Satiety and other core physiological functions are modulated by sensory signals arising from the surface of the gut. Luminal nutrients and bacteria stimulate epithelial biosensors called enteroendocrine cells. Despite being electrically excitable, enteroendocrine cells are generally thought to communicate indirectly with nerves through hormone secretion and not through direct cell-nerve contact. However, we recently uncovered in intestinal enteroendocrine cells a cytoplasmic process that we named neuropod. Here, we determined that neuropods provide a direct connection between enteroendocrine cells and neurons innervating the small intestine and colon. Using cell-specific transgenic mice to study neural circuits, we found that enteroendocrine cells have the necessary elements for neurotransmission, including expression of genes that encode pre-, post-, and transsynaptic proteins. This neuroepithelial circuit was reconstituted in vitro by coculturing single enteroendocrine cells with sensory neurons. We used a monosynaptic rabies virus to define the circuit’s functional connectivity in vivo and determined that delivery of this neurotropic virus into the colon lumen resulted in the infection of mucosal nerves through enteroendocrine cells. This neuroepithelial circuit can serve as both a sensory conduit for food and gut microbes to interact with the nervous system and a portal for viruses to enter the enteric and central nervous systems.
Neuroepithelial circuit formed by innervation of sensory enteroendocrine cells

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Introduction

Satiety, food preference, and even mood behaviors are a few of the functions modulated by gut chemosensation (1). Ingested nutrients and bacterial by-products contacting the gut epithelium stimulate enteroendocrine cells (2). These are electrically excitable biosensors essential for normal life (3). The sensory mechanisms of enteroendocrine cells have recently been elucidated using transgenic fluorescence-reporter mice. For instance, cholecystokinin-GFP mice have enabled scientists to uncover how digested fats modulate metabolism. The mechanisms involve lipid stimulation of enteroendocrine cells through receptors such as GPR41 (4). Once stimulated, enteroendocrine cells secrete several neuropeptides, including cholecystokinin (CCK) and peptide YY (PYY), best known for their ability to induce satiety (5, 6).

Despite their recognized sensory function, how enteroendocrine cells relay sensory signals from the gut lumen onto nerves is poorly understood. Transmission has been regarded as paracrine, but not through direct enteroendocrine cell–nerve contact. Hormones secreted from enteroendocrine cells are thought to diffuse throughout the lamina propria until they reach the bloodstream or act on intrinsic sensory neurons or vagal afferent nerves (7, 8).

Although this is still a possibility, we recently uncovered a prominent cytoplasmic process in enteroendocrine cells of the small intestine and colon that we called neuropod (9, 10). This neuropod is escorted by enteric glia and elongates in the presence of neurotrophins; in addition, its tip almost invariably resembles a synaptic-like bouton, which suggests a physical connection to a nerve (9, 10).

Here, we studied such a possibility by using Cck-GFP, Ppy-GFP, and Pyy-Cre mice in combination with molecular tools for the study of neural circuits, such as monosynaptic rabies neurotracing and a Cre-dependent rabG mouse. We uncovered a new neuroepithelial circuit that has the potential to serve as a conduit between the lumen of the gut and the nervous system.

Results and Discussion

The contact with nerves. Because of their endocrine attribution, we first determined whether neuropods in enteroendocrine cells are associated with blood vessels. We revealed the vasculature of the small intestine and colon by perfusing Pyy-GFP transgenic mice with a buffer solution containing the lipophilic dye DiI. The technique is known as blood vessel painting (11). The results showed that blood vessels are found within 5.6 μm (SEM ± 0.4, n = 3), but do not come into contact with enteroendocrine cells. We then immunolabeled the vessel-painted tissue with the panneuronal marker PGP 9.5 to determine the proximity of neuropods to nerve fibers innervating the mucosa. Nerves were observed penetrating the basal lamina and directly contacting the enteroendocrine cell neuropod (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI78361DS1). Figure 1, A–C, shows 3 examples of enteroendocrine cells contacting individual nerve fibers innervating the mucosa of the ileum and colon. The fre-
mental Video 1. At 11 hours, 55 minutes, an enteroendocrine cell (Cck-GFP, green) is observed lying next to a neuron (lipophilic DiI, red). Next, the neuron extends a small neurite (arrowhead) toward the enteroendocrine cell (12 hours, 4 minutes), and the enteroendocrine cell responds by elongating a cytoplasmic process toward the neuron (13 hours, 14 minutes) that connects to the neuron’s neurite (14 hours, 25 minutes). The sensory neuron continues extending a putative axon, while the enteroendocrine cell remains connected (23 hours, 18 minutes). In this example, the enteroendocrine cell and the neuron remained connected until the experiment was ended after 88 hours. An additional example is shown in Supplemental Video 2. In this case, a neuron clearly elongates an axon that connects with a distant enteroendocrine cell. The footage in both examples is unique because it contains information that can only be appreciated by observing the sequence in motion. This kind of interaction was not evident in control experiments when enteroendocrine cells were cocultured with non-GFP epithelial cells or HEK immortal cells. Two days after plating, it was estimated that about 15% of all Cck-GFP enteroendocrine cells were in contact with a neuron or a neuronal fiber. It is noteworthy that the viability of single Cck-GFP enteroendocrine cells in culture is low. One day after plating, only 5 Cck-GFP cells for every 1,000 remained alive (0.50% ± 0.17% at d1 to 0.10% ± 0.02% at d6; average of 3 wells per replicate, n = 6). Nonetheless, the fact that the connectiv-
Supplemental Figure 2. These data suggest that at least a population of enteroendocrine cells may have a comparable life span to that of other innervated biosensors, such as olfactory receptor neurons or taste cells (17, 18).

**Synaptic features in enteroendocrine cells.** Electron microscopy studies have revealed that, besides hormone-containing large dense-core vesicles, enteroendocrine cells also have small clear synaptic vesicles (19), suggesting that enteroendocrine cells have an essential organelle for neurotransmission. Thus, we considered that the enteroendocrine cell–neuron connection could have features of a synapse. We purified Pyy-GFP enteroendocrine cells by FACS and analyzed the gene expression encoding for defined proteins found in pre- and postsynapses by quantitative reverse-transcriptase PCR (qRT-PCR) (20). Indeed, compared with non-GFP intestinal epithelial cells, intestinal and colonic enteroendocrine cells expressed a number of genes encoding for presynaptic proteins, including: synapsin 1, piccolo, bassoon, MUNC13B, RIMS2, latrophilin 1, and transsynaptic neurexin 2 (21). Pyy-GFP enteroendocrine cells also expressed DOPA decarboxylase and tyrosine hydroxylase — both essential enzymes in synthesis of the neurotransmitter dopamine (Figure 2A and Supplemental Table 1).

Pyy-GFP enteroendocrine cells also expressed a cohort of postsynaptic genes, including the transsynaptic neuroligins 2 and 3, homer 3, and postsynaptic density 95 (Psd95) (22). Using immunohistochemistry, we found that, of all colonic Pyy-GFP enteroendocrine cells, 96.4% (SEM ± 0.5%) of Pyy-GFP cells immunoreacted with the presynaptic marker SYN1 (Figure 2B). The presynaptic marker synapsin 1 (SYN1) immunoreacted with 96.4% (SEM ± 0.5%) of Pyy-GFP cells. (C) 35.3% (SEM ± 3.5%) of Pyy-GFP cells immunoreacted with the postsynaptic marker PSD95. (D) In vitro, Cck-GFP cells connecting to trigeminal neurons expressed the postsynaptic marker PSD95 (cyan). Blue, DAPI nuclear stain. Scale bars: 10 μm.

Figure 2. Enteroendocrine cells express pre- and postsynaptic proteins. (A) Gene expression analysis of FAC-sorted Pyy-GFP enteroendocrine cells shows their expression of presynaptic and postsynaptic proteins. Fold expression represents log_{10} \left[ \frac{\Delta Ct_{\text{Pyy-GFP-positive cells}}}{\Delta Ct_{\text{Pyy-GFP-negative epithelial cells}}} \right] \text{ and error bars represent the SEM. Significant differences were separated by Student’s t test using 2(−ΔΔCt) values at } \alpha = 0.05. (B) The presynaptic marker synapsin 1 (SYN1) immunoreacted with 96.4% (SEM ± 0.5%) of Pyy-GFP cells. (C) 35.3% (SEM ± 3.5%) of Pyy-GFP cells immunoreacted with the postsynaptic marker PSD95. (D) In vitro, Cck-GFP cells connecting to trigeminal neurons expressed the postsynaptic marker PSD95 (cyan). Blue, DAPI nuclear stain. Scale bars: 10 μm.

A note on the enteroendocrine cells’ life span. It has been proposed that because epithelial cells, including enteroendocrine cells, turn over every 5 days, the window for enteroendocrine cells to connect to neurons is restricted (7). However, some reports have suggested that enteroendocrine cells may live longer than epithelial enterocytes (16). We wondered whether Pyy-GFP enteroendocrine cells may live longer than 5 days and used BrdU pulse labeling in Pyy-GFP mice to determine their life spans compared with other intestinal epithelial cells. Although the initial number of labeled Pyy-GFP cells was low (6.3% ± 1.2%; \( n = 3 \)), there was evidence of labeled Pyy-GFP enteroendocrine cells 60 days after labeling, indicating that long-lived epithelial cells constitute a portion of the enteroendocrine cell population. Examples are presented in Figure 2. Enteroendocrine cells express pre- and postsynaptic proteins: (A) Gene expression analysis of FAC-sorted Pyy-GFP enteroendocrine cells shows their expression of presynaptic and postsynaptic proteins. Fold expression represents log_{10} \left[ \frac{\Delta Ct_{\text{Pyy-GFP-positive cells}}}{\Delta Ct_{\text{Pyy-GFP-negative epithelial cells}}} \right] \text{ and error bars represent the SEM. Significant differences were separated by Student’s t test using 2(−ΔΔCt) values at } \alpha = 0.05. (B) The presynaptic marker synapsin 1 (SYN1) immunoreacted with 96.4% (SEM ± 0.5%) of Pyy-GFP cells. (C) 35.3% (SEM ± 3.5%) of Pyy-GFP cells immunoreacted with the postsynaptic marker PSD95. (D) In vitro, Cck-GFP cells connecting to trigeminal neurons expressed the postsynaptic marker PSD95 (cyan). Blue, DAPI nuclear stain. Scale bars: 10 μm.

ity is recapitulated in vitro is of great significance because it demonstrates the specific affinity between enteroendocrine cells and extrinsic sensory neurons in the absence of any other cell input.

Using immunohistochemistry, we found that, of all colonic Pyy-GFP enteroendocrine cells, 96.4% (SEM ± 0.6) expressed the presynaptic marker synapsin 1 (Figure 2B) and 35.2% (SEM ± 3.5) the postsynaptic marker PSD95 (Figure 2C). In vitro, enteroendocrine cells cocultured with neurons also expressed synaptic markers. Figure 2D shows an example of 2 separate fibers from neurons in culture that connect to a Cck-GFP enteroendocrine cell immuno-reactive for PSD95. It is possible that, by comparing the expression to non-GFP cells, other synaptic genes may have been overlooked because the population of non-GFP epithelial cells also contains unsorted enteroendocrine cells that do not express PYY.

These data show that enteroendocrine cells have the necessary elements to engage in afferent and efferent synaptic transmission. Enteroendocrine cells are already thought to relay afferent signals from nutrients to underlying visceral nerves via hormones (23), but their expression of postsynaptic proteins raises the possibility of
enteroendocrine cells (Supplemental Table 1). Immunoreactivity with a PYY antibody confirmed that some of these cells are PYY-secreting enteroendocrine cells (Figure 3A). No cells below the epithelium, including nerves, were infected by rabies (Figure 3A). These data indicate that SAD\(\Delta G\)-GFP rabies infects enteroendocrine cells, but in the absence of rabG, cannot spread further.

We then engineered rabG in a subset of enteroendocrine cells to allow SAD\(\Delta G\)-GFP rabies to travel 1 synapse. Using Cre-loxP recombination, we bred R\(\Phi\)GT (27) and Pyy-Cre (28) mouse lines to develop a Pyy-Cre-rabG mouse. In this mouse, only PYY-secreting enteroendocrine cells express rabG. Then we delivered SAD\(\Delta G\)-GFP by enema into the lumen of the distal colon of P3 Pyy-Cre-rabG mice. The results showed that 7 days after infection, SAD\(\Delta G\)-GFP rabies infects enteroendocrine cells (dotted lines) and underlying connecting nerve fibers. These immunoreacted with the neuronal marker PGP 9.5. (D) Model of afferent and efferent innervation of enteroendocrine cells. Scale bars: 10 \(\mu\)m.

**Figure 3. Enteroendocrine cell–neuron circuit revealed by monosynaptic rabies virus.** (A) When delivered by enema into the lumen of the colon of wild-type mice, SAD\(\Delta G\)-GFP (\(\Delta G\)-GFP) rabies infects enteroendocrine cells, as confirmed by their PYY immunoreactivity. (B) To enable the monosynaptic rabies to spread, we engineered rabG glycoprotein in PYY-containing enteroendocrine cells by breeding Pyy-Cre with R\(\Phi\)GT mice. (C) When delivered into the colon of Pyy-Cre-rabG (Pyy-rabG) mice, SAD\(\Delta G\)-GFP infected enteroendocrine cells (dotted lines) and underlying connecting nerve fibers. These immunoreacted with the neuronal marker PGP 9.5. (D) Model of afferent and efferent innervation of enteroendocrine cells. Scale bars: 10 \(\mu\)m.

efferent neurotransmission. In other sensory epithelial cells, such as hair cells of the inner ear, the responsiveness to the mechanical stimulus of sound is toned down through efferent feedback (24). Likewise, efferent neurotransmission in enteroendocrine cells might serve to modulate the responsiveness of enteroendocrine cells to nutrients and bacterial by-products in the lumen of the intestine.

Rabies infection and neurocircuit connectivity. We sought to define this neural circuit in vivo. For this, we used an established neurotracing method based on a monosynaptic rabies virus. The virus is known as SAD\(\Delta G\)-GFP rabies because its envelope glycoprotein rabG has been replaced with GFP (25). rabG is responsible for the spread of rabies from neuron to neuron through synapses. Thus, a monosynaptic neural circuit can be defined by expressing rabG into the target cell to allow the virus to jump 1 synapse and reveal the 2 connected neurons by fluorescence. Rabies has an affinity for neurons, and despite the neuronal features of enteroendocrine cells (9), it has been unknown whether rabies could infect enteroendocrine cells. We first tested to determine whether SAD\(\Delta G\)-GFP rabies virus could infect enteroendocrine cells when delivered by enema into the lumen of the distal colon in P3 wild-type mice. The results showed that 7 days after infection, SAD\(\Delta G\)-GFP rabies infected enteroendocrine cells when delivered by enema into the lumen of the distal colon in P3 wild-type mice. The results showed that 7 days after infection, SAD\(\Delta G\)-GFP rabies infected enteroendocrine cells (Figure 3A). Rabies virus has been shown to infect neurons through 3 molecular receptors; one of those is NCAM (26), which is expressed in Pyy-GFP enteroendocrine cells (Supplemental Table 1). Immunoreactivity with a PYY antibody confirmed that some of these cells are PYY-secreting enteroendocrine cells (Figure 3A). No cells below the epithelium, including nerves, were infected by rabies (Figure 3A). These data indicate that SAD\(\Delta G\)-GFP rabies infects enteroendocrine cells, but in the absence of rabG, cannot spread further.

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mice that served as controls. Because rabies virus spreads through synapses in a retrograde fashion, these data highlight that enteroendocrine cells are synthetically connected to efferent neurons in vivo.

Although paracrine transmission remains a possibility, we believe that the physical innervation of sensory enteroendocrine cells opens the following possibilities: (a) precise temporal transmission of sensory signals from the gut lumen; (b) real-time modulatory feedback onto enteroendocrine cells; (c) localized plasticity to sensory stimuli in the gut; (d) precise topographical representation of sensory signals from the gut; and (e) given the conditions, a potential physical path for viruses in the lumen of the gut to gain access to the peripheral or central nervous system.

Methods
The Supplemental Methods section contains procedures for cocolutions, BrdU pulse labeling, rabies neurotracing, and imaging.

Study approval. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Duke University Medical Center.

Statistics. Statistical differences were determined using a 2-tailed t test analysis at α = 0.05.

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