestinal lumen (Williams et al., 2016). However, we observed no effect on food intake following optogenetic stimulation of either *Vip⁺* or *Gpr65⁺* neurons (Figures 5F–5H). To confirm this result, we targeted the chemogenetic actuator hM3Dq to these two cell types and treated mice with clozapine-N-oxide (CNO). This increased *Fos* expression in the brainstem targets of these vagal neurons (Figure S5D) but again had no effect on food intake in either fasted or fed animals (Figures 5J–5N and 5S). Thus, activation of *Vip⁺* or *Gpr65⁺* mucosal-ending neurons is not sufficient to modulate feeding.

Gastric distension is thought to be an important signal that promotes meal termination (Phillips and Powley, 1996). A subset of *Glp1r⁺* neurons are IGLE mechanoreceptors that innervate the stomach and are activated by gastric distension (Williams et al., 2016), suggesting that these cells may contribute to stretch-induced meal termination. Consistent with this, we observed a partial reduction in food intake following either optogenetic (Figures 5F–5H) or chemogenetic (Figure 5L, 5M, and 5S) stimulation of *Glp1r⁺* neurons. Although *Glp1r* is also expressed in intestine-innervating cells that form mucosal endings (*Vip/Uts2b⁺* t06 cluster, Figures 2G, S4A, and S4G–S4H), these cells are unlikely to account for the suppression of feeding by *Glp1r⁺* neurons, as stimulation of *Vip⁺* neurons alone had no effect on behavior (Figures 5F–5H and 5L–5N).

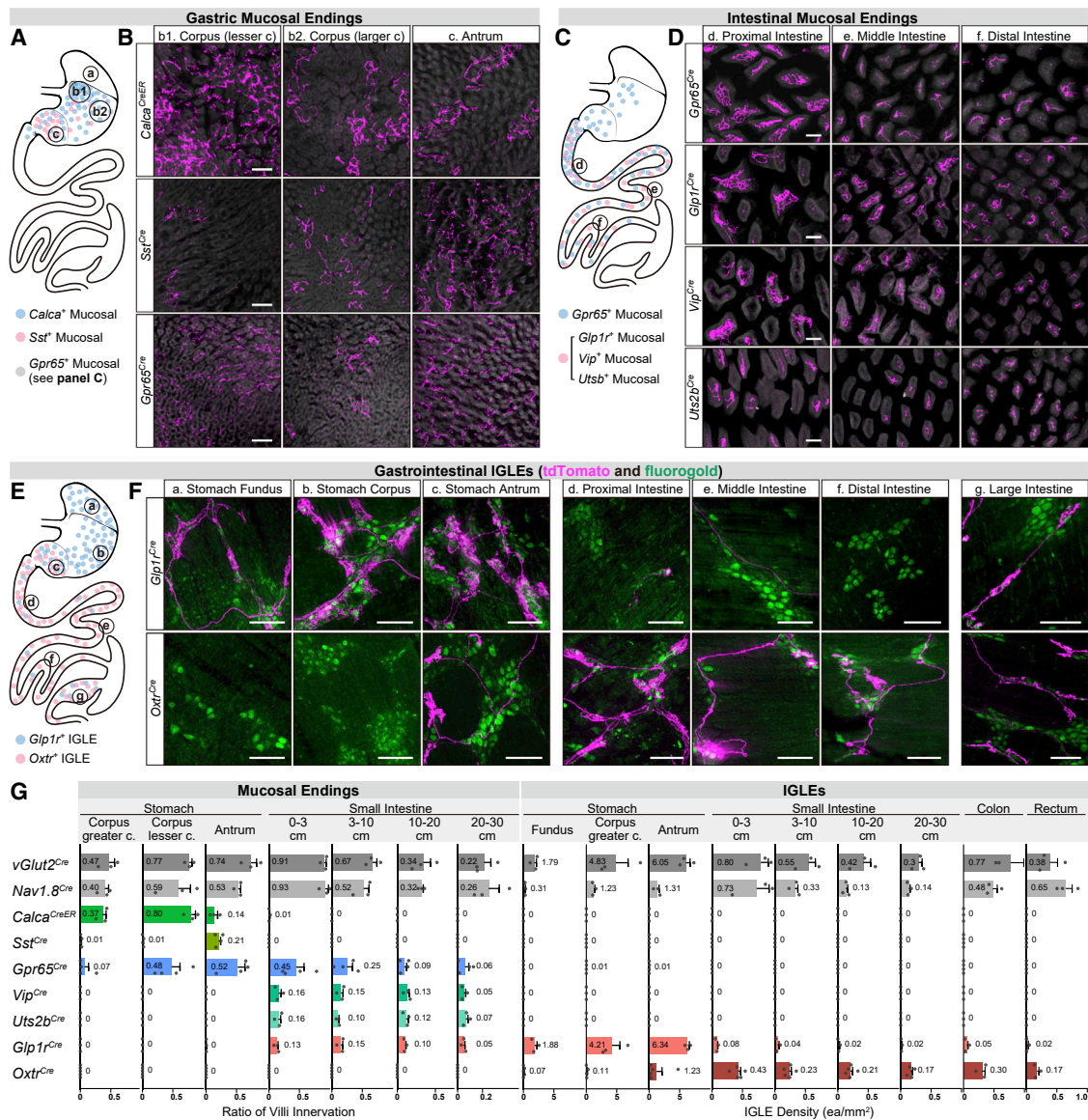


Figure 4. Genetic Markers Identify Vagal Subtypes with Unique Morphologies and Innervation Patterns

(A–F) Distribution of gastric mucosal endings (A–D), intestinal mucosal endings (C and D), and gastrointestinal IGLEs (E and F) labeled by seven nodose-subtype *Cre* lines.

(A, C, and E) Schematic of distribution.

(B, D, and F) Top-down view of whole-mount GI tissue. Magenta, *tdTomato*⁺ vagal sensory terminals. Gray, villi visualized by the autofluorescence in UV channel. Green, FluoroGold-labeled enteric neurons.

(G) Quantification of mucosal ending- and IGLE-distributions labeled by *vGlut2^{Cre}*, *Nav1.8^{Cre}*, and the seven nodose-subtype *Cre* lines (mean ± SEM).

Scale bar, 100 μ m. See also Figure S4.

In addition to the stomach, IGLE mechanoreceptors also innervate the intestine (Figure 1), but their potential role in feeding has received little attention (Berthoud, 2008; Cummings and Overduin, 2007). The identification of *Oxtr* as a specific marker for intestinal IGLEs (Figures 4E–4G) permits us to directly examine their function *in vivo*. To our surprise, we found that both optogenetic and chemogenetic stimulation of vagal *Oxtr* neurons dramatically reduced food intake (Figures 5F, 5G, 5L, and 5M). This effect was rapidly reversible, as feeding rebounded shortly

after optogenetic stimulation was terminated (Figure 5H). This suggests that mechanical signals from the intestine, such as distension, may regulate feeding by acting through *Oxtr*⁺ neurons.

Food intake can also be influenced by a variety of indirect or non-specific mechanisms. To probe the specificity of the feeding suppression induced by *Oxtr*⁺ and *Glp1r*⁺ neurons, we characterized the effect of activating these and other neurons in a battery of behavioral and physiological tests. First, we investigated

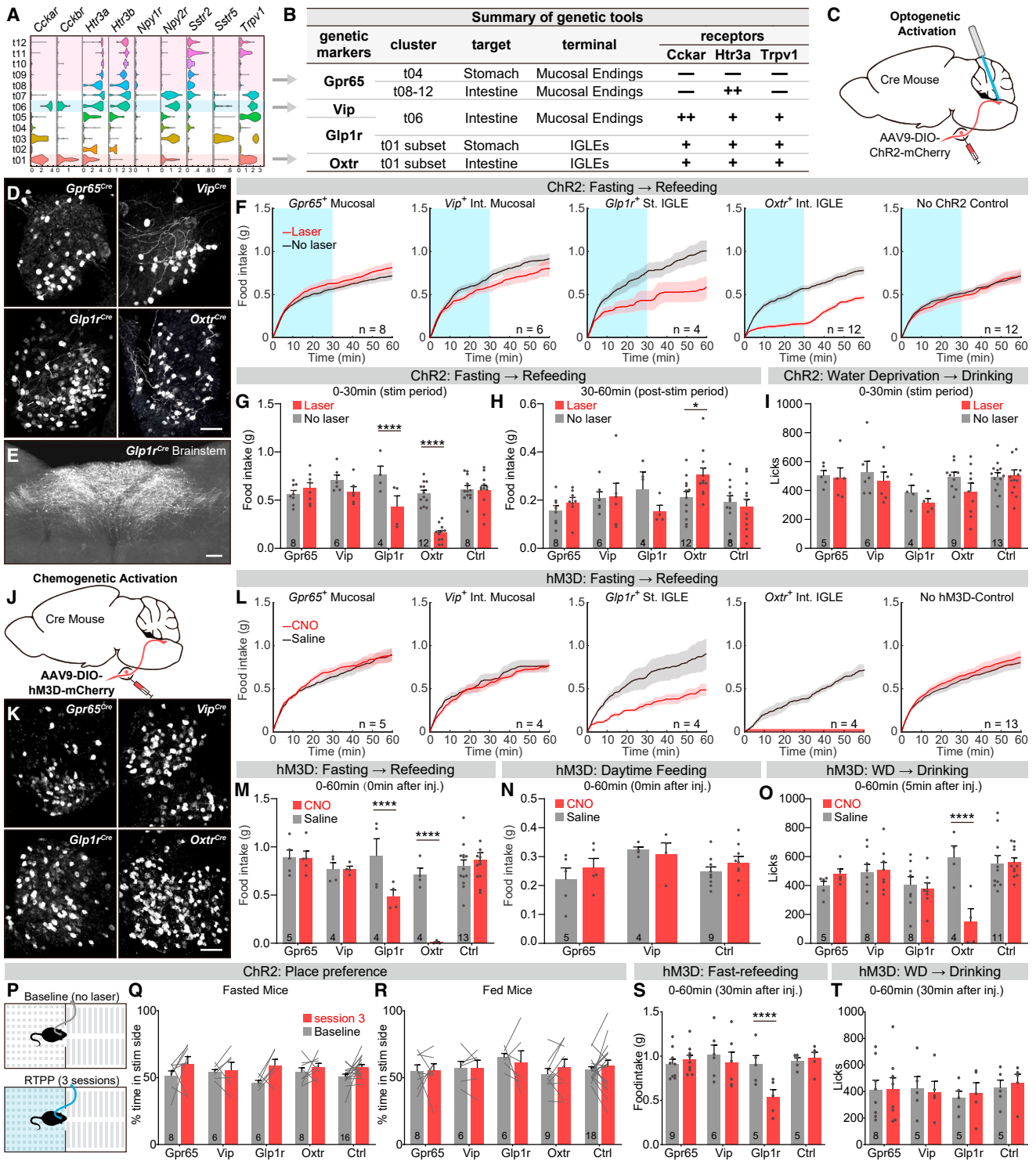


Figure 5. Activation of Gastrointestinal Mechanoreceptors Potently Inhibits Food Intake

(A) Expression of hormone receptors and *Trpv1* across target-scSeq clusters.
 (B) Summary of the four vagal Cre lines used for functional analysis.
 (C) Optogenetic activation strategy.
 (D and E) *ChR2-mCherry* expression in vagal sensory cell bodies (D) and their central terminals in the NTS and AP (E).

(legend continued on next page)

the valence of these vagal sensory subtypes using a closed-loop place-preference assay, in which optogenetic activation is coupled with occupancy of one side of a two-chamber arena. Mice failed to develop preference for either side of the chamber throughout multiple days of testing in either fasted or fed conditions, indicating that activation of these neurons induces neither real-time nor conditioned place preference (Figures 5P–5R).

In addition to feeding, vagal afferents have also been implicated in the regulation of thirst, digestion, body temperature, and blood pressure (Hanyu et al., 1990; Madden et al., 2016; Masclee et al., 1990; Székely, 2000; Watanabe et al., 2010; Zimmerman et al., 2019). We found that optogenetic activation of *Oxtr*⁺ and *Glp1r*⁺ neurons had no effect on drinking after overnight water deprivation (Figures 5I and S5E), whereas chemogenetic stimulation of *Oxtr*⁺ neurons did cause a decrease in water intake (Figures 5O, 5T, and S5F). This suggests that the ability of these cells to reduce drinking depends on the strength of stimulation. We also failed to observe any changes in body temperature or blood pressure following optogenetic stimulation of any of these vagal subtypes (Figures S5G–S5I). CCK is well known for regulating bile acid secretion from the gallbladder (Spellman et al., 1979), and three of these subtypes express the *Cckar*, but we observed no effect on gallbladder emptying following chemogenetic stimulation of any of these neurons (Figures S5B and S5C). Taken together, these data suggest that the effects of *Oxtr*⁺ and *Glp1r*⁺ neuron stimulation on feeding are unlikely to be secondary to malaise or broader physiological changes.

Stimulation of Gastrointestinal Mechanoreceptors Modulates Feeding Centers in the Brain

We next investigated how these vagal signals of GI distension are represented in the brain. The caudal NTS and area postrema (AP) receive direct innervation from vagal sensory neurons and are known to be critical for satiation (Figure S4L, Berthoud and Neuhuber, 2000; Grill and Hayes, 2012). Consistent with this, we observed robust induction of *Fos* expression in both regions following stimulation of *Oxtr*⁺ neurons (Figures 6E, 6G, S6A, and S6B). The *Fos* expression induced in the NTS co-localized extensively with tyrosine hydroxylase (*Th*), which marks a subset of NTS neurons that promote satiation (Figures 6A, 6B, and 6F) (Kreisler et al., 2014; Maniscalco and Rinaman, 2013; Roman et al., 2016). The parabrachial nuclei (PBN) also control food intake and receive ascending sensory inputs from the NTS/AP. Consistently, we observed extensive *Fos* expression in the PBN following stimulation of *Oxtr*⁺ neurons (Figures S6C and

S6D). A subset of *Fos*⁺ neurons were localized to the external lateral PBN (PBNel) and overlapped with appetite-inhibiting *Calca*⁺ neurons (Figures 6C–6F) (Campos et al., 2016). Additionally, we noticed many *Fos*⁺ neurons in the dorsal lateral PBN (PBNdl) (Figures 6E, S6C, and S6D), a region that shows high *Fos* expression following treatment with CCK but not LiCl (Han et al., 2018).

In addition to the hindbrain, the hypothalamus is also critical for the regulation of food intake. Hunger-promoting Agouti-related Peptide (AgRP) neurons in the arcuate nucleus (ARC) are activated by food deprivation and inhibited by GI nutrients (Beutler et al., 2017; Hahn et al., 1998; Su et al., 2017). To monitor AgRP neurons while simultaneously manipulating vagal neuron subtypes, we targeted expression of *GCaMP6m* to AgRP neurons by using *Npy*^{Flp} mice (which labels AgRP neurons in the ARC) and independently targeted expression of *hM3Dq* to vagal sensory neurons by using the corresponding *Cre* drivers (Figures 6H–6J).

Although AgRP neurons are potently inhibited by intragastric nutrients (Beutler et al., 2017; Su et al., 2017), we observed no change in their activity following stimulation of nutrient-activated *Gpr65*⁺ neurons (Figure 6K). We likewise observed no response of AgRP neurons to stimulation of *Vip*⁺ neurons, a distinct population of cells that innervate the intestinal mucosa and highly express *Cckar* (Figure 6L). In contrast, AgRP neurons were rapidly inhibited by activation of IGLE mechanoreceptors innervating the stomach (*Glp1r*⁺) and intestine (*Oxtr*⁺) (Figures 6M–6O). Interestingly, these two mechanoreceptor subtypes inhibited AgRP neurons with different kinetics. Stimulation of stomach-innervating *Glp1r*⁺ neurons caused a rapid but transient reduction of AgRP neuron activity, whereas stimulation of intestine-innervating *Oxtr*⁺ neurons induced a rapid and sustained response. Stimulation of *Oxtr*⁺ neurons also strongly attenuated the response of AgRP neurons to the sensory detection of food, whereas stimulation of other vagal subtypes including *Glp1r*⁺ had no effect (Figures S6E–S6H). The relative inhibition of AgRP neurons by these vagal subtypes mirrored their ability to inhibit food intake (Figure S6F). Thus, these data reveal that hypothalamic feeding circuits are modulated specifically by mechanoreceptors in the stomach and intestine.

Gastrointestinal Distension Is Sufficient to Inhibit Food Intake and AgRP Neuron Activity

Prior work has emphasized the importance of nutrients rather than stretch in regulating AgRP neurons (Beutler et al., 2017; Su et al., 2017). More broadly, studies of the role of stretch in ingestive behavior have traditionally focused on the stomach,

(F–I) Food intake (F–H) or water intake (I) comparing trials with and without photostimulation across the four vagal-*ChR2* lines and control (no *ChR2*). Data are plotted as cumulative intake over time (F) or total intake within the stimulation period (G and I) or post-stimulation period (H). Blue indicates the period of photostimulation.

(J) Chemogenetic activation strategy.

(K) *hM3D-mCherry* expression in vagal sensory cell bodies.

(L–O) Food intake (L–N) or water intake (O) comparing trials with CNO or saline treatment across the four vagal-*hM3D* lines and control (no *hM3D*). Data are plotted as cumulative intake over time (L) or total intake (M–O).

(P) Place-preference assay.

(Q and R) Percentage of time spent in photostimulation-paired chamber, comparing baseline and the third stimulation session, using fasted (Q) or fed (R) mice.

(S and T) Food intake (S) or water intake (T) tested 30 min after CNO or saline treatment.

Error bars and shaded areas represent mean \pm SEM. N mice is annotated within figures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA, Sidak correction. Scale bar, 100 μ m. See also Figure S5.

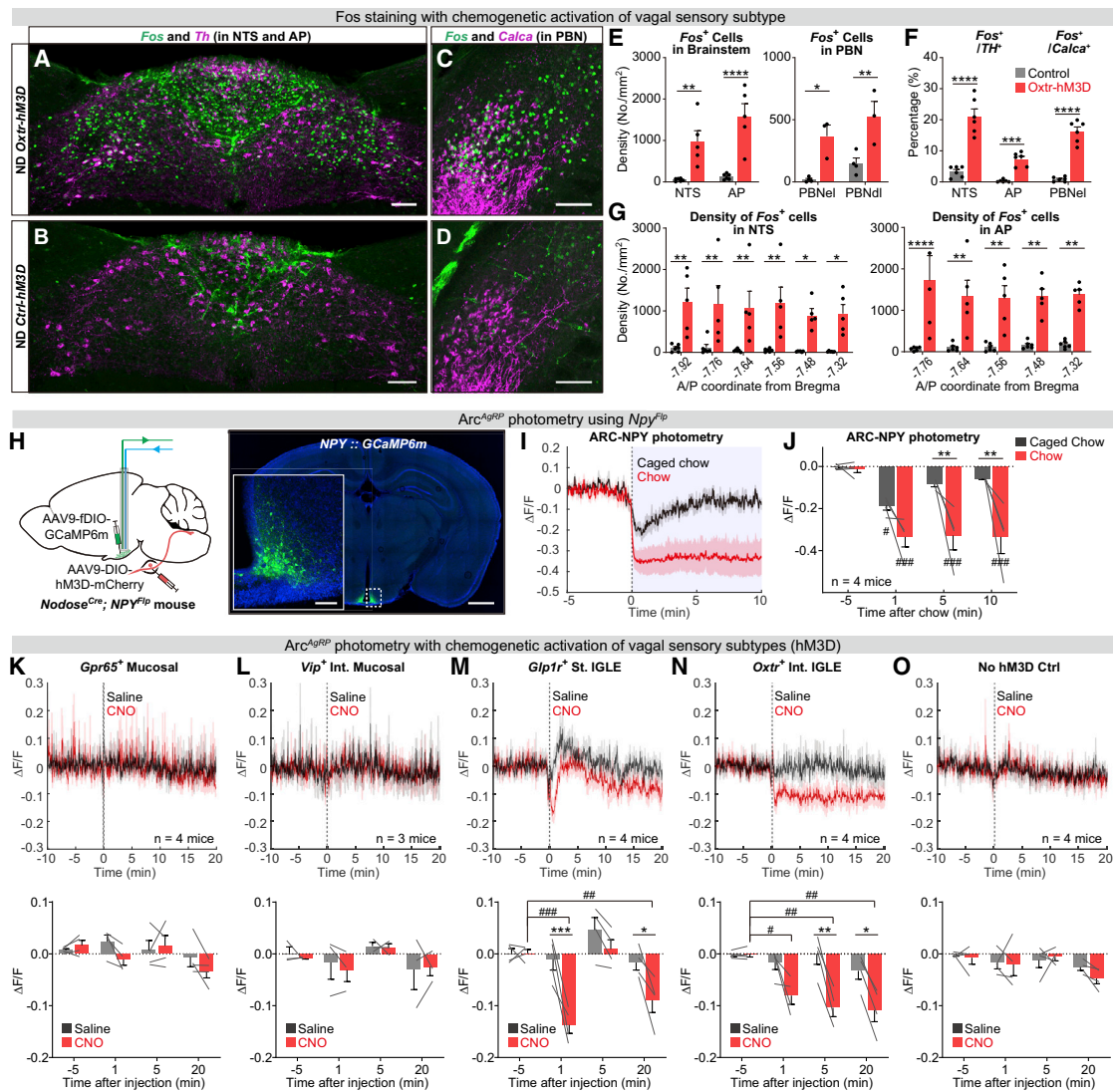


Figure 6. Stimulation of Gastrointestinal Mechanoreceptors Modulates Feeding Centers in the Brain

(A–G) Immunostaining of *Fos* and *Th* in the NTS/AP (A and B), *Fos* and *Calca* in the PBN (C and D), and quantification (E–G), comparing *Oxtr*-hM3D and control mice after CNO treatment. (E) density of *Fos*⁺ cells. (F) Percentage of *Fos*⁺ cells in the *TH*⁺ NTS or *Calca*⁺ PBN neurons. (G) anterior-posterior distribution of *Fos*⁺ cells.

(H) Concurrent Arc-photometry recording and vagal sensory neuron activation (left). The expression of GCaMP6m is restricted within the Arc (right).

(I and J) Normalized AgRP neuron calcium signal in fasted mice presented with chow and caged chow (I) and quantification (J).

(K–O) Normalized AgRP neuron calcium signal in fasted mice after CNO or saline treatment (top) and quantification (bottom) across the four vagal-hM3D lines or control.

Values are reported as mean ± SEM. Comparisons were made between groups (**p* < 0.05, ***p* < 0.01, ****p* < 0.001) or from baseline (#*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001), two-way ANOVA, Sidak correction. Scale bar, 100 μm (A–D) or 1 mm (H). See also Figure S6.

not the intestine (Cummings and Overduin, 2007; Phillips and Powley, 1996). To find conditions that would allow us to manipulate stomach or intestinal volume preferentially in awake animals, we considered the possibility that the level of gastric versus intestinal distension may differ depending on the substance ingested and its kinetics of distribution in the GI tract. To explore this, we delivered a panel of solutions to the stomach by oral gavage and then measured the contents of the stomach and different segments of the intestine five minutes later

(Figures 7A and S7A–S7E). We found that lipid and glucose induced a significant increase of fluid content of the stomach and/or proximal intestine, whereas hyperosmolar, non-nutritive solutions, including hypertonic saline and mannitol, induced a more dramatic increase in the fluid content of the middle intestine. By contrast, methylcellulose, a highly viscous solution, only increased the contents of the stomach. Thus, gastric delivery of these non-nutritive substances makes it possible to differentially introduce stomach versus intestinal fill.

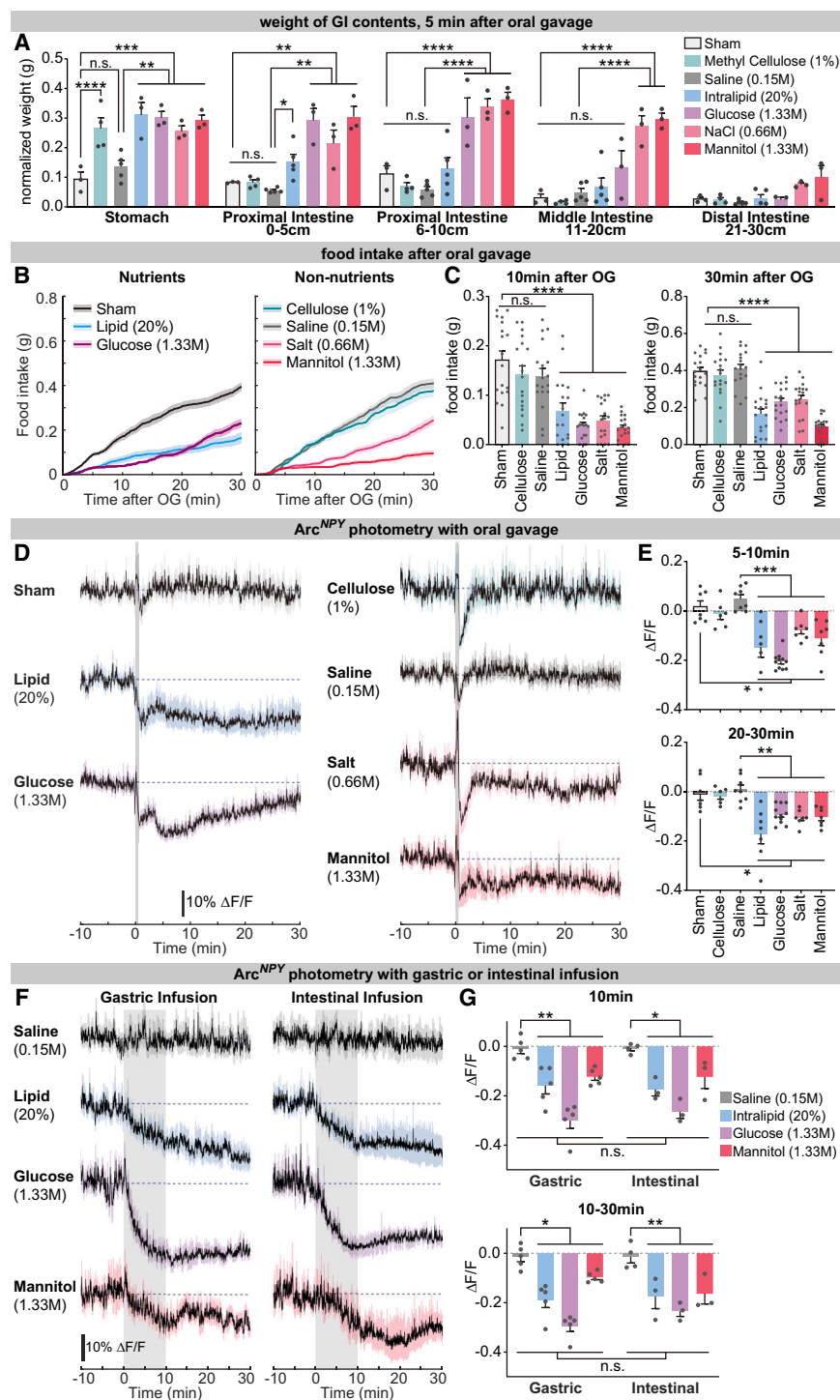


Figure 7. Gastrointestinal Distension Is Sufficient to Inhibit Food Intake and Regulate AgRP Neuron Activity

(A) Normalized weight of GI contents measured 5 min after sham treatment or oral gavage of 500 μ L of various solutions.

(B and C) Cumulative (B) and total (C) food intake of fasted mice after oral gavage of 500 μ L of various solutions.

(D and E) Average of normalized AgRP neuron calcium signal of fasted mice following oral gavage of 500 μ L of various solutions (D) and quantification (E). Gray bars indicate the period of oral gavage. (F and G) Average of normalized AgRP neuron calcium signal in fasted mice after gastric or intestinal infusion of 1 mL of various solutions (F) and quantification (G). Gray bars indicate the infusion period.

Values are reported as mean \pm SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, two-way ANOVA, Sidak correction. See also Figure S7.

of food intake and AgRP neuron activity following gastric delivery of hypertonic saline and mannitol, which induce marked intestinal distension but have no nutritive value. In contrast, gavage of cellulose, which induces gastric but not intestinal distension (Figure 7A), failed to modulate food intake or AgRP neuron activity. These effects of hyperosmotic solutions were not due to dehydration, because subcutaneous hypertonic saline had no effect on AgRP neuron activity or food consumption (Figures S7G–S7I), consistent with previous work showing that AgRP neurons are not modulated by water deprivation or drinking (Zimmerman et al., 2016). Thus, the ability of non-nutritive substances to inhibit food intake and AgRP neurons correlates with their ability to fill the intestine, further suggesting a role for intestinal distension in these processes.

To test directly whether infusions that bypass the stomach can modulate AgRP neurons, we generated mice for photometry recordings of AgRP neurons and then equipped these mice with catheters that enable infusion into either the stomach or the duodenum. As before, we observed robust inhibition of AgRP neurons following intragastric infusion of

We then tested whether filling the stomach or intestine with these substances would have a differential effect on food intake or AgRP neuron activity (Figures 7B–7E and S7F). As expected, gavage of lipid and glucose inhibited AgRP neurons, as well as subsequent food intake, relative to controls (Beutler et al., 2017; Su et al., 2017). Strikingly, we also observed an inhibition

lipid or glucose, as well as significant but weaker inhibition by mannitol (Figures 7F and 7G). Infusion of these three substances into the intestine also inhibited AgRP neurons, including importantly pronounced inhibition by non-nutritive mannitol (Figures 7F and 7G). Intestinal infusion also mimicked the ability of intragastric infusion to inhibit subsequent food intake and to

attenuate the response of AgRP neurons to the sensory detection of food (Figures S7J–S7N). This demonstrates that gastric signals are not required for inhibition of AgRP neurons and, together with our optogenetic and chemogenetic findings, suggests a key role for intestinal distension in the regulation of feeding.

DISCUSSION

A central task of physiology is to explain how food intake reduces hunger. While many studies have emphasized the role of mechanical and chemical signals originating from the gut (Cummings and Overduin, 2007; Dockray, 2013), the identity and properties of the key sensory neurons that detect these signals have remained unclear. Here, we have used an approach for target-guided, single-cell RNA sequencing to catalog the molecular identity of the vagal sensory neurons innervating the abdominal viscera. We then annotated these cell types by comprehensively characterizing their innervation patterns, sensory endings, and behavioral function. We found that food intake was most potently inhibited by vagal afferents that innervate the intestine and form IGLEs—the putative mechanoreceptors that sense intestinal stretch. Stimulation of these intestinal mechanoreceptors was sufficient to activate satiety-promoting pathways in the brainstem and inhibit hunger-promoting AgRP neurons in the hypothalamus. Consistently, increasing intestinal volume was sufficient to inhibit food intake and AgRP neuron activity even in the absence of nutrients. These findings demonstrate an unexpected role for intestinal distension in satiation and provide genetic access to key sensory neurons that regulate food intake.

A Genetic Map of Vagal Afferents Innervating the GI Tract

Early anatomical and electrophysiological studies subdivided GI vagal afferents into three major types: the IGLEs, which innervate the muscle layer and respond to GI distension; the mucosal afferents, which innervate the mucosal membrane and are therefore adjacent to sites of hormone release; and the IMAs, a less-common subtype that may be activated by GI stretch but has not been functionally characterized (Berthoud and Neuhuber, 2000; Brookes et al., 2013). However, it has remained unclear to what extent subsets of these broad classes of vagal afferents exist that have distinct molecular identities, innervation patterns, and physiological functions. Addressing this question requires not only cataloging the molecular diversity of vagal neurons (Kupari et al., 2019) but also systematically linking that molecular heterogeneity to anatomy and function.

Here, we have used a combination of target-specific and whole-nodose scSeq to comprehensively identify the vagal sensory neurons that innervate the abdominal viscera. We then used genetic tools to characterize their morphology, innervation pattern, and function. This revealed a diversity of molecularly and functionally distinct cell types. For example, we have identified three novel vagal cell types that form mucosal endings: *Sst*⁺ and *Calca*⁺ neurons that innervate different parts of the stomach and *Vip*⁺ neurons that innervate the intestine. These three cell types are distinct from the previously identified *Gpr65*⁺ mucosal

ending neurons that innervate the proximal intestine (Williams et al., 2016), and all four of these cell types express different combinations of receptors for nutritionally regulated hormones. We similarly show that major subsets of the IGLEs within the stomach and intestine can be defined by the molecular markers *Glp1r* and *Oxtr*, respectively. These findings provide a roadmap for the use of genetic tools to monitor and manipulate vagal cell types with high specificity, thereby allowing systematic analysis of their physiologic function.

Regulation of Feeding by IGLE Mechanoreceptors

The vagus nerve is thought to be critical for satiation, yet the causal role of specific vagal cell types in the control of feeding behavior has not been tested. To investigate this, we selected four vagal subtypes that differ in their innervation pattern (stomach versus intestine), sensory endings (IGLE versus mucosal), and hormone receptor profiles (including receptors for CCK, PYY, and 5HT). We manipulated the activity of these cells using optogenetics and chemogenetics and measured the response in a battery of behavioral and physiological assays.

We found that feeding was potently inhibited by stimulation of *Oxtr*⁺ vagal neurons, which represent IGLE mechanoreceptors innervating the intestine. Stimulation of *Glp1r*⁺ neurons, which cover the majority of gastric IGLE mechanoreceptors, also attenuated feeding, but to a lesser extent. This weaker inhibition of feeding by *Glp1r*⁺ neurons was somewhat unexpected, given that artificial distension of the stomach is well known to promote meal termination (Phillips and Powley, 1996). However, this discrepancy could reflect differences in the intensity of stimulation or the involvement of compensatory autonomic reflexes that could attenuate the effect of neural stimulation but not mechanical distension of the tissue.

In contrast to the mechanoreceptors, we observed no phenotype following stimulation of two intestine-innervating, mucosal-ending vagal subtypes predicted to be important for the regulation of food intake: *Gpr65*⁺ neurons, which are activated by intestinal nutrients (Williams et al., 2016), and *Vip*⁺ neurons, which had the highest expression of the *Cckar* in our sequencing data. This suggests that *Gpr65*⁺ and *Vip*⁺ mucosal afferents may be more involved in other aspects of GI physiology. In future studies, it will be important to further investigate these cell types in additional assays and following loss-of-function manipulations.

We have emphasized in this study the fact that *Glp1r*⁺ and *Oxtr*⁺ neurons are IGLE mechanoreceptors, but this does not exclude a role for additional, nutrient-specific mechanisms of satiation that act through either these cells or other vagal cell types. Electrophysiological recordings have shown that the vagal neurons activated by gastric and intestinal distension are also activated by CCK (Schwartz et al., 1991, 1995), and our sequencing data show that both *Glp1r*⁺ and *Oxtr*⁺ IGLE neurons express the *Cckar* (Figures 5A and S5A). We have also shown that subthreshold levels of CCK can potentiate the inhibition of AgRP neurons by GI distension (Figures S7O–S7R). These synergistic interactions between CCK and GI distension could serve as a mechanism for the integration of GI mechanical and chemical signals.

Central Circuits Underlying the Vagal Regulation of Food Intake

A number of key nodes in the brain that regulate feeding have been identified, but how these nodes encode sensory information, especially signals from the abdominal visceral, remains poorly understood. In this study, we chemogenetically stimulated the four vagal sensory subtypes above and then recorded the response of AgRP neurons *in vivo*. Stimulation of *Oxtr*⁺ intestinal mechanoreceptors rapidly and durably inhibited AgRP neurons in hungry mice, whereas activation other vagal subtypes had lesser (*Glpr1r*⁺) or no (*Gpr65*⁺, *Vip*⁺) effect. Consistently, AgRP neurons were inhibited by delivery of a volumetric (non-nutritive) load to the intestine but not to the stomach. This reveals that hypothalamic hunger circuits, which traditionally have been studied in the context of long-term nutritional signals such as leptin, also receive real-time information about the mechanical status of the gut.

The ascending pathway that transmits this information from vagal mechanoreceptors to AgRP neurons is unknown, but it likely involves NTS neurons that project to the hypothalamus either directly (D'Agostino et al., 2016; Roman et al., 2017) or via a relay in the PBN (Rinaman, 2007; Roman et al., 2016). Indeed, we found that stimulation of *Oxtr*⁺ intestinal mechanoreceptors activated cells in the NTS and AP as well as their downstream target, the PBN, including two cell types that are known to inhibit food intake: *Th*⁺ neurons in the NTS and *Calca*⁺ neurons in the PBN. These two cell types are activated by food ingestion, and NTSTh neurons directly project to and stimulate PBN^{Calca} neurons (Rinaman, 2007; Roman et al., 2016). This suggests that *Oxtr*⁺ vagal mechanoreceptors inhibit feeding, at least in part, by activating the NTSTh → PBN^{Calca} satiation pathway.

The Role of Intestinal Distension in Satiation

Prior work has focused on the role of gastric stretch in regulating feeding, in part because gastric distension can be readily manipulated using a balloon or pyloric cuff (Phillips and Powley, 1996). However, we have shown that hyperosmotic solutions can introduce intestinal distension, which may explain earlier findings showing that intraduodenal osmolytes can potently inhibit feeding (Davis and Collins, 1978; Houpt et al., 1983). Despite the lower density of intestinal IGLs compared to the stomach, the intestine receives similar total IGL innervation due to its larger surface area (Figure 1J). Perhaps the strongest prior evidence implicating intestinal IGLs in satiation comes from neurotrophin overexpression or knockout mouse models (Chi and Powley, 2007; Fox, 2006). While interpretation is complicated by potential pleiotropic effects, these mutants exhibit selective alterations in intestinal IGL innervation that correlate with changes in meal patterns in a way that is consistent with our data from optogenetic and chemogenetic manipulations.

A final important question regards when and how intestinal distension is naturally triggered. During normal feeding, the intestinal load is determined by the rate of gastric emptying, which can vary greatly depending on the properties of the food consumed (including liquid versus solid, caloric density, and osmolarity) (Janssen et al., 2011; Kaplan et al., 1992). However, one setting in which intestinal distension is exaggerated is following bariatric surgeries, such as Roux-en-Y and vertical sleeve gas-

trectomy, that reduce food intake. While these procedures are surgically diverse, they share the common property that they result in extraordinarily high rates of gastric emptying (Chambers et al., 2014). This is thought to promote satiety by over-activating intestinal nutrient sensors, thereby causing exaggerated release of gut peptides, but it has been challenging to confirm this model experimentally (Abdeen and le Roux, 2016; Woods et al., 2018). Our data suggest an alternative explanation for this phenomenon: that mechanical distension of the intestine may itself be the signal that triggers the profound reduction in hunger caused by bariatric surgery. The tools described in this paper provide a means to test this and related hypotheses.

STAR★METHODS

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ACKNOWLEDGMENTS

We thank the René Remie Surgical Skills Center for surgical training, the UCSF Genomics Core Facility and UCSF Gladstone Transgenic Gene-Targeting Core Laboratory for facility supports, John Wood for *Nav1.8^{Cre}* mice, and David Ginty and members of the Knight lab for comments on the manuscript. L.B. is supported by the Jane Coffin Childs Memorial Fund. Z.A.K. is a Howard Hughes Medical Institute Investigator and is supported by the New York Stem Cell Foundation, the American Diabetes Association, the Rita Allen Foundation, the McKnight Foundation, the Alfred P. Sloan Foundation, the Brain and Behavior Research Foundation, the Esther A. and Joseph Klingenstein Foundation, the UCSF Program for Breakthrough Biomedical Research, the UCSF Diabetes Center, and the UCSF Nutrition Obesity Research Center. This work was also supported by an NIH New Innovator Award (DP2-DK109533), R01-DK106399, and R01-NS094781.

AUTHOR CONTRIBUTIONS

L.B. and Z.A.K. conceived the project and designed the experiments. Y.L. and M.A.K. developed target-scSeq and shared the technique. L.B. and E.L.H. conducted scSeq. L.B. generated *Uts2b^{Cre}* mouse. H.Z. and L.M.

provided the *Oxtr^{Cre}* and *Npy^{Flp}* mice. L.B. performed nodose and visceral injection. L.B., S.M., and E.L.H. performed stereotaxic surgery. L.A.G., L.R.B., and J.S.A. performed intragastric surgery. L.B., K.S.R., S.M., and T.J.A. performed histology and quantification. Y.C. set up photometry system. L.B., S.M., and E.L.H. conducted behavior and photometry experiments. L.B. conducted the experiments, analyzed the data and generated the figures. L.B. and Z.A.K. prepared the manuscript with input from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: June 10, 2019

Revised: September 2, 2019

Accepted: October 23, 2019

Published: November 14, 2019

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STAR★METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|--|---------------------------------|-----------------------------------|
| Antibodies | | |
| mouse anti-cFos (1:1000, IHC) | Biosensis | Cat# M-1752-100 |
| Rabbit anti-CGRP (1:1000, IHC) | Immunostar | Cat# 24112; RRID: AB_572217 |
| Chicken anti-GFP (1:1000, IHC) | Aves | Cat# GFP 1020; RRID: AB_10000240 |
| Goat anti-mCherry (1:500, IHC) | Sicgen | Cat# Ab0040-200; RRID: AB_2333092 |
| Rabbit anti-NeuN (1:1000, IHC) | Millipore | Cat# ABN78; RRID: AB_10807945 |
| Rabbit anti-TH (1:1000, IHC) | Millipore | Cat# AB152; RRID: AB_390204 |
| Bacterial and Virus Strains | | |
| AAV9-CAG-DIO-tdTomato-WPRE-bGH | Addgene | Cat# 51503 |
| AAV9-hSyn-DIO-hM3D(Gq)-mCherry | Boston Children Viral Core | N/A |
| AAV9-EF1-DIO-hChr2(H134R)-mCherry-WPRE-hGH | Addgene Viral Core | Cat# 20297 |
| AAV9-CAG-DIO-ChR2(H134R)-mCherry-WPRE-hGH | Boston Children Viral Core | N/A |
| AAV9-CAG-DIO-ChR2(H134R)-mCherry-WPRE-hGH | Janelia Viral Core | N/A |
| AAV8-EF1a-fDIO-GCAMP6m-WPRE-SV40 | Stanford Viral Core | N/A |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Cholecystokinin octapeptide (sulfated) ammonium salt (CCK) | Bachem | Cat# 4033010.0001, Lot# 1068721 |
| Clozapine N-oxide (CNO) | Fisher Scientific | Cat# A3317-50 |
| Tamoxifen | Sigma | Cat# T5648-1g |
| Critical Commercial Assays | | |
| Nextera DNA Library Prep Kit | Illumina | Cat# FC-121-1031 |
| RNAscope Fluorescent Multiplex Kit | Advanced Cell Diagnostics (ACD) | Cat# 320850 |
| RNAscope® Probe- Mm-Glp1r-C2 | ACD | Cat# 418851-C2 |
| RNAscope® Probe- Mm-Vip-C1 | ACD | Cat# 415961 |
| RNAscope® Probe- Mm-Oxtr-C1 | ACD | Cat# 412171 |
| RNAscope® Probe- Mm-Gpr65-C2 | ACD | Cat# 431431-C2 |
| RNAscope® Probe- Mm-Dbh-C1 | ACD | Cat# 407851 |
| RNAscope® Probe- Mm-Edn3-C1 | ACD | Cat# 505841 |
| RNAscope® Probe- Mm-Uts2b-C1 | ACD | Cat# 468331 |
| Deposited Data | | |
| Target-scSeq raw and analyzed data | This paper | GEO: GSE138651 |
| Whole-nodose scSeq raw and analyzed data | This paper | GEO: GSE138651 |
| Experimental Models: Organisms/Strains | | |
| Mouse: wild-type: C57BL/6J | The Jackson Laboratory | JAX: 000664 |
| Mouse: <i>Calca</i> ^{CreER} ; <i>Calca</i> ^{tm1.1(cre)ERT2} Ptch | (Song et al., 2012) | MGI: 5460801 |
| Mouse: <i>Glp1r</i> ^{Cre} ; <i>Glp1r</i> ^{tm1.1(cre)Lbrl} /RcngJ | The Jackson Laboratory | JAX: 029283 |
| Mouse: <i>Gpr65</i> ^{Cre} ; <i>Gpr65</i> ^{tm1.1(cre)Lbrl} /RcngJ | The Jackson Laboratory | JAX: 029282 |
| Mouse: <i>Nav1.8</i> ^{Cre} ; <i>Scn10a</i> ^{tm2(cre)Jnw} | (Nassar et al., 2004) | MGI: 3053096 |
| Mouse: <i>Npy</i> ^{Flp} ; B6.Cg- <i>Npy</i> ^{tm1.1(flpo)Hze} /J | (Daigle et al., 2018) | JAX: 030211 |
| Mouse: <i>Oxtr</i> ^{Cre} ; B6.Cg- <i>Oxtr</i> ^{tm1.1(cre)Hze} /J | (Daigle et al., 2018) | JAX: 031303 |
| Mouse: <i>vGlut2</i> ^{Cre} ; <i>Slc17a6</i> ^{tm2(cre)Lowl} /J | The Jackson Laboratory | JAX: 016963 |
| Mouse: <i>Sst</i> ^{Cre} ; B6N.Cg- <i>Sst</i> ^{tm2.1(cre)Zjh} /J | The Jackson Laboratory | JAX: 018973 |

(Continued on next page)

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|------------------------|------------------|
| Mouse: <i>Uts2b</i> ^{Cre} ; <i>Uts2b</i> ^{2A-Cre} | This paper | NA |
| Mouse: <i>Vip</i> ^{Cre} ; <i>Vip</i> ^{tm1(cre)Zjh} /J | The Jackson Laboratory | JAX: 010908 |
| Oligonucleotides | | |
| <i>Uts2b</i> sgRNA sequence: TGTTTCAAGCTCTAAGAACTG | This paper | N/A |
| Recombinant DNA | | |
| pAAV-hSyn-DIO-hM3D(Gq)-mCherry | Addgene | Cat. 44361 |
| pAAV-CAG-DIO-ChR2(H134R)-mCherry | Addgene | Cat. 18916 |
| pAAV8-EF1a-fDIO-GCAMP6m-WPRE-SV40 | This paper | N/A |
| Software and Algorithms | | |
| ArrayStar Version 11.0 | DNASTAR Inc. | N/A |
| ImageJ | NIH | RRID: SCR_003070 |
| MATLAB 2016b | MathWorks | RRID: SCR_001622 |
| PRISM 7.01 | GraphPad | RRID: SCR_002798 |
| RStudio version 3.4.3 | RStudio | RRID:SCR_000432 |
| Seurat 2.0 and 3.0 | Satija Lab | RRID:SCR_007322 |

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for reagents and resources should be directed to the Lead Contact, Zachary A. Knight (zachary.knight@ucsf.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals were maintained in temperature- and humidity-controlled facilities with 12 h light-dark cycle and *ad libitum* access to water and standard chow (PicoLab 5053). All transgenic mice used in these studies were on the C57BL/6J background, except *Uts2b*^{Cre} mice that were maintained on a mixed FVB/C57BL/6J background. Mice were at least six weeks old at the time of surgery. All studies employed a mixture of male and female mice and no differences between sexes were observed. All experimental protocols were approved by the University of California, San Francisco IACUC following the National Institutes of Health guidelines for the Care and Use of Laboratory Animals.

METHOD DETAILS**Mouse Strains**

The *Uts2b*^{Cre} allele was generated by homologous recombination at the endogenous *Uts2b* locus, aided by targeted CRISPR endonuclease activity. Briefly, a sgRNA (TGTTTCAAGCTCTAAGAACTG) was selected to introduce CRISPR double strand breaks within the 3' UTR. The targeting vector contained a T2A-Cre cassette inserted immediately upstream of the endogenous stop codon, a 1kb upstream homology arm, and a 2kb downstream homology arm with a TG to GT mutation introduced into the 3' UTR (114-115bp after stop codon) to avoid cleavage by the sgRNA. Super-ovulated female FVB/N mice were mated to FVB/N stud males, and fertilized zygotes were collected from oviducts. Cas9 protein (100 ng/μL), sgRNA (250 ng/μL), and targeting vector DNA (100 ng/mL) were mixed and injected into the pronucleus of fertilized zygotes. 125 injected zygotes were implanted into oviducts of pseudopregnant CD1 female mice. 4 out of 16 pups contained the Cre cassette, with one of these also containing the targeting vector inserted randomly as a transgene. The other three independent founder lines were crossed to reporter mice, and reporter expression patterns from these lines were identical and similar to the ISH result from Allen brain atlas. All *Uts2b*^{2A-Cre} mice used here were maintained on mixed FVB/C57BL/6J background. Founder pups and offspring were genotyped for the presence of the knock-in allele by qPCR.

Wild-type mice were obtained from Jackson Laboratories (Bar Harbor, ME). Genotypes and sources of transgenic animals used are listed in the Key Resources Table.

Target-ScSeq Methods**Nodose ganglion dissociation**

Target-ScSeq was developed by Y.L. and M.A.K. and will be described in more detail elsewhere. Briefly, thirty-four 8-12 week old C57BL/6JN mice (18 males and 16 virgin females) were used for the target-scSeq preparation. 7-14 days following WGA555 visceral

organ injection, mice were anesthetized under isoflurane and then transcardially perfused with HBSS containing 10 U/mL Heparin. Nodose ganglia were rapidly dissected out within ice-cold HBSS, and chopped into 2-3 pieces, and digested in papain solution (Worthington, LS003119) 10 min at 37°C, followed by collagenase/dispase solution (Worthington, LS004188 and LS02109) for 30 min at 37°C. Tissue was centrifuged at 400 g for 4 min, resuspended in L15 medium (GIBCO, 11415), then triturated with a P1000 pipette. The dissociated cell suspension was loaded on top of a Percoll/L15 solution (1:4) (Sigma, P1644-25ML) and centrifuged for 9 min at 400 g. The cell pellet was washed with L15 medium, resuspended in L15 medium, and then kept on ice until use.

Lysis buffer preparation

A similar protocol has been described before (Tabula Muris Consortium et al., 2018). Briefly, a 4 μ L lysis buffer was aliquoted into PCR tubes and included the following reagents: nuclease free water (Thermo Fisher, AM9937), 1 Unit RiboLock RNase Inhibitor (Thermo Fisher, EO0381), 0.05% Triton X-100 (Sigma, 93443-100ML), 2.5 mM dNTP (Thermo Fisher, R0193), 2.5 μ M dT (Integrated DNA Technologies, 5'-AAGCAGTGGTATCAACGCAGAGTACT30VN-3'). The lysis buffer was freshly prepared and kept on ice before cell picking.

Cell picking

The cell suspension was placed on microscope slides (Fisher Scientific, 12-550-15) and examined under the epifluorescence microscope (Nikon, Eclipse Ts2). WGA-555 labeled cells were then individually collected by glass micropipettes (Sutter Instrument, B100-58-10) and transferred to a 2- μ L drop of L15 medium to wash and confirm the presence of a single cell. Each cell was then collected again by a glass micropipette and transferred to a separate PCR tube containing 4 μ L lysis buffer. The cell picking was done within 1.5 h after the preparation of cell suspension. The single-cell lysates were kept on ice until the end of cell picking, then frozen at -80°C until processed for cDNA synthesis and library preparation.

cDNA synthesis

cDNA synthesis was performed using the Smart-seq2 protocol. Briefly, PCR tubes containing single-cell lysates were thawed on ice and followed by first-strand synthesis. Primer annealing was carried out by incubating lysates on thermal-cycler at 72°C for 3 min, and hold at 4°C. Immediately after, reverse transcription reaction mix (6 μ L) was added to each well. Each 6 μ L of reaction mix contained 1 units SMARTScribe Reverse Transcriptase (Takara, 639538), 1.67 U/ μ L Recombinant RNase Inhibitor (Takara, 2313B), 1.67X First-Strand Buffer (Takara, 639538), 1.67 μ M TSO (Exiqon, 5'-AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG-3'), 8.33 mM dithiothreitol (Thermo Fisher, P2325), 1.67 M Betaine (Sigma, B0300-5VL), and 10 mM MgCl_2 (Thermo Fisher, AM9530G). Reverse transcription was carried out by incubating wells on a thermal-cycler at 42°C for 90 min, stopped by heating at 70°C for 5 min, and held at 4°C.

Subsequently, 15 μ L of DNA amplification mix was added to each well. Each amplification mix contained 1.67X KAPA HiFi HotStart ReadyMix (Kapa Biosystems, KK2602), 0.17 μ M IS PCR primer (IDT, 5'-AAGCAGTGGTATCAACGCAGAGT-3'), and 0.038 U/ μ L Lambda Exonuclease (NEB, M0262L). Second-strand synthesis was performed on a thermal-cycler by using the following program: 1) 37°C for 30 min, 2) 95°C for 3 min, 3) 17 cycles of 98°C for 20 s, 67°C for 15 s and 72°C for 4 min, 4) 72°C for 5 min, and 5) held at 4°C.

The DNA amplicon was cleaned up using Ampure XP beads (Beckman-Coulter, A63880). 18 μ L Ampure XP beads was added to each amplicon (0.7:1 ratio), mixed by pipetting, incubated at RT for 5 min, and placed on magnetic stand for 3 min. The supernatant was carefully removed. Beads were washed twice with 80 μ L of 80% EtOH (freshly prepared), then air-dried for 5 min. Beads with amplicon were then removed from magnetic stand, added with 18 μ L TE buffer (Thermo Fisher, AM9849), mixed by pipetting, incubated at RT for 5 min, and placed on magnetic stand for 3 min. The supernatant was carefully moved to the elution plate, and frozen at -20°C until use.

The concentration and quality of cDNA was examined by running 1 μ L on a High Sensitivity DNA chip (Agilent, 5067-4627), using Agilent Technology 2100 Bioanalyzer. cDNA with a concentration lower than 50 pg/ μ L or abnormal peak distribution was not used. 449 out of 501 samples passed this criterion and were used for sequencing library synthesis.

Sequencing library synthesis

The cDNA was diluted in TE buffer to a final concentration of 200 pg/ μ L. cDNA samples with a concentration between 50 pg/ μ L to 200 pg/ μ L were directly used without any dilution. Sequencing libraries were prepared using Nextera DNA Library Prep Kit (Illumina, FC-121-1031). Briefly, 1.25 μ L (diluted) cDNA was mixed with 2.5 μ L tagmentation DNA buffer and 1.25 μ L Amplification Tagment, Tagmentation reaction was carried out at 55°C for 10 min and then held at 10°C. The reaction was stopped by adding 1.25 μ L Neutralize Tagment Buffer and centrifuging at room temperature at 2,250 g for 5 min. Indexing PCR reactions were performed by adding 3.75 μ L NPM buffer, 1.25 μ L of 5 μ M i5 indexing primer, and 1.25 μ L of 5 μ M i7 indexing primer. PCR amplification was carried out on a thermal cycler using the following program: 1) 72°C for 3 min, 2) 95°C for 30 s, 3) 12 cycles of 95°C for 10 s, 55°C for 30 s and 72°C for 60 s, 4) 72°C for 5 min, and 5) held at 10°C.

Library pooling, quality control and sequencing

After library preparation, wells of each library plate were pooled and purified twice using 0.8x AMPure XP beads (Beckman-Coulter, A63880). Library quality was assessed using a High Sensitivity DNA chip (Agilent, 5067-4627) on an Agilent Technology 2100 Bioanalyzer. Libraries were sequence on the HighSeq 4000 Sequencing System (Illumina) using 2 \times 150 bp paired-end reads. Raw and analyzed data deposited at Gene Expression Omnibus (GEO) database (accession number GSE138651).

Whole-nodose ScSeq Methods

Generation of nodose ganglion neuron suspension

Seven 6-week-old C57BL/6JN mice (4 males and 3 virgin females) were used to collect nodose ganglion neurons, following the same dissociation protocol described above (for target-scSeq). All nodose ganglia were collected within an hour and were pooled into two eppendorf tubes for digestion. At the end, cells were resuspended in L15 medium and kept on ice prior to sequencing. 10 μ L of cell suspension was loaded on a cell counter to estimate the density. Before sequencing, cells were centrifuged at 400 g for 5 min and resuspended in PBS with 0.1% BSA to reach a density of 1000 cell/ μ L.

Droplet-based scSeq

Single cells were processed through the GemCode Single Cell Platform using the GemCode Gel Bead, Chip and Library Kits (10X Genomics, Pleasanton) as per the manufacturer's protocol. Briefly, a nodose neuron suspension (1000 cell/ μ L in PBS with 0.1% BSA) was loaded. The cells were then partitioned into Gel Beads in Emulsion in the GemCode instrument where cell lysis and bar-coded reverse transcription of RNA occurred, followed by amplification, shearing, and 5' adaptor and sample index attachment. Libraries were sequenced on the Illumina Highseq 4000. Raw and analyzed data deposited at Gene Expression Omnibus (GEO) database (accession number GSE138651).

Analysis of Target-scSeq data

Reads alignment

Sequences from the HighSeq 4000 were de-multiplexed to generate a fastq file. Reads were aligned to annotated mRNAs in the mouse genome (UCSC, *Mus musculus* assembly mm10) and the read count for each gene was calculated using ArrayStar software (DNAstar). Sequence of 449 picked cells were collected and used for further analysis.

Cluster analysis and identification of injured cells

The Seurat (v2) package was used to perform the clustering analysis of the target-scSeq dataset as described (Satija et al., 2015). Gene expression was normalized for each cell by the total expression (global-scaling method), multiplied by a scale factor (10,000) to create TPM-like values (TPM: transcripts per million), and finally computed as $\ln(\text{TPM}+1)$ to be used for all following data analysis. 5633 variable genes (mean 0.1-10, dispersion > 0.5) were used to compute principle components (PCs). The first 40 PCs were significant based on the jackstraw analysis and were used further for graph-based clustering and tSNE visualization. Cluster specific marker genes were identified by comparing the mean gene expression within the cluster to the median gene expression in all other clusters.

Of note, cells in cluster 1 highly expressed injury induced genes (Figures S2D and S2E). This was likely due to peripheral axonal injury in the tracer-injection surgeries. Consistent with this, most of the injured cells were collected from the stomach or portal vein tracing. Those tracings required injection or manipulation near vagal nerve bundles and were likely to introduce axonal injury.

Cluster analysis without injured cells

To analyze neurons without injury (Figures 2 and S2F–S2L), we removed 54 putative injured cells using cutoffs *sprr1a* < 10 UMI and *Ecel1* < 10 UMI. Gene expression of the remaining 395 cells was normalized using the method described above and used for further clustering analysis. 3912 variable genes (mean 0.1-10, dispersion > 0.5) were used to compute principle components (PCs). The first 36 PCs were significant based on the jackstraw analysis and were used for graph-based clustering and tSNE visualization (resolution = 3). 395 cells were assigned into 12 cell type clusters.

Average expression of the variable genes for each cluster was determined and used as an input for constructing phylogenetic tree (dendrogram). Cluster specific marker genes were identified by comparing the average gene expression of one cluster to the median gene expression of all other clusters. Pearson's correlation between cells or clusters was calculated using average expression of the variable genes.

Analysis of whole-nodose scSeq data

Reads alignment

De-multiplexing alignment to the mm10 transcriptome and unique molecular identifier (UMI)-collapsing were performed using Cell Ranger version 2.0.1, available from 10x Genomics with default parameters. A gene-barcode matrix was generated. 1108 cells were captured with 0.3 million mean reads per cell and 7.7 thousand median genes per cell.

Cluster analysis and identification of glial/endothelial cells

The Seurat (v2) package was used to perform the clustering analysis of whole-nodose scSeq dataset as described (Satija et al., 2015). Gene expression of a total of 1108 cells was normalized using the method described above. $\ln(\text{TPM}+1)$ was calculated and used for the following analysis. 2438 variable genes were used to compute the principle components (PCs). The first 20 PCs were used for graph-based clustering and tSNE visualization (resolution = 0.5). Cluster specific marker genes were identified by comparing the mean gene expression within the cluster to the median gene expression in all other clusters (min.pct = 0.25, thresh.use = 0.25).

Cluster 1 (41 cells) highly expressed marker genes for either satellite glial cells (*ApoE*, *Fabp7*, *Dbi*, and *Plp1*) or endothelial cells (*Emcn*, *Ecscr*, *Cdh5*, and *Igfbp7*) and did not express neuronal marker genes (*Nefl*, *Nefm*, *Snap25*, and *Tubb3*), similar to nodose and jugular ganglion scSeq results described previously (Kupari et al., 2019). All other cells (1067 cells within cluster 2-11) contained neurons which highly expressed *Nefl*, *Nefm*, *Snap25*, and *Tubb3*. Of note, subsets of neuronal cells also weakly expressed the glial cell marker genes (enriched within cluster 3, 4, 6, 9). This is likely derived from samples containing neurons that are tightly attached to

satellite glial cells. Marker genes for cluster 1 (1204 genes) were used to define genes that are enriched in non-neuronal cells and will be used for later analysis.

Analysis of combined scSeq datasets

The Seurat (v3) package was used to perform the data integration and clustering analysis of the combined scSeq datasets as described (Stuart et al., 2019). The target-scSeq dataset and whole-nodose scSeq dataset were filtered and normalized separately, then integrated for clustering analysis (Figure S3). Briefly, 54 putative injured cells were removed from target-scSeq (*sprr1a* < 10 UMI and *Ece1* < 10 UMI), leaving 395 uninjured target-scSeq cells for further analysis. 115 low quality cells (nGene < 6000, percentage of mitochondrial genes > 18%) and 63 non-neuronal cells (endothelial marker *Ecscr* > 0.5, satellite glial cell marker *ApoE* > 400) were removed from the whole-nodose scSeq dataset, leaving 956 cells for further analysis.

Of note, samples that contained both neuron and satellite glial cells were included for analysis, since neurons are much larger than satellite cells and contain more reads per cell (Kupari et al., 2019). However, to avoid the contaminated glial cell from biasing the cluster analysis and cell type identification, we removed non-neuronal cell marker genes (1204 genes enriched in the satellite glial cells and endothelial cells, described above) from the variable gene list for clustering analysis. Briefly, 4000 variable genes were identified in each dataset, using the default method of Seurat v3. After removing the non-neuronal marker genes, 3591 and 3520 variable genes were used for the target-scSeq and whole-nodose scSeq dataset, respectively.

Lastly, the filtered target-scSeq and whole-nodose scSeq dataset were integrated (anchor.features = 2000, dims = 1:30). Graph-based clustering was performed using the first 40 PCs and a resolution = 3.5. Cluster specific marker genes were identified by comparing the mean gene expression within the cluster to the median gene expression in all other clusters.

Surgeries

Stereotaxic injection and implantation

Animals were anesthetized with 2% isoflurane and placed in a stereotaxic head frame on a heating pad. Ophthalmic ointment was applied to the eyes and subcutaneous injections of meloxicam (5 mg/kg) and sustained-released buprenorphine (1.5 mg/kg) were given to each mouse prior to surgery. The scalp was shaved, scrubbed (betadine and alcohol three times), local anesthetic applied (Bupivacaine 0.25%), and then incised through the midline. A craniotomy was made using a dental drill (0.5 mm). Virus was injected at a rate of 50 nL/min using glass pipette connected with 10 uL Hamilton syringe (WPI, Sarasota, FL), controlled by a micro-injector (Drummond, Nanoject II injector). The needle was kept at the injection site for 3 min before withdrawal. Fiberoptic cannulas were implanted after virus injection in the same surgery, and were secured to the skull using adhesive dental cement (a-m systems 525000 and 526000). At the end of surgery, the skin incision was closed using 5-0 nylon sutures (Henry Schein, 101-7137).

For AgRP neuron photometry (Figures 6, 7, S6, and S7), *NPY^{Flp}* mice were injected with 200 nL of AAV8-fDIO-G6m virus in the Arc (−1.8 mm AP; −0.3 mm ML; −6.0 mm DV relative to the bregma). Photometry cannula (Doric Lenses, MFC_400/430-0.48_6.1mm_MF2.5_FLT) with sleeve (Doric Lenses, SLEEVE_BR_2.5) were implanted unilaterally in the Arc 0.1mm above the virus injection site (−1.8 mm AP; −0.3 mm ML; −5.9 mm DV relative to the bregma). Mice are allowed to recover for a minimum of three weeks before the first photometry experiment.

For vagal afferent optogenetic experiments (Figures 5 and S5), custom-made fiberoptic implants (Thorlabs; 0.39 NA Ø200 mm core FT200UMT and CFLC230-10) were placed unilaterally above the NTS (+ or −0.2mm AP; +1.2mm ML; −4.0mm DV relative to the occipital crest with 20° in the AP direction). Mice are allowed to recover for a minimum of two weeks before optogenetic experiments.

Nodose ganglion injection

Animals were anesthetized with ketamine/xylazine (100mg/kg ketamine + 10mg/kg xylazine, IP injection). Ophthalmic ointment was applied to the eyes and subcutaneous injections of meloxicam (5 mg/kg) and sustained-released buprenorphine (1.5 mg/kg) were given to each mouse prior to surgery. The skin under neck was shaved and scrubbed (betadine and alcohol three times). A midline incision (~1.5 cm) was made and nodose ganglia were exposed. 200 nL virus containing 0.02 mg/mL Fast Green (Sigma, F7252-5G) was injected at a rate of 100 nL/min using glass pipette. At the end of surgery, the skin incision was closed using 5-0 nylon sutures (Henry Schein, 101-7137).

For histology analysis (Figures 1 and 4), mice were injected with AAV9-DIO-tdTomato and recovered for a minimum of four weeks before perfusion. For chemogenetic experiments (Figures 5 and 6), mice were injected with AAV9-DIO-hM3D-mCherry and were allowed to recover for a minimum of two weeks before behavior tests. For optogenetic experiments (Figure 5), mice were injected with AAV9-DIO-ChR2-mCherry and were allowed to recover for one to two weeks before the stereotaxic optic-implant surgery.

Retrograde tracing from visceral organs

Animals were anesthetized with ketamine/xylazine (100 mg/kg ketamine + 10 mg/kg xylazine, IP injection). Ophthalmic ointment was applied to the eyes and subcutaneous injections of meloxicam (5 mg/kg) and sustained-released buprenorphine (1.5 mg/kg) were given to each mouse prior to surgery. The abdominal skin was shaved and scrubbed (betadine and alcohol three times). A midline abdominal incision (2 cm) was made along the linea alba running approximately 1 cm caudal from the xiphisternum. Blunt glass probe was used to position the internal organ. A sharp glass needle was used to inject retrograde tracer (Thermo Fisher, WGA555 or WGA647, 5 mg/mL), which was controlled by air pressure applied from a 5 mL syringe. For gastric or intestinal injections, 3 uL tracer was injected at multiple sites into the layer between muscularis externa and serosa layer. For the portal vein injection, 0.5-1 uL tracer was injected to the connective tissue wrapping the portal vein. After injection, the skin incision was closed using 5-0 nylon sutures.

2-14 days following surgery, animals were euthanized and labeled tissue was harvested for histology or sequencing experiments.

Intragastric or intrainestinal catheter implantation

Mice were anesthetized deeply with ketamine/xylazine and surgical sites were shaved and cleaned with betadine and ethanol. Subcutaneous injections of meloxicam (5 mg/kg) and sustained-released buprenorphine (1.5 mg/kg) were given to each mouse prior to surgery. A midline abdominal skin incision was made, extending from the xyphoid process about 1.5 cm caudally, and a secondary incision of 1 cm was made between the scapulae for externalization of the catheter. The skin was separated from the subcutaneous tissue using blunt dissection, such that a subcutaneous tunnel was formed between the two incisions along the left flank to facilitate catheter placement. A small incision was made in the abdominal wall and the catheter (Instech, C30PU-RGA1439) was pulled through the intrascapular skin incision and into the abdominal cavity using a pair of curved hemostats. The stomach was externalized using atraumatic forceps and a purse string stitch was made in the middle of the forestomach using 7-0 non-absorbable Ethilon suture. A puncture was then made in the center of the purse string and the end of the catheter was inserted and secured by the purse string suture. For gastric implant, 2-5 mm of catheter end was fixed within the stomach. While for the intestinal catheter implant, the end of catheter was gently advanced and positioned distal to the pyloric sphincter, and about 2.5 cm of catheter end was fixed within the gastrointestinal tract.

At the end of surgery, the abdominal cavity was irrigated with ~1 mL of sterile saline and the stomach was replaced. The abdominal incision was closed in two layers, and the catheter was sutured to the muscle layer at the interscapular site. The interscapular incision was then closed and the external portion of the catheter capped using a 22-gauge PinPort (Instech, PNP3F22). Mice received baytril (5 mg/kg) and warm saline at the end of surgery, and were allowed to recover for 7-10 days prior to behavioral experiments.

Post-surgical care

Post-surgery mice were placed over a heating pad and monitored for their recovery from anesthesia. The health of the mice is monitored daily post-surgery. All mice were given post-surgery analgesia by a subcutaneous injection of meloxicam (5 mg/kg) on post-surgery day 2 and day 3.

Treatments

Intragastric or intrainestinal infusions

All animals were fasted overnight before infusion. All solutions were infused via intragastric catheters using a syringe pump (Harvard Apparatus, 70-2001). Each infusion was delivered at a rate of 100 μ L/min with a total volume of 1 mL. Infusion solutions were prepared accordingly using deionized water: 0.240 g/mL glucose (1.33 M), 0.256 g/mL mannitol (1.33 M), 0.039 g/mL hypertonic salt (0.66 M), 0.009 g/mL saline (0.15 M, 0.9%), and 0.45 g/mL collagen peptide (Sports Research). 20% intralipid (Sigma, I141-100ML) was used without dilution. All solutions were prepared freshly at the beginning of the day.

Oral Gavage

Oral gavage experiments were performed using a 24-gauge reusable feeding tube (FST, 18061-24). All animals were fasted overnight before oral gavage. The tube was introduced through the esophagus to the stomach and 500 μ L of various solution was manually infused within 20 to 30 s. Solutions for oral gavage were prepared the same as those for intragastric infusions, except 1% methylcellulose (Sigma, M0512-100G) prepared using deionized water.

Tamoxifen treatment

Tamoxifen (Sigma, T5648-1g) was dissolved in ethanol (20 mg/mL), mixed with equal volume of sun flower seed oil (Sigma), vortexed for 5-10 min, and centrifuged under vacuum for 20-30 min to remove the ethanol. The solution was kept at -20°C and delivered via intraperitoneal injection treatment. Two weeks after AAV nodose injection, the *Calca*^{CreER} mice received 4 mg tamoxifen each day for five days and were euthanized for histology at least four weeks after tamoxifen treatment.

FluoroGold treatment

FluoroGold (Fluorochrome) was injected 20 mg/kg intraperitoneally to either label enteric neurons (Figures 1C and 4F), or to retrogradely label vagal efferent neurons in the DMV (Figure S1C). 3-7 days following surgery, animals were euthanized and labeled tissue was harvested for histology.

Optogenetic stimulation

Construction of the photostimulation system has been previously described (Chen et al., 2016). For RTPP experiments, the laser modulated at 10 Hz with a 10 ms pulse width. For other optogenetic experiments (feeding, drinking, and blood pressure tests), the laser was modulated at 20 Hz for a 2 s ON and 3 s OFF cycle with a 10 ms pulse width. Laser power was set to 15-18 mW, measured at the tip of each patch cable before each day's experiments. To prepare mice for optogenetic experiments, AAV9-DIO-ChR2-mCherry was bilaterally injected into the nodose ganglion of the indicated *Cre* line (experimental group) or *Cre*⁻ litter mates (control group), and an opto-fiber was implanted over the NTS to activate the central terminals of *ChR2*⁺ vagal sensory neurons.

Chemogenetic stimulation

Clozapine N-oxide (CNO) (Fisher Scientific, A3317-50) stock solution was prepared in DMSO as 40 mg/mL. Before experiments, CNO was diluted 1:100 in saline (0.4 mg/mL with 1% DMSO). Mice received an intraperitoneal injection (50 μ L for 20 g animal) of either CNO (1 mg/kg) or the control vehicle (saline with 1% DMSO). To prepare mice for chemogenetic experiments, AAV9-DIO-hM3D-mCherry was bilaterally injected into the nodose ganglion of the indicated *Cre* line (experimental group) or *Cre*⁻ litter mates (control group)

Behavior Tests

Fiber photometry recording

Mice were tethered to a patch cable (Doric Lenses, MFP_400/460/900-0.48_2m_FCM-MF2.5). Continuous 6 mW blue LED (470 nm) and UV LED (405 nm) served as excitation light sources, driven by a multichannel hub (Thorlabs), modulated at 211 Hz and 511 Hz respectively, and delivered to a filtered minicube (Doric Lenses, FMC6_AE(400-410)_E1(450-490)_F1(500-540)_E2(550-580)_F2(600-680)_S) before connecting through optic fibers (Doric Lenses, MFP_400/460/900-0.48_2m_FCM-MF2.5). GCaMP calcium GFP signals and UV isosbestic signals were collected through the same fibers back to the dichroic ports of the minicube into a femto-watt silicon photoreceiver (Newport, 2151). Digital signals sampled at 1.0173 kHz were then demodulated, lock-in amplified, and collected through a processor (RZ5P, Tucker-Davis Technologies). Data was then collected through the software Synapse (TDT), exported via Browser (TDT), and analyzed in MATLAB.

All photometry recordings were collected at least 6 weeks after surgery to allow stable expression GCaMP6m. All experiments were performed during the light cycle to control for circadian factors. All mice were fasted overnight (16-23 h) prior to tests. Before each recording, photometry implants were cleaned with 70% ethanol using connector cleaning sticks (MCC-S25), and connected to photometry patch cable immediately afterward. For intragastric or intrainestinal infusion experiments, the catheters of the mice were connected to the infusion tubes. All of these connections were done before experiments and started without anesthetization. Experiments were performed in behavioral chambers (Coulbourn Instruments, Habitest Modular System) without water or food access unless otherwise specified. For all experiments mice were acclimated to the behavior chamber for 20 min with recording. Photometry data collected in the later 10 min were used to calculate the baseline activity.

For the chemogenetic experiments (Figures 6K–6O and S6E–S6H), mice were given an intraperitoneal injection of CNO (1 mg/kg) or saline after acclimation, and presented with chow (PicoLab 5058) at 30 min after the injection. Photometry recording was continued until 10 min after chow presentation (40 min after injection). Chow intake within the 10 min was measured.

For the oral gavage experiments (Figures 7D, 7E, S7H, and S7I), mice received an oral gavage (500 μ L, lasting 20-30 s) of various contents, or as a control for dehydration, subcutaneous injection of hypertonic NaCl solution (0.66 M), and were recorded for 30 min after those treatments.

For the intragastric or intrainestinal infusion experiments (Figures 7F, 7G, and S7J–S7N), all mice received 10 min of infusion (1 mL total, 100 μ L/min), and were presented with chow (PicoLab 5058) at 30 min after the infusion started. Photometry recording was continued until 10 min after chow presentation, and chow intake within the 10 min was measured.

For experiments testing synergistic effects of CCK and gastrointestinal stretch (Figures S7O and S7P), mice were given intraperitoneal injection of CCK (2 μ g/kg), oral gavage of 500 μ L Mannitol (1.33M), or both treatments (injection immediately followed by oral gavage). Recording were only collected for 15 min due to the short half-life of CCK.

Feeding and drinking behavior

All experiments were performed in behavioral chambers (Coulbourn, Habitest Modular System). Feeding experiments were performed using a pellet dispensing system (Coulbourn, H14-01M-SP04 and H14-23M) with free water access. Food pellets (20 mg Bio-Serv F0163) were dispensed at the beginning of trials, or after pellet removal with a 10 s interval. After each experiment, pellet consumption was measured by deducting the quantity of pellets left on the ground from the total food count. Water consumption was monitored with contact lickometers. Mice were habituated for one night to the chambers, water bottle, food pellets, and pellet dispensing systems before experiments. Prior to the test, mice were fasted (for fast-refeeding test) or water-deprived (for drinking test) overnight (15-19 h), except Figure 5N in which mice were *ad libitum* fed (to examine whether activation of vagal afferents can increase food intake). All feeding and drinking tests were performed during the light cycle.

To measure food intake under photostimulation (Figures 5F–5H), mice were habituated in chambers for 20 min before given 60 min of food access. Photostimulation started 5 min before food access and lasted for 35 min (30 min stimulation during food access).

To measure water intake under photostimulation (Figures 5I and S5E), mice were habituated in chambers for 5 min before given water access. Photostimulation started 4 min before water access and lasted for 34 min (30 min stimulation during water access).

To measure food or water intake under chemogenetic activation (Figures 5L–5O, 5S, and S5F), mice were habituated in chambers for 5 min, injected with CNO or saline intraperitoneally, and given food or water access 0 min (Figures 5L–5N), 5 min (Figures 5O and S5F) or 30 min (Figures 5S and 5T) after injection.

To measure food intake after oral gavage (Figures 7B, 7C, S7F, and S7G), mice were habituated in chambers for 5 min, received oral gavage of 500 μ L solution, and given food access immediately after oral gavage.

To measure synergistic effects of CCK and gastrointestinal stretch (Figures S7Q and S7R), mice were given an intraperitoneal injection of CCK (2 μ g/kg), oral gavage of 500 μ L Mannitol (1.33 M), or both treatments (injection immediately followed by oral gavage). Food was delivered immediately after treatment.

Real-Time Place Preference (RTPP)

A custom two-chamber apparatus (30 cm x 15 cm) was used with each side differing in floor texture and wall markings. Mice were allowed to move freely between each side throughout the experiment. A custom MATLAB script was used to track the mice and pair stimulation to a specified chamber. Mice were first tested under a fed condition with no photostimulation to assess for a baseline chamber preference. Preferences were calculated as the perfect time spent in the chamber paired with photostimulation. Mice were next tested under a fed or fasted condition. Under the fed condition mice were tested on consecutive days while fasted mice were tested every other day, for a total of 3 trials per condition.

Blood pressure measurement

Mice were sedated with medetomidine (50 ug/kg, ApexBio) and then restrained and placed on a warming platform. Blood pressure was measured using the CODA-HT4 Noninvasive Blood Pressure System (Kent Scientific). Measurements were taken once per minute for a total of 10 measurements per cycle. Mice were first habituated to apparatus in the first cycle, data were then collected from the following three cycles that consisted of a pre-stim and stim period. All animals were tested twice with each test separated by at least three days. Each measurement from the two test cycles was normalized to the average blood pressure from the pre-stim period and then averaged between the two tests.

Temperature measurement

Core temperature was measured using a thermocouple rectal probe and thermometer (Braintree Scientific, MA). The brown adipose tissue (BAT) temperature was measured using an implanted transponder. One week prior to the start of the experiment, mice were anesthetized under isoflurane and a transponder (IPTT-300, Biomedic Data Systems, DE) was implanted subcutaneously at the midline of the intrascapular region. Temperature was then measured by a non-contact DAS-7007 reader.

Gallbladder emptying

All mice were fasted overnight (20 h). Prior to perfusion, mice were weighed and given a CNO injection intraperitoneally in their home cage. An h later, mice were anesthetized under isoflurane and transcardially perfused with 10 mL PBS followed by 15ml 10% formalin. Immediately after perfusion, the bile ducts were carefully ligated using 6-0 silk suture. Gallbladders were dissected out and kept on ice within eppendorf tubes, and measured weight within an h. The gallbladder weight was normalized to their body weight and presented as mg/kg (Figures S5B and S5C).

Measuring gastrointestinal content

To test GI contents after natural ingestion (Figures S7C and S7D), mice were fasted (for glucose ingestion) or water-deprived (for water ingestion) for 20 h overnight and measured for body weight. Mice were given access to 1.33 M glucose or water, monitored using a lickometer, and euthanized 10 min after the first lick.

For other GI content measurements (Figures 7A and S7A–S7E), mice were fasted for 20 h overnight, measured for body weight, received various treatments, and then euthanized 5 min after the treatment. Various solutions were delivered to the stomach within 20–30 s through oral gavage, including 1% methyl cellulose, 0.15 M NaCl (saline), 20% intralipid, 1.33 M glucose, 1.33 M mannitol, or 0.66 M NaCl. For the sham control, mice were fed with oral gavage needle and held for 30 s. For the dehydration control, mice were injected with 500 μ L of 0.66 M NaCl subcutaneously.

To measure the GI contents, mice were anesthetized under isoflurane and transcardially perfused with 5 mL PBS followed by 10ml 10% formalin. The pyloric sphincter was ligated using 6-0 silk suture (Henry Schein, 101-2636) during the perfusion. After perfusion, the gastrointestinal tract was gently dissected out, and the intestine was ligated at about 5cm, 10cm and 20cm distal to the pyloric sphincter. The whole procedure was performed quickly within 10min. The weight of GI contents within each segment was measured by the total weight subtracted by the tissue weight after removing the inner content. The stomach content was normalized to the body weight and presented as weight per 20 g animal (Figure 7A). The intestinal contents were normalized to body weight and length of GI tract, and presented as weight per 20 g animal, per 10 cm intestine (Figure 7A).

Histology

Perfusion and tissue preparation

Mice were anesthetized under isoflurane and then transcardially perfused with 10ml PBS followed by 15 mL formalin (10%). Brains, ventral aspect of skulls, and visceral organs were dissected, post-fixed in 10% formalin overnight at 4°C, and washed 3x20 min with PBS at RT. Nodose ganglion were further dissected out. All tissues were kept in PBS at 4°C before imaging, sectioning, or staining.

Immunostaining of sections

Tissues were cryoprotected with 30% sucrose in PBS overnight at 4°C, embedded in OCT, frozen and stored at –20°C. Sections (20 μ m for nodose ganglion or vagal nerve, or 50 μ m for the brain) were prepared with a cryostat and collected in PBS or on Superfrost Plus slides. Sections were washed 3 \times 10 min with 0.1% PBST (0.1% Triton X-100 in PBS), blocked (5% NGS or NDS in 0.1% PBST) for 30 min at RT, and incubated with primary antibodies (1:1000 diluted in blocking solution) overnight at 4°C. The next day, sections were washed 3x10 min with 0.1% PBST, incubated with secondary antibodies (1:500 diluted in blocking solution) for 2 h at RT, washed again 3 \times 10 min with 0.1% PBST, and mounted using fluoromount-G with or without DAPI (Southern Biotech).

Primary antibodies used were: mouse anti-cFos (Biosensis, M-1752-100), chicken anti-GFP (Abcam, ab13970, 1:1000), goat anti-mCherry (ACRIS, AB0040-200), rabbit anti-CGRP (Immunostar, 24112), rabbit anti-TH (Millipore, AB152).

Immunostaining of whole-mount preparation

Whole-mount staining was performed to visualize the gastrointestinal innervation by tdTomato⁺ vagal sensory neurons (Figures 1C–1G and 4A–4F). Tissues were washed 3 \times 30 min with 0.1% PBST (0.1% Triton X-100 in PBS), blocked (5% NDS in 0.1% PBST) for 2 h at RT, and incubated with primary antibodies (goat anti-mCherry, 1:1000 diluted in blocking solution) overnight at 4°C. The next day, tissues were washed 3x30 min with 0.1% PBST, incubated with secondary antibodies (1:500 diluted in blocking solution) overnight at RT 4°C, washed again 3 \times 30 min with 0.1% PBST, then washed 3x30 min with 100% MeOH at RT. Tissues were pinned to a glass dish coated with Polydimethylsiloxane (PDMS), cleared in BABB (BABB: 1 part Benzyl Alcohol: 2 parts Benzyl Benzoate), and mounted on slides using BABB as mounting medium.

To visualize WGA555 or WGA647 gastrointestinal injection, nodose ganglion (Figures 1N and S1H) or gastrointestinal tissue (Figures S1F and S1G) were directly washed 3x30 min with 100% MeOH at RT for dehydration, then cleared in BABB and mounted on slides using BABB as mounting medium.

In situ hybridization

To prepare sections for *in situ* hybridization, nodose ganglion was freshly frozen in Tissue-Tek O.C.T Compound. 10 μ m sections were prepared with a cryostat, collected on Superfrost Plus slides, and left to dry in the cryostat for 30 min before staining.

In situ hybridization was performed using RNAscope Fluorescent Multiplex Kit (Advanced Cell Diagnostics, 320850) following the manufacturer's instructions. Briefly, sections were fixed in 4% paraformaldehyde (15 min at 4°C), washed in PBS, dehydrated in a series of ethanol washes, and then dried. A hydrophobic barrier was drawn around the section with an ImmEdge pen (Vector Lab, H-4000). The sections were treated with Protease IV in a HybEZ Humidity Control Tray (30 min at RT), incubated with target probes (see Key Resources Table) in a HybEZ Oven (2 h at 40°C), and then treated with Hybridize Amp 1-4. Slides were mounted using fluoromount-G with DAPI (Southern Biotech).

Image acquisition

All histology images were taken by confocal microscopy (Zeiss, LSM 510).

QUANTIFICATION AND STATISTICAL ANALYSIS

Photometry analysis

Fiber photometry data were analyzed with custom MATLAB scripts, except statistical analyses and bar graphs were generated using Prism. All responses were normalized to baseline using the function: $\Delta F/F = (F_t - F_0) / F_0$, in which F_t is fluorescence at time t , F_0 is the average fluorescence within the baseline period. The baseline period was defined as 10 min before time zero (the moment of the first stimulus). One to two trials of the same experiment for each mouse were combined, averaged, and treated as a single replicate. For quantification, the average of $\Delta F/F$ was calculated within a 1 min window around the indicated time points (Figures 6J–6O, 7G, S6H, and S7K), or within the indicated time window (Figures 7E and 7G bottom, S7I, and S7P).

Animals that showed no response (< 15%) to chow presentation under overnight fasted condition were assumed to be technical failures and were excluded from further experiments. These criteria were pre-established.

Behavioral analysis

Behavior data were analyzed with custom MATLAB scripts and statistical analyses and bar graphs were generated using Prism. Multiple trials of the same experiment for each mouse were combined, averaged, and treated as a single replicate. All optogenetic or chemogenetic trials involved age-matched littermates as controls where possible.

To calculate food intake, consumption of each pellet was defined as the first pellet removal event after each food pellet delivery. The food dropping ratio was calculated by the pellets found dropped divided by the total number of pellets removed at the end of each trial ($R_{\text{drop}} = \text{Food}_{\text{drop}} / \text{Food}_{\text{removal}}$). At each time point, the estimated food intake was calculated by scaling the food removal using the ingestion ratio ($\text{Food}_{\text{intake}} = (1 - R_{\text{drop}}) \times \text{Food}_{\text{removal}}$).

Histology analysis

IGLE quantification

IGLEs were identified using standard criteria (Wang and Powley, 2000) using whole-mount preparation. The number of IGLEs was counted and divided by the counted area to calculate IGLE density. For the stomach, representative images covering 14–26 mm^2 were taken in the gastric fundus, corpus, and antrum, and the IGLE density was estimated based on the image. Intestinal tissue was divided into four segments of small intestine (0–3 cm, 3–10 cm, 10–20cm, 20–30cm) and two segments of large intestine (colon and rectum). For each intestinal segment, the total number of IGLEs of each segment was counted under confocal microscope. The density was calculated by the total number of IGLEs divided by the total area of each segment.

Mucosal ending quantification

Mucosal ending were quantified using whole-mount preparation. The structure of villi or crypts was visualized using the autofluorescence in the 405 channel. The tdTomato⁺ endings within villi or wrapping crypts were considered as mucosal innervation to the corresponding structure. For stomach, representative images covering 14–26 mm^2 were taken within the greater curvature of gastric corpus, lesser curvature of gastric corpus, and gastric antrum. For small intestine, about 20 representative images (1.6 mm^2 each) were taken within each of the four segments of small intestine (0–3 cm, 3–10 cm, 10–20cm, 20–30cm). The ratio of villi or crypt innervation was then quantified from images taken for each gastrointestinal segment. Of note we do not rule out the possibility that a single villi could receive mucosal innervation from multiple neurons, especially in the region with high density of innervation.

Fos analysis

For the Fos analysis (Figures 6A–6G, S5D, and S6A–S6D), all mice were fasted overnight (20 h), measured for body weight, and received CNO injection intraperitoneally in their home cage. An h later, mice were anesthetized under isoflurane and transcardially perfused with 10 mL PBS followed by 15 mL 10% formalin. Brain were harvested, sectioned, and stained with Fos and TH antibodies. To compare between animals, all images were taken using the same setting.

Statistical analysis

All statistical analyses were performed using GraphPad Prism7. All values are reported as mean \pm SEM (error bars or shaded area). Sample size is the number of animal subjects per group and is annotated within figures or legend. P values for comparisons across multiple groups were performed using analysis of variance (ANOVA) and corrected for multiple comparisons using Sidak multiple comparisons test. In figures, asterisks denote statistical significance * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

DATA AND SOFTWARE AVAILABILITY**Data Resources**

RNA-sequencing data (both target-scSeq and whole-nodose scSeq) are available under Gene Expression Omnibus ID GEO: GSE138651.

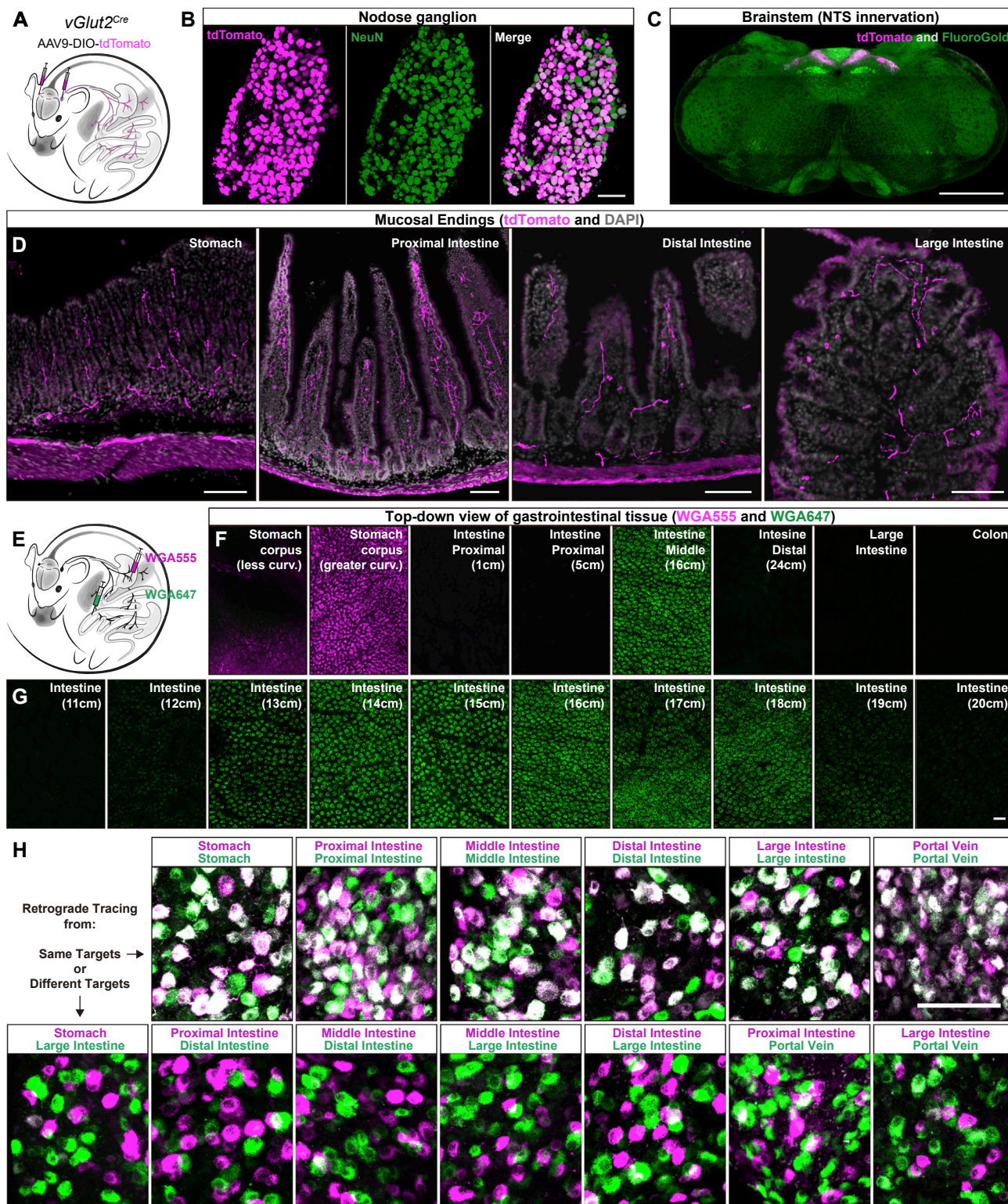


Figure S1. Anterograde and Retrograde Tracing of Vagal Sensory Neurons, Related to Figure 1

(A) Schematic of anterograde tracing strategy.

(B) Immunostaining of nodose ganglion sections reveals that *tdTomato* labels the majority of vagal sensory neurons marked by NeuN ($76 \pm 8\%$, $n = 4$ mice)

(legend continued on next page)

(C) Immunostaining of brainstem sections reveals the central projections of *tdTomato*⁺ vagal sensory neurons within the NTS and AP. Note *tdTomato* is not expressed in the dorsal motor nucleus of vagus nerve (DMV) labeled by FluoroGold (green).

(D) Immunostaining of the transverse cross sections of intestine showing the *tdTomato*⁺ mucosal endings.

(E) Schematic of retrograde tracing strategy.

(F-G) Top-down view of the whole mount preparation shows WGA555 and WGA647 at different regions of the GI tract. Both tracers are restricted near their injection sites. In this case, WGA555 was injected into the stomach antrum and greater curvature of stomach corpus, while WGA647 was injected into the middle region of the small intestine.

(H) Whole-mount nodose ganglion showing vagal sensory neurons that are retrogradely labeled by WGA555 and WGA647 from the same GI targets (top) or different GI targets (bottom).

Scale bar: 100 μ m (B, D, F-H) or 1mm (C).

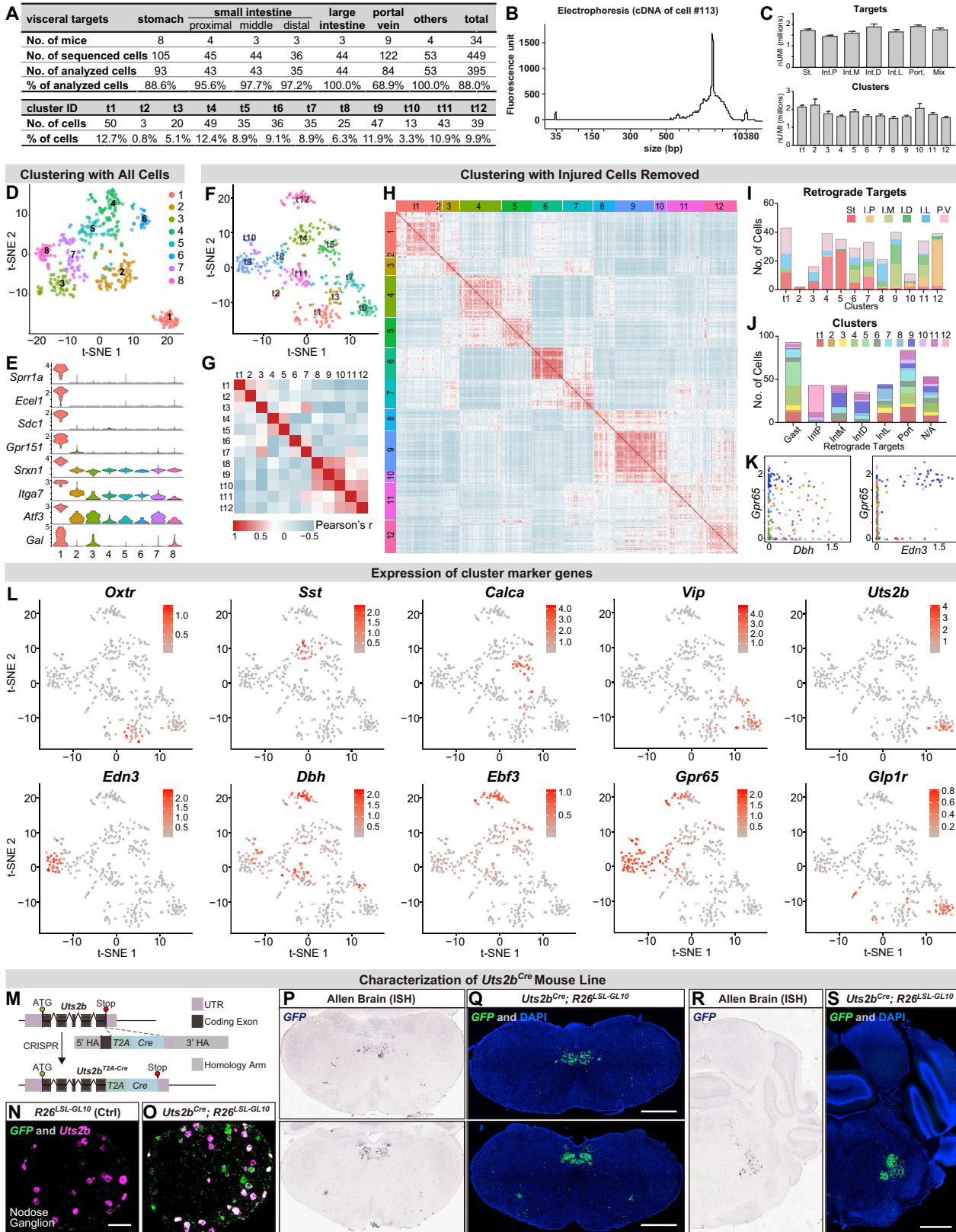


Figure S2. Target-scSeq Procedure and Molecular Identity of Subdiaphragmatic Vagal Sensory Neuron Subtypes, Related to Figure 2

(A) Summary of the number of cells used in the target-scSeq analysis.

(B) Representative electropherogram of a single cell cDNA library.

(C) Number of unique molecular identifier (UMIs) across different target injections or different clusters.

(D-E) Analysis of all 449 cells passing the cDNA QC test reveals the expression of injured cell marker genes in small subsets of cells. This could be due to injury of the peripheral axons in the tracer-injection surgeries. (D) Spectral tSNE plots colored according to gene-based density clustering. (E) Violin plots show injury marker genes are highly enriched in cluster 1.

(F-I) Analysis of 395 cells. 54 Injured cells are removed based on the expression of injury marker genes identified in D-E (cutoff *Sprr1a* < 10 UMI, *Ecel1* < 10 UMI).

(G-H) Heatmap shows cluster-cluster similarities (G) or cell-cell similarities (H) (Pearson's *r*) calculated using the 3912 variable genes.

(I-J) Relationship between clusters and retrograde-tracing targets. Stacked bar graph shows the number of cells that are retrogradely labeled from different targets within individual clusters (I), or vice versa (J). Cells labeled from portal vein injection fell into all clusters with a similar number, which could be due to leakage of tracer, or labeling of axons that bypass the portal vein injection site.

(K) Coexpression of *Gpr65* with *Dbh* or *Edn3*. Values are in ln(TPM+1). Cells are colored according to cluster identity.

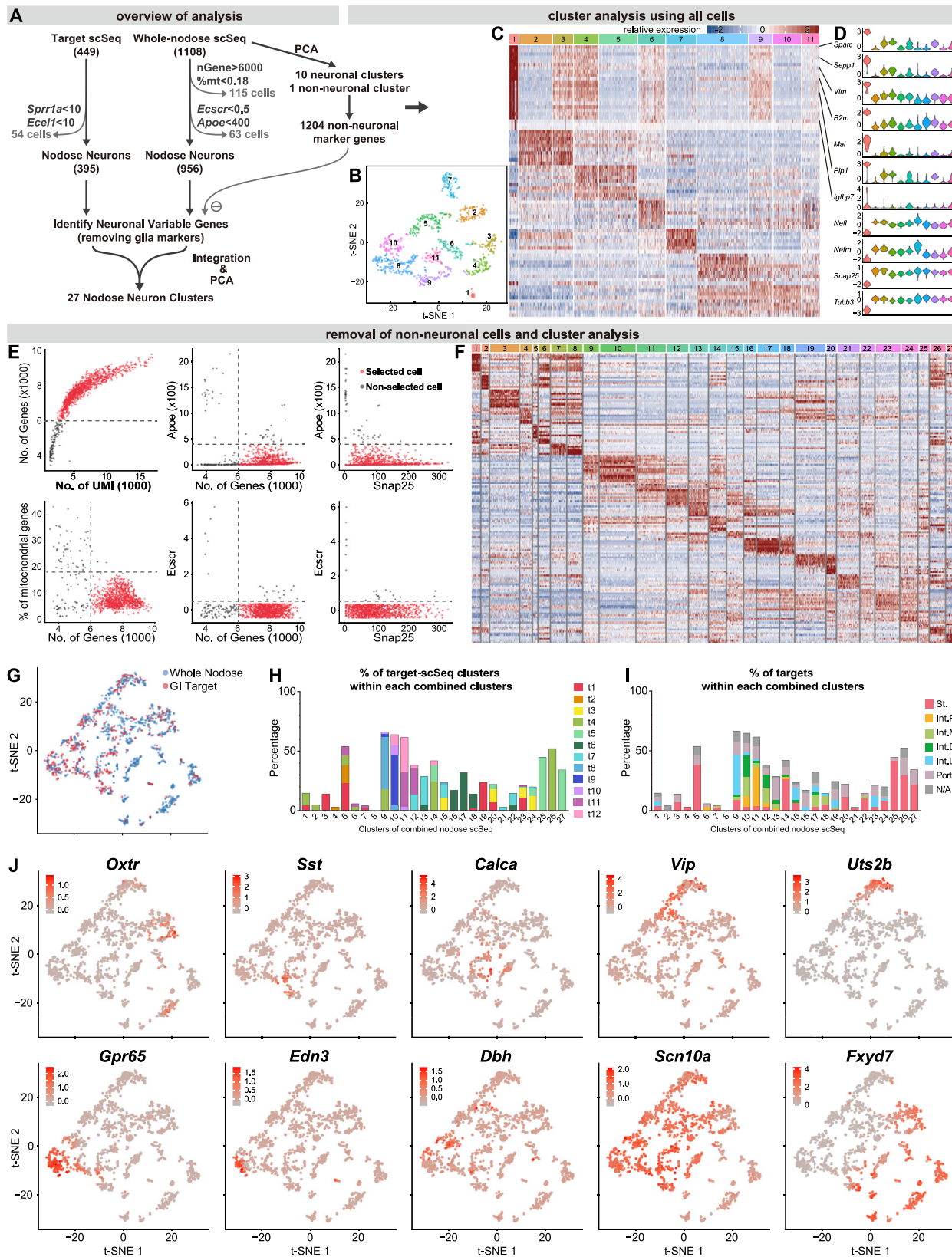
(L) Expression of cluster marker genes across individual cells. t-SNE plot shows cell colored by gene expression (using ln(TPM+1), TPM: transcripts per million).

(M) Schematic for the generation of *Uts2b*^{T2A-Cre} knockin mouse line by CRISPR-mediated homologous recombination.

(N-O) *In situ* hybridization of *Uts2b* and *GFP* on the nodose ganglion section of *Uts2b*^{Cre}; *Rosa*^{LSL-GL10} mice (O) and wild-type control mice (N). In the *Uts2b*^{Cre}; *Rosa*^{LSL-GL10} mice, 68.6 ± 9.5% of *Uts2b*⁺ cells are *GFP*⁺, and 75.6 ± 3.4% of *GFP*⁺ cells are *Uts2b*⁺ (n = 3 mice).

(P-S) The *Uts2b*^{Cre} recombination pattern (Q and S, *Uts2b*^{Cre}; *Rosa*^{LSL-GL10} mice) recapitulates the endogenous *Uts2b* mRNA expression pattern (P and R; Allen Institute for Brain Science ISH #75144621) in the hypoglossal nucleus, dorsal motor nucleus of the vagus nerve, nucleus ambiguus (P-Q), and motor nucleus of trigeminal (R-S).

Values are reported as mean ± SEM. Scale bar: 100 μm (N-O) or 500 μm (P-S).



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Figure S3. Whole-Nodose scSeq Procedure and Molecular Identity of Vagal Sensory Subtypes, Related to Figure 3

(A) Schematic of the combined analysis of GI-target scSeq and unbiased whole-nodose scSeq datasets. 395 Target-scSeq neurons are included after removing injured cells (with filter *Sprr1a* < 10, *Ecel1* < 10). 956 whole-nodose scSeq neurons are included after removing low-quality cells (115 cells with filter no. of genes > 6000, percentage of mitochondrial genes < 18%), satellite glial cells (43 cells, with filter *ApoE* < 400), and endothelial cells (21 cells with filter *Ecscr* < 0.5) (Kupari et al., 2019). To prevent any contaminating glial biasing the cluster analysis and cell type identification, satellite glial and endothelial marker genes (see B-D for the identification of non-neuronal marker genes) are removed from the whole-nodose scSeq dataset before the integration of two datasets and cluster analysis.

(B-D) Identification of non-neuronal marker genes by cluster analysis of all cells in the whole-nodose scSeq. Majority (1067 cells, within cluster 2-11) are neurons (expressing *Nefl*, *Nefm*, *Snap25*, *Tubb3*). A small subset of cells (41 cells, within the cluster 1) are either satellite glial cells (expressing *ApoE*, *Fabp7*, *Dbi*, and *Plp1*), or endothelial cells (expressing *Emcn*, *Ecscr*, *Cdh5*, and *Igfbp7*) (Kupari et al., 2019). Genes enriched in cluster 1 are used as non-neuronal marker genes (480 genes). (B) Spectral tSNE plots colored according to gene-based density clustering. (C-D) Gene expression heatmap (C) and violin plots (D) showing the expression of glial marker genes, endothelial marker genes, and neuronal marker genes across the 11 clusters.

(E-J) Combined analysis of GI-target scSeq and unbiased whole-nodose scSeq datasets.

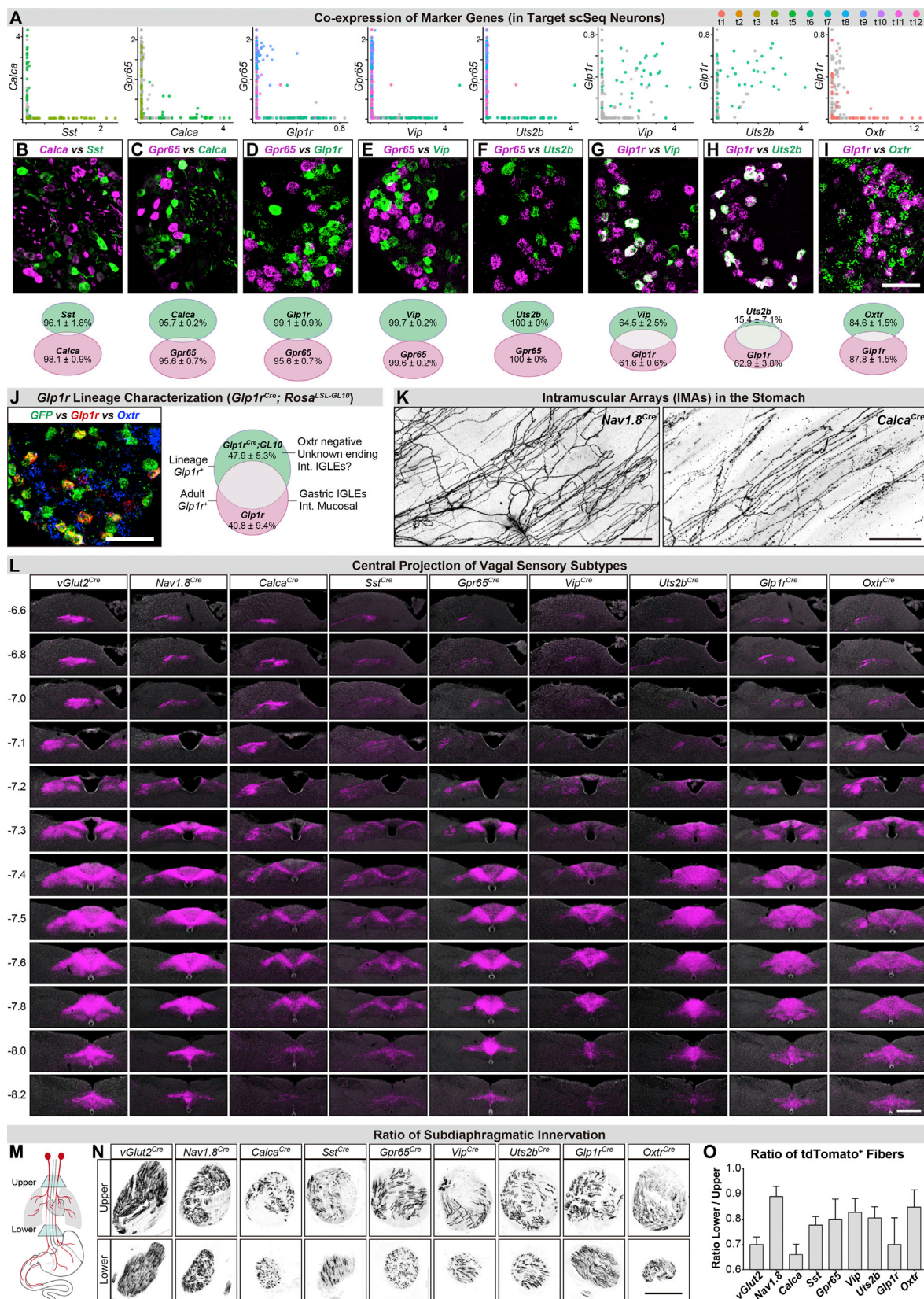
(E) Feature-scatterplots show the filter setup for whole-nodose scSeq dataset.

(F) Signature of vagal sensory neurons shown by the relative expression of subtype-enriched genes (rows) across cells sorted by clusters (columns).

(G) tSNE plots comparing neurons from the GI target-scSeq dataset and the unbiased whole-nodose scSeq dataset.

(H-I) Stacked bar graphs showing the percentage of neurons in each cluster that were originated from individual target-scSeq clusters (H), or labeled from individual GI targets (I).

(J) Spectral tSNE plots colored according to the expression of cluster-specific marker genes (using $\ln(\text{TPM}+1)$).



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Figure S4. Genetic Markers Identify Vagal Subtypes with Unique Morphologies and Innervation Patterns, Related to Figure 4

(A) Comparison of the expression of cluster marker genes. Values are in $\ln(\text{TPM}+1)$. Cells are colored according to their cluster identity.

(B-I) Top: Double immunostaining (B-C) or *in situ* hybridization (D-I) of marker genes on nodose ganglion sections of *Sst^{Cre}*; *Rosa^{LSL-ChR2}* (B), *Gpr65^{Cre}*; *Rosa^{LSL-ChR2}* (C), or wild-type mice (D-I). Bottom: Quantification showing the percentage of cells that only express one of the two marker genes. We observed expression of *Glp1r*, which was previously identified as a marker for IGLEs (Williams et al., 2016), in the mucosal-ending cluster labeled by *Vip/Uts2b* (G-H). Consistent with this, we found that *Glp1r^{Cre}* labeled intestinal mucosal endings in a similar pattern to *Vip* and *Uts2b* (Figures 4D and 4G).

(J) *In situ* hybridization of *GFP*, *Glp1r*, and *Oxtr* on the nodose ganglion sections of *Glp1r^{Cre}*; *Rosa^{LSL-GL10}* mice (left) and the quantification (right). $52.1 \pm 5.3\%$ *GFP⁺* cells are *Glp1r⁺*, and $8.9 \pm 1.7\%$ of *GFP⁺* cells are *Oxtr⁺*.

A recent report showed that vagal neurons labeled by *Glp1r^{Cre}*; *Rosa^{LSL-GCaMP3}* include the majority of neurons that respond to gastric distension (81%) and intestinal distension (67.7%) (Williams et al., 2016). However we observe following injection of *Cre*-dependent AAV into the nodose ganglia of adult mice that *Glp1r^{Cre}* labels only IGLEs in the stomach. This difference could be due to the fact that the developmental expression of *Glp1r* is broader than its expression in the adult, and consequently *Glp1r^{Cre}*; *Rosa^{LSL-GCaMP3}* labels intestinal IGLEs and other cell types that do not express *Glp1r* in the adult. To be consistent in this study, we have performed viral injection into nodose ganglia of adult animals for all anatomical and functional analyses.

(K) Top-down view of whole-mount stomach reveals the *tdTomato⁺* vagal sensory terminals (black) labeled by *Nav1.8^{Cre}* or *Calca^{CreER}*. Within the nine *Cre* lines analyzed in Figures 4 and S4, *vGlut2^{Cre}* and *Nav1.8^{Cre}* densely labeled IMAs in the stomach and large intestine. *Calca^{CreER}* labels IMAs with a lower density (mainly in the gastric antrum and large intestine). Very few IMAs (1-5 terminals per stomach) have been found in *Sst^{Cre}*, *Gpr65^{Cre}*, and *Oxtr^{Cre}* mice, while no IMA was found in *Glp1r^{Cre}*, *Vip^{Cre}*, and *Uts2b^{Cre}*.

(L) Immunostaining of brainstem sections showing the *tdTomato⁺* axonal terminals of vagal sensory subtypes in the NTS and AP. AAV9-DIO-*tdTomato* was injected into the bilateral nodose ganglion of individual *Cre* mouse lines. Number on the left annotates the anterior-posterior coordinate from the Bregma.

(M-O) The ratio of subdiaphragmatic innervation of individual *Cre* mouse lines. Number of *tdTomato⁺* axons within vagal nerves are counted at a level close to the nodose ganglion (N, top) or at a subdiaphragmatic level (close to the stomach) (N, bottom). The ratio was calculated to estimate the percentage of subdiaphragmatic innervation of *tdTomato* labeled vagal sensory subtypes (O). Values are reported as mean \pm SEM. Scale bar: 100 μm (B-J, N) or 500 μm (K-L).

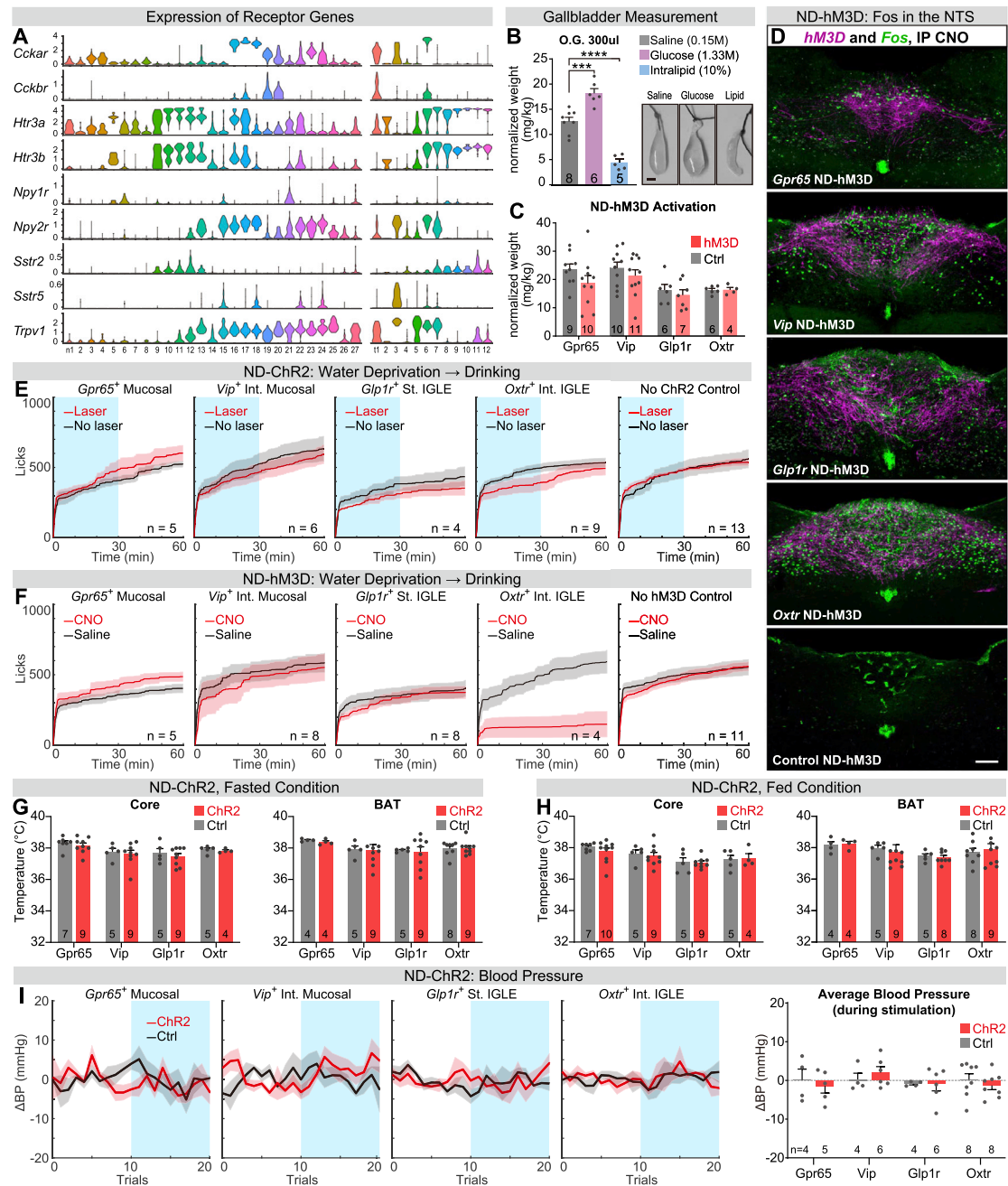


Figure S5. Functional Characterization of Vagal Cell Types, Related to Figure 5

(A) Violin plots of hormonal receptors and *Trpv1* across 27 whole-nodose scSeq clusters as well as 12 target-scSeq clusters.

(B) Weight quantification (left) and representative images (right) of gallbladders one h after oral gavage of 300 μ L saline, lipid, or glucose.

(C) Quantification of gallbladder weight 1 h after CNO injection, across control and four groups of mice that express hM3D within vagal sensory subtypes.

(D) Immunostaining of brainstem sections (50 μ m) shows Fos⁺ cells and axonal terminals of hM3D-mCherry⁺ vagal sensory neurons in the NTS and AP. Mice are intraperitoneally injected with CNO 1 h before euthanizing for Fos analysis.

(E) Cumulative water intake of overnight water-deprived mice, comparing trials with and without photostimulation across the four vagal-ChR2 lines and control without ChR2 expression. Blue shading indicates the period of laser stimulation.

(F) Cumulative water intake of overnight water-deprived mice, comparing trials with CNO or saline treatment.

(G-H) Core body temperature (rectal measurement) and BAT temperature of fasted (G) or fed (H) mice, measured 1.5 h after CNO injection.

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(I) Change in mean arterial blood pressure over time (left) and quantification of average photostimulation-induced change (right) across vagal-ChR2 and control mice. Blue shadow indicates the period of photostimulation (left)
Error bars and shaded areas represent mean \pm SEM n mice is annotated within figures. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA (B) or two-way ANOVA (others), Sidak correction. Scale bar: 1 mm (B) or 100 μ m (D).

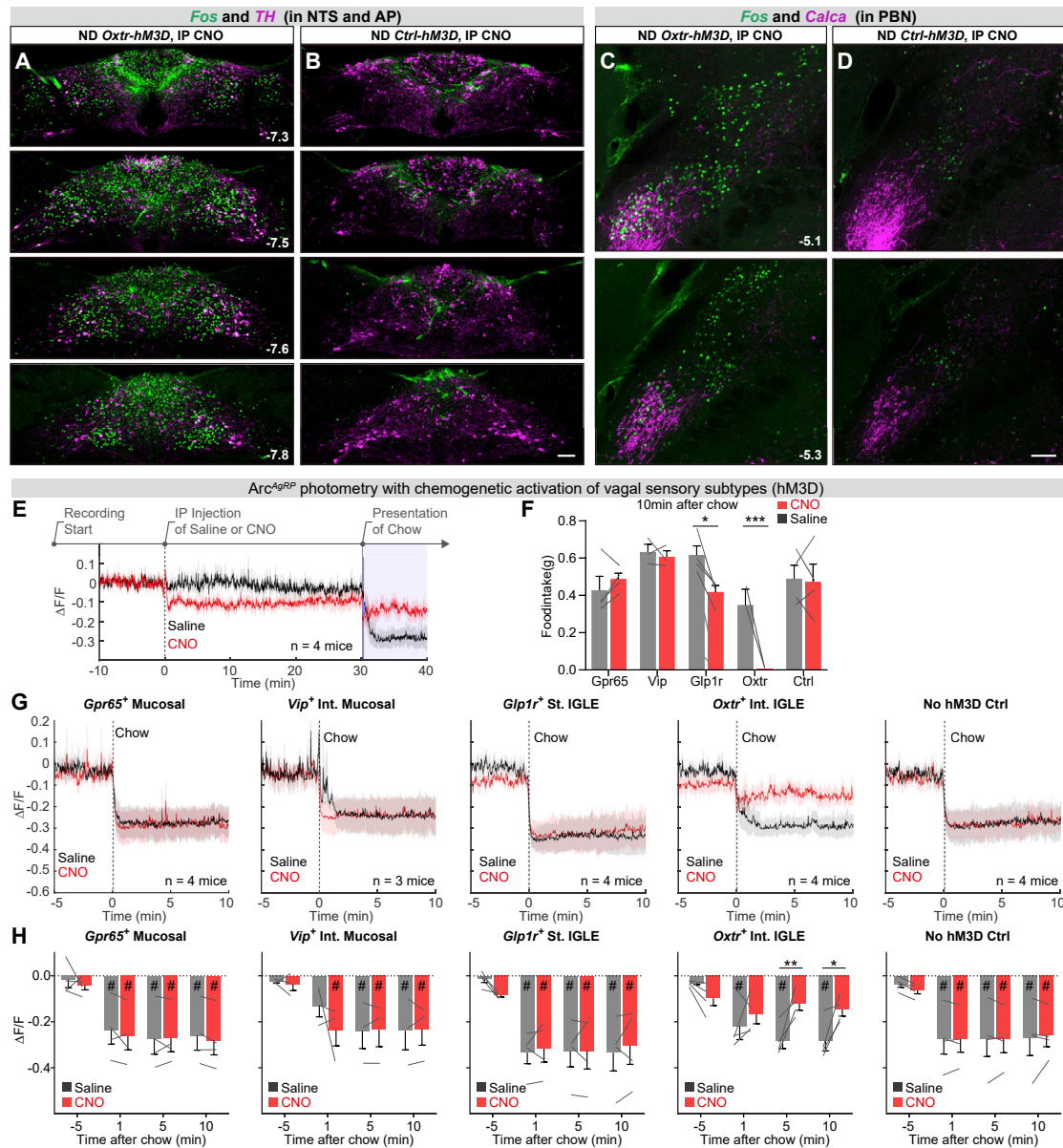


Figure S6. Stimulation of Gastrointestinal Mechanoreceptors Modulates Feeding Centers in the Brain, Related to Figure 6

(A-D) Immunostaining of brain sections showing the overlapping of *Fos*⁺ and *TH*⁺ cells in the NTS/AP (A-B), or *Fos*⁺ and *Calca*⁺ cells in the PBN (C-D), comparing *Oxttr*^{hM3D} mice (A and C) and control mice (B and D). The anterior-posterior coordinate relative to bregma is annotated in the bottom right corner of each image. (E) Overview of the photometry recording paradigm (top) and average AgRP neuron activity in fasted *Oxttr*^{hM3D} mice. Blue shading indicates the period of time with chow presentation.

(F) Quantification of total food intake 10 min after chow presentation, comparing CNO and saline treated trials.

(G) Average AgRP calcium signal in fasted mice after chow presentation. Mice received CNO or saline injection 30 min before receiving the chow. *hM3D* is expressed in vagal sensory neurons labeled by *Gpr65*, *Vip*, *Glp1r*, or *Oxttr*.

(H) Quantification of (G), showing average $\Delta F/F$ within 1 min window around time points indicated in the x axis.

Error bars and shaded areas represent mean \pm SEM n mice is annotated within figures (F) or under the trace (G). Comparisons were made between groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) or from baseline (# $p < 0.05$), two-way ANOVA, Sidak correction.

Scale bar: 100 μ m.

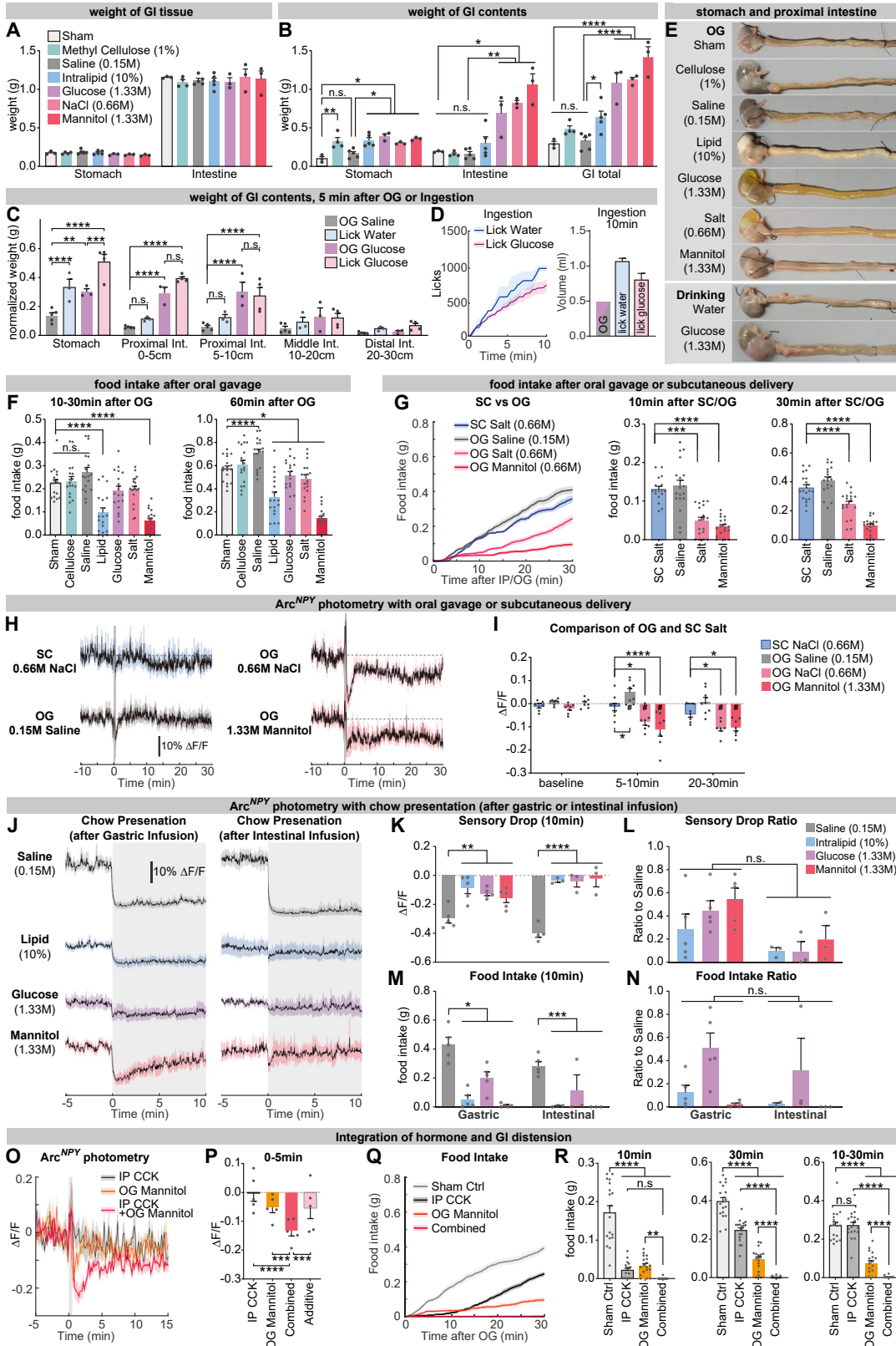


Figure S7. Gastrointestinal Distension Is Sufficient to Inhibit Food Intake and Regulate AgRP Neuron Activity, Related to Figure 7

(A) Weight of GI tissue measured 5 min after sham treatment (n = 3) or 500 uL oral gavage of various contents (n = 3-5 mice). The similarity of tissue weight after oral gavage (A) suggests that the differences in GI contents (Figure 7A) are not due to systematic differences in the GI tract tissue between animals.

(B) Weight of GI contents measured 5-10 min after sham treatment (n = 3) or 500 uL oral gavage of various contents (n = 3-5 mice). Note for the hypertonic solutions (glucose, NaCl, and mannitol), the total weight of GI contents is larger than the infused contents (500 uL, about 0.5g). This suggests that additional fluids have been drawn from the circulation into the GI lumen due the differences in osmotic pressure, which could potentially induce further GI stretch.

(C) Normalized GI contents measured 5-10 min after oral gavage saline (500 uL, n = 5), drinking water (n = 3), oral gavage glucose (500 uL, n = 3), and drinking glucose (n = 4). All mice were fasted before the test, except that the water drinking group was water deprived. For water or glucose drinking, mice were euthanized 10 min after the starting of drinking and the amount ingested is shown in (D).

(D) Cumulative water intake of overnight water-deprived mice, and cumulative glucose intake of overnight fasted mice. The volume is estimated based on the number of licks (1.1 uL/lick) (St. John et al., 2017).

(E) Stomach and proximal intestine collected 5-10 min after sham treatment, 500 uL oral gavage of various solutions, drinking water or glucose.

(F) Total food intake of overnight food-deprived mice (n = 18) within 10-30 min or 0-60 min after oral gavage of 500 uL various solutions.

(G) Cumulative food intake (left) and total food intake (right) of overnight food-deprived mice (n = 18) after subcutaneous injection of 500 uL hypertonic salt solution, or oral gavage of 500 uL various contents.

(H) Averaged AgRP neuron calcium signal in fasted mice (n = 6-11 mice) after subcutaneous injection of 500 uL hypertonic salt solution, or oral gavage of 500 uL various contents. Gray bars indicate the period of oral gavage.

(I) Quantification of (E), showing average $\Delta F/F$ within 5-10 min or 20-30 min time window.

(J-N) AgRP neuron activity or food intake in fasted mice after chow presentation (starting at 0 min). 30 min before the chow presentation, all mice received 1 mL gastric infusion (n = 5, left) or intestinal infusion (n = 3, right) of various contents.

(J) Average AgRP neuron calcium signal. Gray bars indicate the period of chow presentation.

(K) Quantification of (J), showing average change of $\Delta F/F$ after chow presentation (sensory drop) within 10 min.

(L) Ratio of sensory drop in (H), calculated between various solutions and saline infusion.

(M) Quantification of total food intake within 10 min.

(N) Ratio of food intake in (M), calculated between various contents and saline infusion.

(O-R) Synergistic effect between IP injection of subthreshold CCK and oral gavage of mannitol, on the regulation of AgRP neuron activity (O-P) or food intake (Q-R). Mice received one of the following treatments within each trial: 1) IP injection of 2 ug/kg CCK, 2) oral gavage of 500 uL 1.33 M mannitol, 3) Combined treatment with IP injection immediately followed by oral gavage, 4) Sham oral gavage.

(O) Average AgRP neuron activity in fasted mice (n = 5 mice).

(P) Quantification of (O), showing average $\Delta F/F$ within 5 min after treatment. Note AgRP activity after receiving combined treatment is significantly different from individual treatments or the hypothetical additive value.

(Q-R) Cumulative food intake (Q) and total food intake (R) of overnight food-deprived mice (n = 18 mice).

Error bars and shaded areas represent mean \pm SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, two-way ANOVA, Sidak correction. Scale bar: 1 mm