Sector: Transforming Medicine with Synthetic Biology

Use Case for Medicine: Synthetic Tissues and Organs

End Product: Variety of synthetic tissues and organs to replace or compensate for dysfunction

Organism(s) if applicable: Mammalian Cell Lines

3-D printers can assemble raw materials into very complex products, and progress has been made in fabricating tissues and organoids that recapitulate human biology. However, vascularization is essential for in vivo function of these engineered tissues. While simple blood vessels have been fabricated, branching networks that penetrate 3-D printed tissues have remained elusive. Printing tissues with intact vascularization would enable the on-demand repair of injured and/or diseased organs.

Networks of blood vessels branch throughout almost every tissue of the body and are essential to proper organ function. While progress has been made in engineering new tissues and organs using 3-D printers, sufficient vascularization remains a hurdle. The promise of programming 3-D printed tissues to spontaneously vascularize is within reach using synthetic biology approaches. Combining the speed of 3-D printing and the cellular control of synthetic biology could lead to rapid, precision tissues for implantation.

Desired outcome(s) that stretch current capabilities

- Complex, vascularized solid tissues and organs to manage and treat disease
- Rapid single-cell -omics pipelines to understand the molecular and cellular recipes in development and tissue formation
- Advanced modeling of interactions between implant and the host
- Biocompatible allo- and xeno-transplant and implantation of engineered tissues and organs
- Improved parallel and precise genome editing in recipient's immune system (to become tolerant to the donor tissue/organs and immunize against cross-species disease transmission)
- Engineer the recipient's immune system to be tolerant of the implant without excessive immune suppression

Use Case for Medicine: Cells as Physicians

End Product: cells of the gut microbiome decide when to produce neuroactive chemicals
Organism(s) if applicable: in vitro and in vivo mammalian studies

The saying goes that "the route to a one's heart is through one's stomach", but the coordinates of this statement are slightly off: the final destination isn't one's heart, it is one's head. The number of neurons that innervate the gastrointestinal tract is second only to the brain, creating a two-way information highway between them. Specific gastrointestinal cells, dubbed 'neuropods', initiate a single synapse connection from the gut to the brain via the vagal nerve. These neuropods are dotted along the GI tract and can respond to sugars, but their effect on the brain is dependent upon where in the GI tract the sugar is detected: upper GI tract, pleasure; lower GI tract, pain. This creates a distributed surveillance network of gut health along its length that has direct psychological effects (e.g., in mood, cognition, and behavior). Cells of the GI tract are bristling with receptors and so it is highly likely that these neuropod cells respond to many other cues than just sugar. Enter the gut microbiome. We are discovering that the millions of microbes that are the normal residents of the GI tract produce a wealth of metabolites, including those that are neuroactive. Identification of the microbially-produced compounds that induce psychological effects is at the frontier of microbiome research and mapping the synthesis pathway of these compounds is a major push in synthetic biology.

We are standing at the precipice of how engineered biological systems can be utilized in human health and performance. By combining the pointy end of microbiome and synthetic biology research, microbially-produced compounds that have a positive cognitive and psychological effect can be identified and synthesized in vitro and in vivo. This capability, coupled with identifying gut biomarkers that are indicative of specific cognitive and psychological detriments, and in vitro and in vivo modelling capabilities, could lead to smart probiotic interventions leading to passive amelioration. How should we proceed with this goal? There has been a natural convergence of synthetic biologists and microbial ecologists as evidenced by the increasing presence of synthetic biology posters at host-microbe interaction conferences. The tools and the interest are there, but how do we go from lab experiments to a clinically-relevant outcome? What needs to be in place to allow us to launch from the precipice rather than fall from it.

Desired outcome(s) that stretch current capabilities

- Identification and synthesis of microbial compounds that activate neuropod cells
- Mapping of neuronal path from gut to brain regions
- Quantifiable, reproducible cognitive and/or psychological effect of native/engineered microbe/community

Use Case for Medicine: Living Wearable Technology

End Product: Smart Medicines/Wearable Technology integrated with living cells to sense and act upon threats to health

Organism(s) if applicable: Mammalian cells, bacteria

Shifting the paradigm of detecting varied states of health for personalized medicine will require the ability to take continual, multiplexed measurements of an individual. Having reliable and actionable diagnostic information could lead to more precise, more efficacious, and earlier medical interventions. The current state-of-the-art in non-invasive diagnostics are built upon highly sensitive electronic sensors that detect metabolites indicative of states of health; however, if biology was used as the sensor, a more precise interface between human biology and electronic systems could be created to enable continual diagnostics for health.

The ability to engineer sensors and response systems into mammalian cells provides the opportunity to create smart medicines that actively monitor disease and respond accordingly. For example, cells could sense metabolites, which stimulate artificial signaling networks that result in biosynthesis of checkpoint inhibitors that prevent immune systems from clearing cancer cells. In another example, cells could be engineered to detect cancer vasculature, attach and rapidly proliferate causing reduced blood flow in the local vascular. The ability to develop biological sensors (i.e. proteins, RNA, etc) that detect novel molecules and unique responses would significantly increase the number of approaches that could be used to manage or eliminate disease

Limitations of current cutting-edge sensors, which possess high sensitivity, include poor abiotic-biotic interface and issues with general incompatibility between human biology and manufactured sensors. By engineering bacteria and mammalian cells as the active sensing component of a wearable diagnostic platform these limitations can be overcome. Sensing is a naturally occurring and constant event in human and microbial biology, and it happens with high precision and high compatibility. Co-opting biology for precise multiplexed sensing could lead to the development of 'cyborg' devices comprised of engineered living systems that seamlessly interface with human biology and commonly used electronics to augment diagnostics for health, leading to completely novel methods to approach disease management. This requires high-level understanding of biological circuits and components such that rules that enable assembly of the synthetic circuit and a predictive output of the circuit is possible.

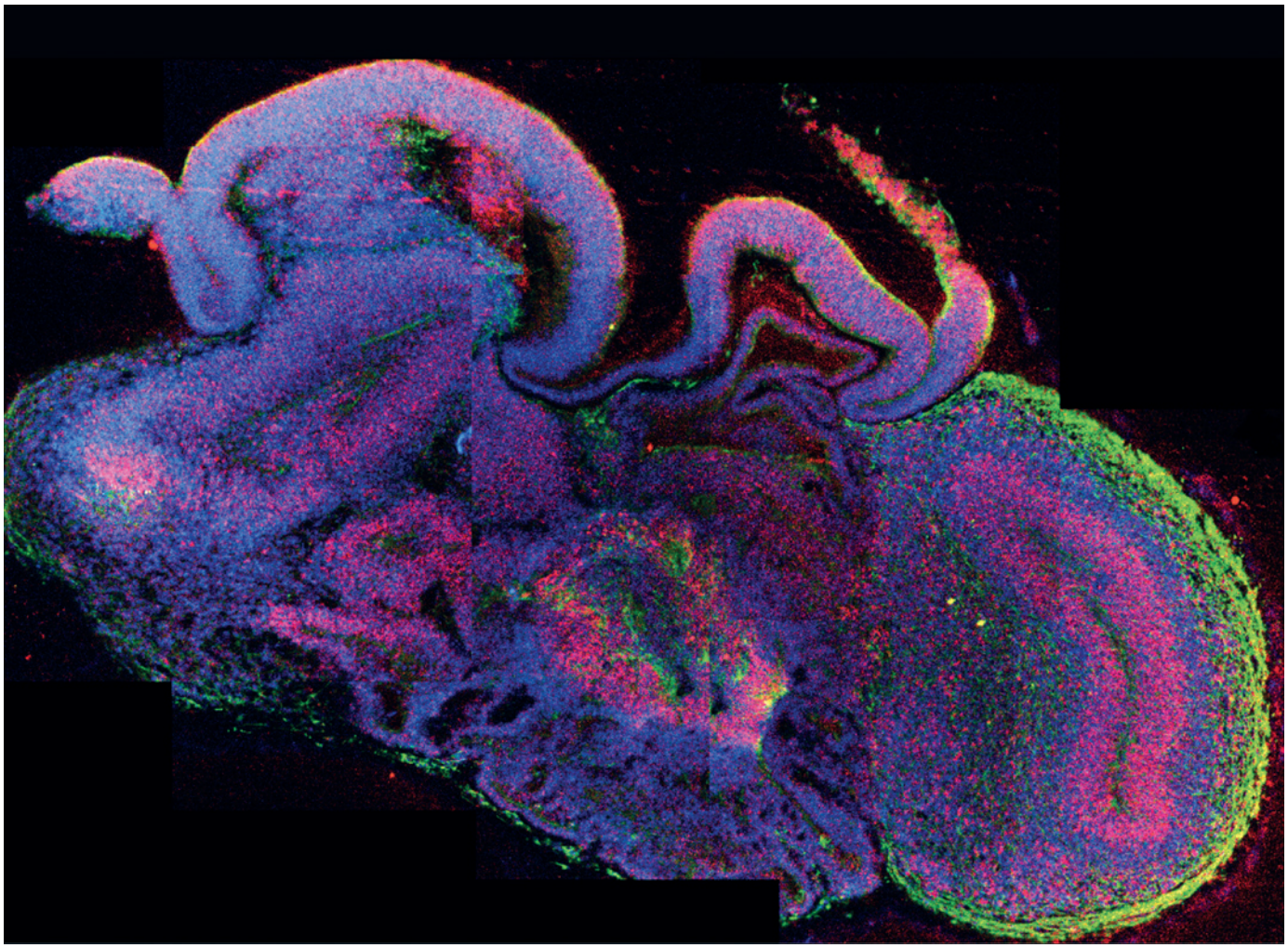
Desired outcome(s) that stretch current capabilities

- Sensors with high biocompatibility
- Sensors that are modular and can be predictably programmed for multiplexing
- Engineered cells to detect and continuously monitor multiple metabolic inputs from skin
- Biocompatible abiotic interface to support engineered cells, communicate with skin, and transduce biological signals into electronic signals
- Understanding of biocircuit components such that they can be considered unit operations where it is known how specific variables affect the output
- Understanding how context/niche variables affect the complex system behavior
- Predictive models for biocircuit components and assemblies of components

TECHNOLOGY FEATURE

ORGANS FROM THE LAB

The body's organs are more complex than any factory. Attempts to mirror their physiology in the laboratory are getting closer to capturing their intricacies.



J. KNOBLICH, M. LANCASTER, AUSTRIAN ACAD. SCI.

Stem cells can be coaxed into forming organized clusters called organoids, such as this brain model.

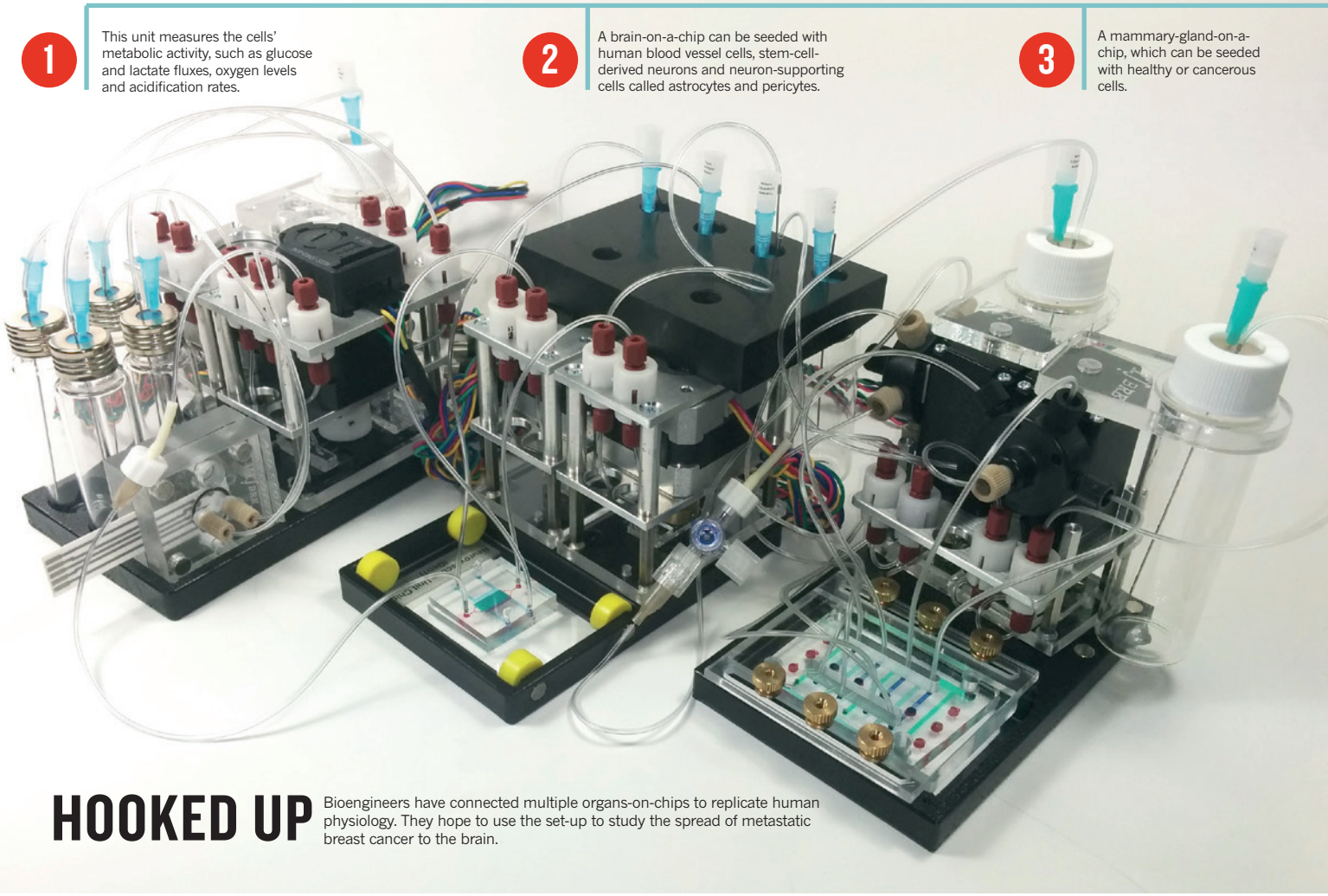
BY VIVIEN MARX

In their quest to create organs in the laboratory, researchers have come a long way. Engineered tissues are already used in medical research and have even entered clinical trials. But they are much simpler than the real thing. To make a stomach, a lab might use 3D printing to create a mould that could be seeded with the appropriate cells. But without cues provided by blood flow and interactions with other tissues, the result would be simply a stomach-shaped statue, unable to digest or growl. An organ is much more than a mass of cells arranged in a particular configuration: it also has support scaffolds, blood vessels to deliver nutrients and signal molecules, and a hierarchy of intricate control functions that can respond to internal and external cues.

All this makes it tough to build a functional, physiologically relevant organ in the lab, says Rosemarie Hunziker at the US National Institutes of Health, who manages the funding of programmes devoted to designing and building artificial organ systems.

But tissue engineers are making inroads into the problem. To try to tackle the biological complexity of organs, they can choose from various fabrication approaches. One method is to place cells into elaborate, but still simplified models of an organ the size of a microscope slide, which can then be connected together to probe how organs interact. These miniature 'organs-on-chips' provide a unique vantage into organ function and disease, and for applications such as toxicity tests of drug candidates. An alternative approach is to foster the ability of cells to self-assemble, in the hope that they will recapitulate actual organ development and reveal insights into the process.

Whatever the strategy, researchers can start with biologically simple approaches, and ▶



HOOKED UP

Bioengineers have connected multiple organs-on-chips to replicate human physiology. They hope to use the set-up to study the spread of metastatic breast cancer to the brain.

D. MARKOV, J. WIKSWO, VANDERBILT UNIV.

then add complexity to the model a little at a time. Just how similar an artificial version of an organ needs to be to its original depends on the questions that are being asked of it, Hunziker says. Artificial organs may look very different from their *in vivo* counterparts but nonetheless be useful for drug testing and basic research. Whether the goal is to understand an organ or to replace it, the eventual aim is an engineered system that functions as reliably as the real thing, Hunziker adds.

Researchers across the world are using these systems to address a wealth of important questions. They can, for example, help to reveal how cancer cells detach from a tumour to invade other tissues, and allow scientists to recapitulate processes in disease and development, such as what might go awry in neurodevelopmental disorders.

SYSTEMS THINKING

The most highly engineered organ models are the organs-on-chips that look the least like organs in the body. They are made using similar manufacturing techniques to those for silicon microchips in computers. First, a photosensitive material is layered onto silicon, and ultraviolet light is used to etch grooves in a desired pattern into silicone rubber. This guides the production of a 3D network of hollow tubes inside a rubbery rectangle the size of a computer memory stick. The tubes are seeded with cells of the desired types and hooked up to pumps and an external fluid source, providing inlets and outlets through which scientists can mimic blood flow and deliver nutrients and environmental signals. Perfusion by continuously flowing liquid mirrors the dynamic environment in organs. The set-up also lets bioengineers modulate a tissue's stiffness as well as mechanical, chemical and electrical cues to reproduce the signals that cells might receive in healthy or diseased states, says John Wikswo of Vanderbilt University in Nashville, Tennessee (see 'Hooked up'). Researchers can replicate inflammation, for example, by adding the molecular messengers known as cytokines and even living immune cells into the chips' channels — they then watch the inflammatory response that is characteristic of most tissues when damaged or infected[1].

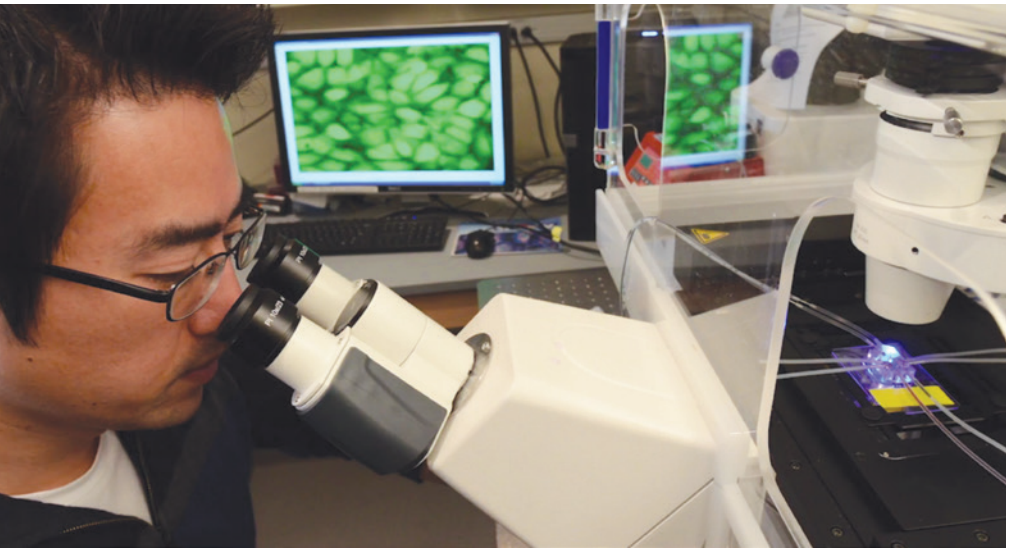
> *"The aim is an engineered system that functions as reliably as the real thing."*

The chips are usually transparent to allow high-resolution, real-time imaging of cells, says Donald Ingber director of Harvard University's Wyss Institute for Biologically Inspired Engineering in Boston, Massachusetts. The liver, kidney, lung, intestine, fat, muscle and the blood–brain barrier have all been rendered into chip form[2].

Now researchers are combining chips into multi-organ systems that can replicate some of the body's physiology. Gordana Vunjak-Novakovic and her team at Columbia University in New York City are building a model of the heart–liver–blood system with which to probe drug toxicity and disease. Wikswo at Vanderbilt, and his colleagues at the University of Pittsburgh, Pennsylvania, are linking organ chips together to predict the effects of potentially toxic chemicals and drugs. He believes that a liver–kidney model could identify safety problems before a drug reaches testing in humans, because these are the organs in which toxicity first becomes apparent. To emulate *in vivo* situations of health or disease, researchers can grow the appropriate cells in 3D support structures and explore their reactions to cues delivered into the system, says Wikswo.

The key to successful mimicry is attention to microstructure, says Hunziker. Careful placement of liver cells across a chip can better replicate real liver tissue, which has different zones close to and away from the main blood supply. These zones differ in the genes that are active, which results in differences in cell development and behaviour, and different responses to chemical stresses, she says.

Microscale organ systems allow experiments that cannot be done in cell cultures,



WYSS INST. HARVARD UNIV.

Dan Dongeun Huh at Harvard University's Wyss Institute tracks cellular events in a lung-on-a-chip.

animals or people, says Ingber. Organ chips lined with cells from individual patients enable the assessment of physiological differences between health and disease, and between people, in more detail and over a longer period than would be practical in people or in animal models, he says. Ingber's team has kept multi-organ chips going for more than a month. With an organ chip, it is also possible to adjust parameters to see what happens in a way that is not possible in a patient.

Several labs have formed spin-out companies to commercialize their model tissues. Emulate, in Cambridge, Massachusetts, founded by Ingber, is developing organ-on-chip systems for high-throughput drug screening and toxicity testing. The company Hepregen in Medford, Massachusetts, co-founded by bioengineer Sangeeta Bhatia at the Massachusetts Institute of Technology in Cambridge, uses the technique of 'micropatterning' to develop liver models in which different cell types are precisely placed to produce a platform that more closely mimics the complexity of the liver. These are being developed as drug-screening assays. HemoShear Therapeutics in Charlottesville, Virginia, founded by two University of Virginia scientists, has developed several organ modelling systems, including one that specifically mimics blood flow in tissues. In January, HemoShear began a collaboration with pharmaceutical company Pfizer of New York to find better ways to predict injuries to blood vessels, such as inflammation, that drug candidates might cause.

Right now, microfabrication is out of reach for many labs. However, there are some companies that offer services to make chips for labs that do not have the necessary equipment or expertise. And many universities offer microfabrication capabilities through core service centres. Meanwhile, labs at the cutting edge are working to make engineered chips better homes for living cells. One challenge is seeding cells evenly throughout the devices and maintaining their growth within the tiny channels, says Ingber. Another is that bubbles in the system can injure the cells.

THREE-DIMENSIONAL HELP

In contrast to organs on chips, soft scaffolds seeded with cells can result in artificial organs that look much more like the real thing. This approach blends a variety of synthetic materials to make a support system. It is then seeded with cells that grow and develop throughout the scaffold and thus become arranged in the desired configuration. In one well-known example from the early days of the field, Linda Griffith at Massachusetts Institute of Technology and Charles Vacanti at Massachusetts General Hospital in Boston and their colleagues used such a scaffold implanted under the skin of a mouse to guide bovine cartilage-forming cells to grow tissue in the form of a human outer ear[3,4]. The polymers in the scaffold degraded as the tissue formed, leaving behind the structure made of cartilage.

Today, Griffith and her team use a custom-built 3D printer to create highly intricate tissue scaffolds. A stream of photoreactive polymer spurts out of the instrument's nozzle and one layer at a time is exposed to ultraviolet light to stabilize the structure. Material is removed in an iterative process to a build micrometre-scale substructure.

Scientists have also developed ways of mimicking the mechanical stimuli that seem crucial to tissue development. For example, the early development of teeth in a mammalian embryo involves embryonic cells packing closely together. To mimic this process, Ingber's lab has developed a polymer that acts like shrink-wrap at certain temperatures[5]. When the polymer is warmed to body temperature, it shrinks and compacts the cells it encloses, which activates genes responsible for tooth development. Bioengineers could potentially use this material to induce tissue development for a variety of therapies, Ingber says, because cartilage and other internal organs (such as the lungs and kidneys) also undergo cellular compaction as they develop.

Incorporating blood flow into a model organ is particularly challenging, especially when trying to mimic the heart, which pumps rhythmically for a lifetime. Nevertheless, tissue engineers are well under way in their search for therapies to help heal injured hearts, and eventually perhaps, to find alternatives to heart transplants. Starting with a cell-sheet technology that does not incorporate a scaffold, Teruo Okano, a biomedical engineer at Tokyo Women's Medical University and his colleagues have made vascularized heart-tissue patches. The experiments start with thin layers of cells, which they can grow from a variety of cell types, including rat neonatal cardiac cells, human muscle cells and induced pluripotent stem (iPS) cells. These sheets are grown in dishes coated with a temperature-sensitive polymer. When the temperature is lowered, researchers can harvest sheets of cells that remain connected to each other without any kind of scaffold, says Okano's colleague Tatsuya Shimizu. In ongoing clinical trials, the team is evaluating 30 patients with heart problems who have received implanted tissue patches made from muscle-cell-derived sheets. These sheets secrete several types of cytokine, which promote blood-vessel formation and inhibit cell death in the patient's heart tissue. In the future, Shimizu and his colleagues hope to transplant tissues with beating cells.

But these sheets are not yet optimal. The ideal grafts need to be thick, especially because events such as heart attacks lead to thin heart tissue. The team has returned to the lab to engineer thicker patches that will be infiltrated with even more blood vessels and should remain viable for longer than the previous versions. They have grown cell sheets from human iPS cells and transplanted them into rats just under the skin on their backs, building up a patch 1 millimetre thick made of 30 cell sheets[6]. After implantation, small blood vessels from the rat sprouted through the layers. By moving smaller stacks into more vascularized areas, the researchers were able to cause more and more blood vessels to grow and eventually to connect the stack directly to larger blood vessels, such as the jugular vein. The heart-muscle cells continued to beat during six months of observation.

"Engineered tissues are starting to allow incisive experiments and even replacement therapies."

However, similar multiple surgical interventions could not be carried out in people. So the researchers have developed a technique that relies on a gel on which they can grow multiple layers of rat-cell sheets in the lab.

One day, Okano and his team hope, it will be possible to engineer such grafts for use in

M. LEVIN/J. MOROKUMA/A. WESTFALL/TUFTS UNIV.

humans with severe heart failure. The general approach could also be applied to engineer tissue to mimic the liver or kidneys.

SELF-ASSEMBLY

Other teams rely even more heavily on the intrinsic ability of cells to assemble into complex structures. Stem cells grown in suspension can be coaxed to form organized clusters called organoids, and these have been made for diverse tissues, including intestine, kidney and retina. Organoids are usually much smaller than the actual organ, just a few millimetres across, and with a much simpler assortment of cells, but some teams are now making organoids with more cell types and more complex structures, and even attempting to model the most daunting organ — the brain.

In 2013, Madeline Lancaster and Juergen Knoblich at the Institute of Molecular Biotechnology of the Austrian Academy of Sciences in Vienna generated human brain-tissue organoids about the size of a lentil[7]. They started from groups of human pluripotent stem cells, which are differentiated into neural tissue. Part of the protocol is to let the biology unfold.

Under the right conditions, the differentiating cells self-organize into a tight swirl of neural tissue with multiple cell types, including radial glial stem cells that give rise to cells in the brain such as neurons. The swirls even include rudimentary brain structures such as the beginning of a forebrain and retina. "We pretty much recapitulate the formation of neural tissue in a dish, letting it develop as it does in the embryo," says Knoblich.

These cerebral organoids have helped them to address questions that are hard to answer when growing neurons flat on the surface of a culture dish. The team studies the human neurodevelopmental disorder microcephaly, in which infants have markedly small brains. Although mice can be used to model the disorder, the animals do not show the extreme difference in brain size. But when the team reprogrammed skin cells from a patient with microcephaly into iPS cells that developed into cerebral organoids, the resulting structures bore clear characteristics of the disease. In these organoids, the radial glial cells proliferated less and, in some regions, differentiated into neurons prematurely. Even under normal conditions, radial glial cells do not proliferate in developing mice the way they do in humans, and so human organoids are a promising way to study how these neural precursor cells might be involved in the disorder.

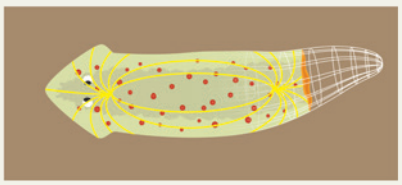
Lancaster and Knoblich also used organoids to assess the effects of a gene called *CDK5RAP2* that helps to guide cell division. The patient with microcephaly had a mutation in this gene that probably results in an aberrant protein. When the team introduced an undamaged protein into the organoid, some cells developed into types akin to radial glial cells, indicating that the loss of function of this gene contributes to microcephaly[7].

ORGAN BUILDING

Instead of replacing damaged or diseased organs, some labs have attempted to stimulate organ regeneration. Researchers have tapped into the innate chemical and bioelectric signalling of flatworms to induce the development of a second head. The regeneration occurs because the altered pattern is stored across the animal's bioelectric network.

1 An adult normal flatworm.

2 Manipulation of the worm's bioelectric network.



3 The worm is permanently altered by rewriting the memory of what should be regenerated.

There are still plenty of challenges for organoid technology. Lancaster and Knoblich point out that their organoids lack a blood supply and the interaction that neural tissue normally has with surrounding tissue. Over time, the organoids begin to die and lose resemblance to early brain tissue. The team has managed to keep them alive for as long as a year, but how useful late-stage organoids are for disease modelling remains to be seen, says Knoblich.

Another challenge is consistency, because the organoids take on different shapes from one batch to the next, he says. The lab is continuing to tinker with the growth conditions in the hope of overcoming these problems and being able to model more complex neurodevelopmental disorders.

LIKE AN EMBRYO

Bioengineers intent on building organ models can tap into the complexity of intercellular signals — the wealth of biochemical and bioelectrical messages that tell cells to differentiate, migrate, change shape or clump together to form an organ. This approach has already been used to regenerate legs on frogs that are normally too old to naturally regrow an amputated limb. That work, led by Michael Levin, a biomedical engineer at Tufts University in Medford, Massachusetts, might translate more readily to humans than many expect: children can regenerate fingertips, but adults cannot.

To get the frogs' legs to regrow, Levin and his team chemically tinkered with the pattern of electrical charge in the limb so that it matched the bioelectrical gradient found in the limbs of young animals[8], and this induced the cells at the tip of the amputated limb to grow. They also induced the formation of a two-headed flatworm (see 'Organ building'). The alterations the team had made caused a permanent change in the memory of what to form, which was encoded in an electrical circuit just like memories in our brains. Levin describes the process as "manipulating information" within the tissues in ways that cause predictable, large-scale changes in growth and form. That, he says, "makes the job of growing anything much easier".

Instead of trying to micromanage organ building, Levin believes in leveraging the body's own processes. He and his team are developing a physiological 'phrase book' of mathematical models and software. Scientists can use these software tools to search for factors to manipulate in their experiments, and so find ways to tell cells what tissues to build. The goal is to link data sets about genes, proteins and signalling pathways to knowledge about how organ shape and function is regulated. "These are the kinds of tools that will be indispensable as bioengineers confront the complexity barrier facing the creation of even simple organs," says Levin.

Ultimately, the usefulness of the tool is what is important, not the specific approach that is chosen. Engineered tissues are starting to allow incisive experiments and even replacement therapies. And perfectly mirroring nature may not, in all cases, be what is needed. "What is critical is that the organ has enough complexity to accomplish its function," says Hunziker.

Whether it be a patch for damaged hearts, a better toxicity test or an insight into a devastating brain disease, tissue engineering delivers what scientists crave: more understanding, and the potential to help people. ■

Vivien Marx *is technology editor for* Nature *and* Nature Methods

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RESEARCH ARTICLE SUMMARY

NEUROSCIENCE

A gut-brain neural circuit for nutrient sensory transduction

Melanie Maya Kaelberer, Kelly L. Buchanan, Marguerita E. Klein, Bradley B. Barth, Marcia M. Montoya, Xiling Shen, Diego V. Bohórquez*

INTRODUCTION: In 1853, Sydney Whiting wrote in his classic *Memoirs of a Stomach*, "...and between myself and that individual Mr. Brain, there was established a double set of electrical wires, by which means I could, with the greatest ease and rapidity, tell him all the occurrences of the day as they arrived, and he also could impart to me his own feelings and impressions." Historically, it is known that the gut must communicate with the brain, but the underlying neural circuits and transmitters mediating gut-brain sensory transduction still remain unknown. In the gut, there is a single layer of epithelial cells separating the lumen from the underlying tissue. Dispersed within this layer reside electrically excitable cells termed enteroendocrine cells, which sense ingested nutrients and microbial metabolites. Like taste or olfactory receptor cells, enteroendocrine cells fire action potentials in the presence of stimuli. However, unlike other sensory epithelial cells, no synaptic link between enteroendocrine cells and a cranial nerve has been described. The cells are thought to act on nerves only indirectly through the slow endocrine action of hormones, like cholecystokinin. Despite its role in satiety, circulating concentrations of cholecystokinin peak only several minutes after food is ingested and often after the meal has ended. Such a discrepancy suggests that the brain perceives gut sensory cues through faster neuronal signaling. Using a mouse model, we sought to identify the underpinnings of this neural circuit that transduces a sense from gut to brain.

RATIONALE: Our understanding of brain neural circuits is being propelled forward by the emergence of molecular tools that have high topographical and temporal precision. We adapted them for use in the gut. Single-cell quantitative real-time polymerase chain reaction and single-cell Western blot enabled the assessment of synaptic proteins. A monosynaptic rabies virus revealed the neural circuit's synapse. The neural circuit was recapitulated in vitro by using nodose neurons cocultured with either minigut organoids or purified enteroendocrine cells. This system, coupled to optogenetics and whole-cell patch-clamp recording, served to determine the speed of transduction. Whole-nerve electrophysiology, along with optical excitation and silencing, helped to uncover the neurotransmission properties of the circuit in vivo. The underlying neurotransmitter was revealed by using receptor pharmacology and a fluorescent reporter called iGluSnFR.

RESULTS: Single-cell analyses showed that a subset of enteroendocrine cells contains presynaptic adhesion proteins, including some necessary for synaptic adhesion. Monosynaptic rabies tracing revealed that enteroendocrine cells synapse with vagal nodose neurons. This neuroepithelial circuit connects the intestinal lumen with the brainstem in one synapse. In coculture, this connection was sufficient to transduce a sugar stimulus from enteroendocrine cells to vagal neurons. Optogenetic activation of enteroendocrine cells elicited excitatory postsynaptic potentials in connected nodose neurons within milliseconds. In vivo recordings showed that enteroendocrine cells are indeed necessary and sufficient to transduce a sugar stimulus to the vagus. By using iGluSnFR, we found that enteroendocrine cells synthesize the neurotransmitter glutamate, and pharmacological inactivation of cholecystokinin and glutamate receptors revealed that these cells use glutamate as a neurotransmitter to transduce fast, sensory signals to vagal neurons.

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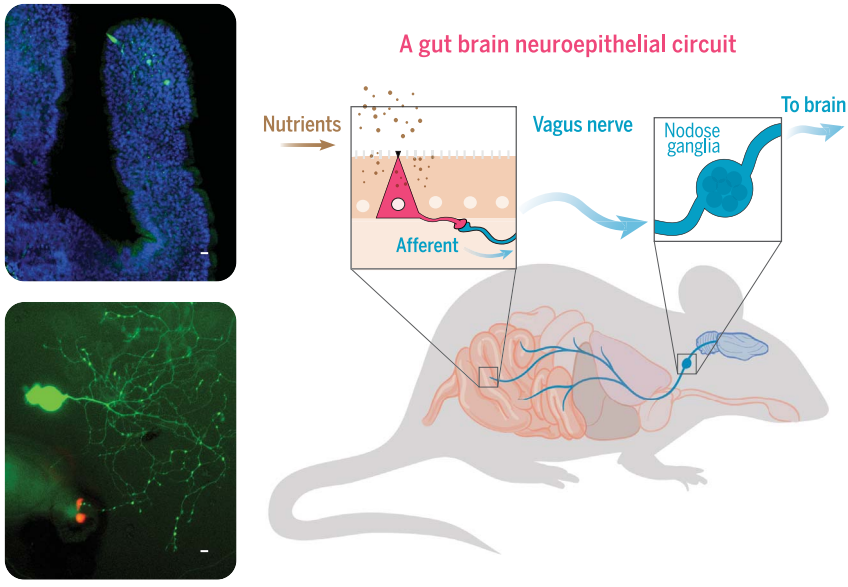
CONCLUSION: We identified a type of gut sensory epithelial cell that synapses with vagal neurons. This cell has been referred to as the gut endocrine cell, but its ability to form a neuroepithelial circuit calls for a new name. We term this gut epithelial cell that forms synapses the neuropod cell. By synapsing with the vagus nerve, neuropod cells connect the gut lumen to the brainstem. Neuropod cells transduce sensory stimuli from sugars in milliseconds by using glutamate as a neurotransmitter. The neural circuit they form gives the gut the rapidity to tell the brain of all the occurrences of the day, so that he, too, can make sense of what we eat. ■



The neuropod cells. (Top left) Neuropod cells synapse with sensory neurons in the small intestine, as shown in a confocal microscopy image. Blue indicates all cells in villus; green indicates green fluorescent protein (GFP) in neuropod cell and sensory neurons. (Bottom left) This neural circuit is recapitulated in a coculture system between organoids and vagal neurons. Green indicates GFP in vagal neuron; red indicates tdTomato red fluorescence in neuropod cell. (Right) Neuropod cells transduce fast sensory signals from gut to brain. Scale bars, 10 μm.

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RESEARCH ARTICLE

NEUROSCIENCE

A gut-brain neural circuit for nutrient sensory transduction

Melanie Maya Kaelberer[1], Kelly L. Buchanan[2], Marguerita E. Klein[1], Bradley B. Barth[3], Marcia M. Montoya[3], Xiling Shen[3], Diego V. Bohórquez[1,4,5]*

The brain is thought to sense gut stimuli only via the passive release of hormones. This is because no connection has been described between the vagus and the putative gut epithelial sensor cell—the enteroendocrine cell. However, these electrically excitable cells contain several features of epithelial transducers. Using a mouse model, we found that enteroendocrine cells synapse with vagal neurons to transduce gut luminal signals in milliseconds by using glutamate as a neurotransmitter. These synaptically connected enteroendocrine cells are referred to henceforth as neuropod cells. The neuroepithelial circuit they form connects the intestinal lumen to the brainstem in one synapse, opening a physical conduit for the brain to sense gut stimuli with the temporal precision and topographical resolution of a synapse.

Whereas touch, sight, sound, scent, and taste are transduced to the brain by innervated epithelial sensor cells (*1*), perception of gut stimuli is thought to occur only indirectly, through the slow action of hormones (*2*). The putative gut epithelial sensor cells—enteroendocrine cells—are assumed to lack synapses with the cranial nerve that innervates the viscera—the vagus (*3*).

Coined in the 1930s (*4*), the term enteroendocrine is rooted in the notion that nutrients stimulate the release of hormones. These neuropeptides either enter the bloodstream or act on nearby nerves minutes to hours after ingesting a meal (*5*). But enteroendocrine cells have several features of epithelial transducers: They have mechanical (*6*), olfactory (*7*), and taste (*8*) receptors; their membranes contain voltage-gated ion channels that render them electrically excitable (*9*); and they are capable of forming synapses (*10*). Almost two-thirds of enteroendocrine cells synapse with adjacent nerves in the intestinal and colonic mucosa (*10*). Similar features have been confirmed in a subset of colonic enteroendocrine cells known as enterochromaffin (*11*). Therefore, we hypothesized that enteroendocrine cells synapse with the vagus to transduce a sense from gut to brain.

Innervated epithelial sensors in the gut

Using mass spectroscopy (see methods and table S1), we confirmed that enteroendocrine cells express multiple neuropeptides (*12*, *13*), including both cholecystokinin (CCK) and peptide YY (PYY). Thus, we identified these cells using CCK and

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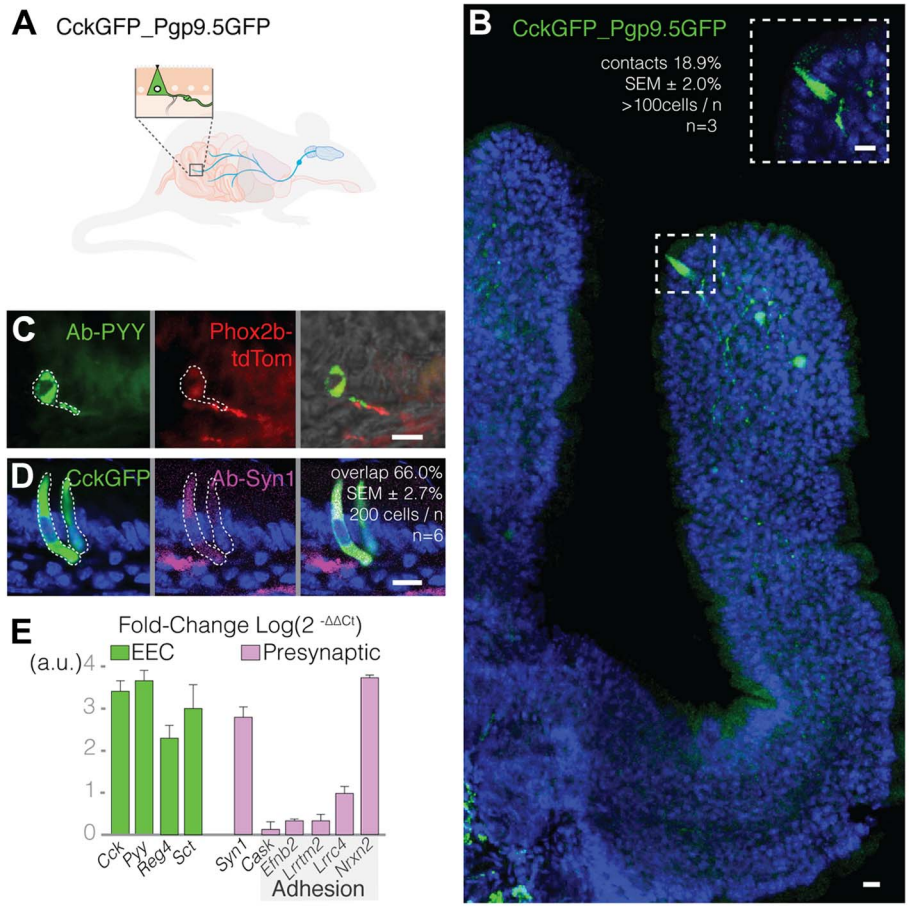

Fig. 1. Enteroendocrine cells contact sensory nerve fibers. (**A**) CckGFP_Pgp9.5GFP mice express GFP in CCK-enteroendocrine cells and Pgp9.5 sensory nerve fibers. The two cell types are shown in the enlarged view, with the CCK-enteroendocrine cell represented by a triangle. (**B**) Confocal microscopy image of proximal small intestine villus showing a GFP-labeled CCK-enteroendocrine cell and GFP-labeled Pgp9.5 nerve fibers; 18.9 ± 2.0% SEM of CckGFP cells contact Pgp9.5 fibers (n = 3 mice, >100 cells per mouse). (**C**) PYY-stained enteroendocrine cells (left, green) in the colon contact Phox2b vagal nerve fibers (center, red) in a Phox2bCRE_tdTomato mouse; merged image is shown on the right. (**D**) Two-thirds of CckGFP (green) enteroendocrine cells colocalize with the presynaptic marker synapsin-1 (purple) (n = 6 mice, 200 cells per mouse). (**E**) Real-time quantitative polymerase chain reaction (qPCR) expression levels of presynaptic transcripts, including genes encoding for synaptic adhesion proteins (n = 3 mice, >10,000 cells per cell type per mouse; error bars indicate mean ± SEM; a.u., arbitrary units; EEC, enteroendocrine cell). All scale bars, 10 μm.

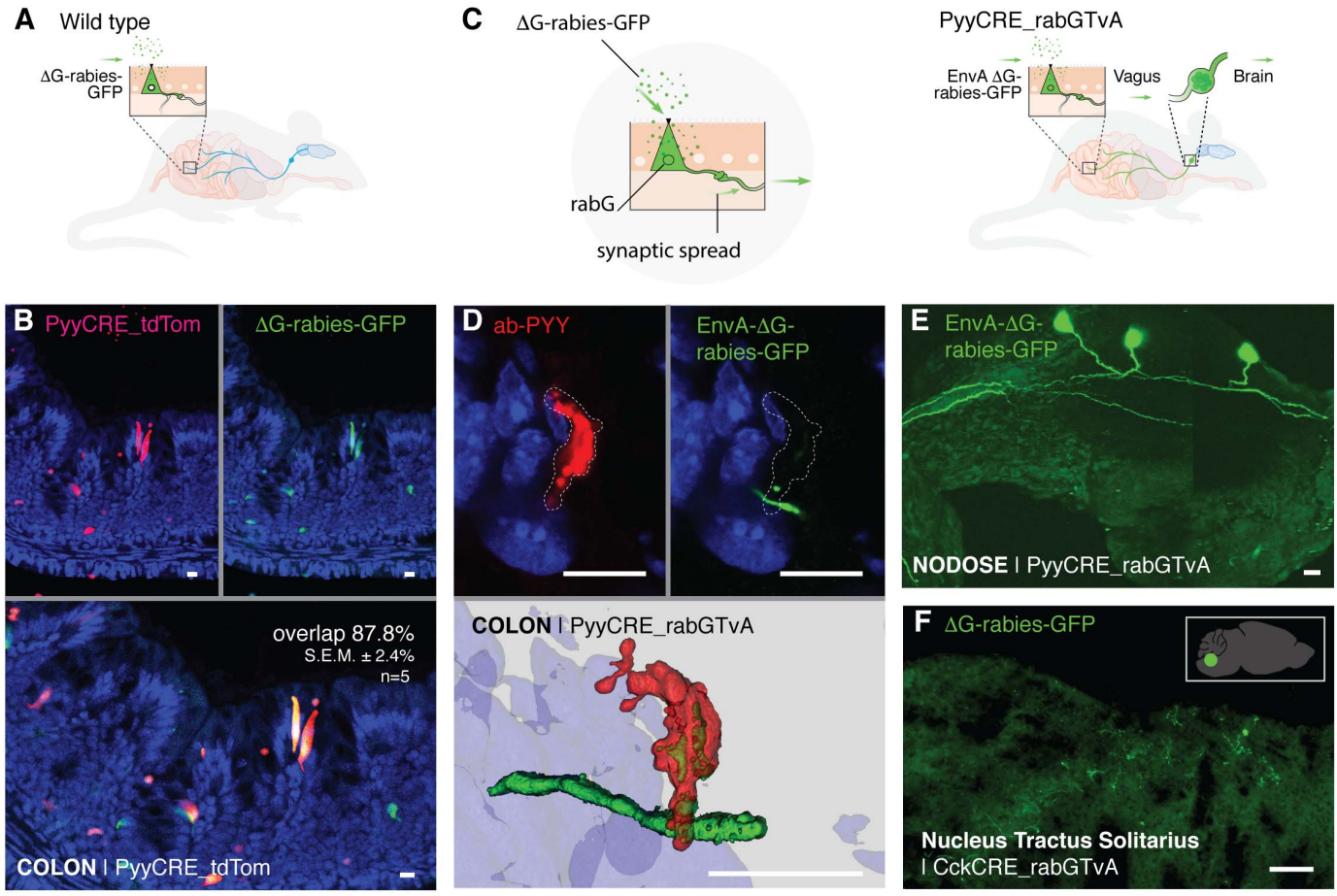

Fig. 2. Enteroendocrine cells of the colon and small intestine synapse with vagal nodose neurons. (**A**) Model of ΔG-rabies-GFP enema delivery. (**B**) PYY cells expressing tdTomato (top left, red) are infected by ΔG-rabies-GFP (top right, green). Overlay (bottom) shows overlap of 87.8 ± 2.4% SEM (n = 5 mice). In the absence of G glycoprotein (ΔG), ΔG-rabies-GFP does not spread beyond the infected PYY cell. (**C**) EnvA-ΔG-rabies-GFP virus enters cells via the TvA receptor and spreads by using the rabG protein within specific cells. (**D**) EnvA-ΔG-rabies-GFP (top right, green) infects PYY cells (top left, red) and spreads synaptically to underlying colon nerve fibers. Three-dimensional reconstruction (bottom) shows EnvA-ΔG-rabies-GFP–infected PYY cell and monosynaptically labeled nerve fiber. (**E**) EnvA-ΔG-rabies-GFP enema infects colonic enteroendocrine cells and spreads onto vagal neurons in the nodose ganglion (green). (**F**) In additional experiments, ΔG-rabies-GFP delivered by oral gavage spreads in the intestinal lumen of CckCRE_rabG-TvA mice to label the nucleus tractus solitarius (green). This neuroepithelial circuit links the intestinal lumen with the brainstem. The inset shows the location of the nucleus tractus solitarius in the mouse brain. All scale bars, 10 μm.

PYY. In the mouse small intestine and colon, enteroendocrine cells contacted sensory nerve fibers (Fig. 1, A to C). About one in five CCK-expressing enteroendocrine (CCK-enteroendocrine) cells contacted Pgp9.5 sensory nerve fibers that express green fluorescent protein (Pgp9.5GFP nerve fibers) (18.9 ± 2.0% SEM, >100 cells per mouse, n = 3 mice) (Fig. 1B). CCK-enteroendocrine cells immunoreact with an antibody against the presynaptic protein synapsin-1 (Fig. 1D), showing that these connections have synaptic features. Furthermore, using single-cell Western blot, we found that 83% of enteroendocrine cells contain synapsin-1 (164 of 198 CckGFP cells analyzed) (fig. S1). Compared with other intestinal epithelial cells, purified CCK-enteroendocrine cells express the synaptic adhesion genes *Efnb2*, *Lrrtm2*, *Lrrc4*, and *Nrxn2* (Fig. 1E), showing that these epithelial sensors have the machinery to form synapses.

From gut lumen to brainstem in one synapse

To determine the source of neurons synapsing with enteroendocrine cells, we used a modified rabies virus (ΔG-rabies-GFP) (*10*). This rabies virus infects neurons but lacks the G glycoprotein necessary for transsynaptic spread (Fig. 2A) (*14*). In intestinal organoids, rabies prefers to infect enteroendocrine cells over other epithelial cells (fig. S2A). In the mouse, when introduced into the lumen of the colon by enema, almost 9 out of 10 infected cells are PYY-enteroendocrine cells (87.8 ± 2.4% SEM, n = 5 mice) (Fig. 2B) (*10*). The lack of fluorescence in the underlying mucosa shows that, in the absence of its G glycoprotein, the rabies virus does not spread beyond infected enteroendocrine cells.

To trace the neural circuit, we bred a mouse (strain PyyCRE_rabG-TvA) in which enteroendocrine cells express the G glycoprotein (rabG) (Fig. 2C). In these mice, rabies delivered by enema infects enteroendocrine cells and spreads through synapses onto nerves. Some of the nerve fibers can be traced to vagal nodose neurons (control group: 0 positive out of 3 PyyCRE_tdTomato mice; experimental group: 4 positive out of 5 PyyCRE_rabG-TvA mice). Furthermore, an enema of the chemical tracer dye Fast Blue labeled both nodose ganglia, confirming that the vagus indeed innervates the distal colon (*15*). In control experiments in which the right cervical vagus was severed, the Fast Blue enema labeled the left (intact) but not the right (vagotomized) nodose (fig. S3).

Because ΔG-rabies-GFP can infect any neuronal cell it contacts, we restricted its entrance to enteroendocrine cells only by using an EnvA-coated rabies (EnvA-ΔG-rabies-GFP) (Fig. 2C). EnvA is an envelope glycoprotein of the avian sarcoma leukosis virus that binds to the avian

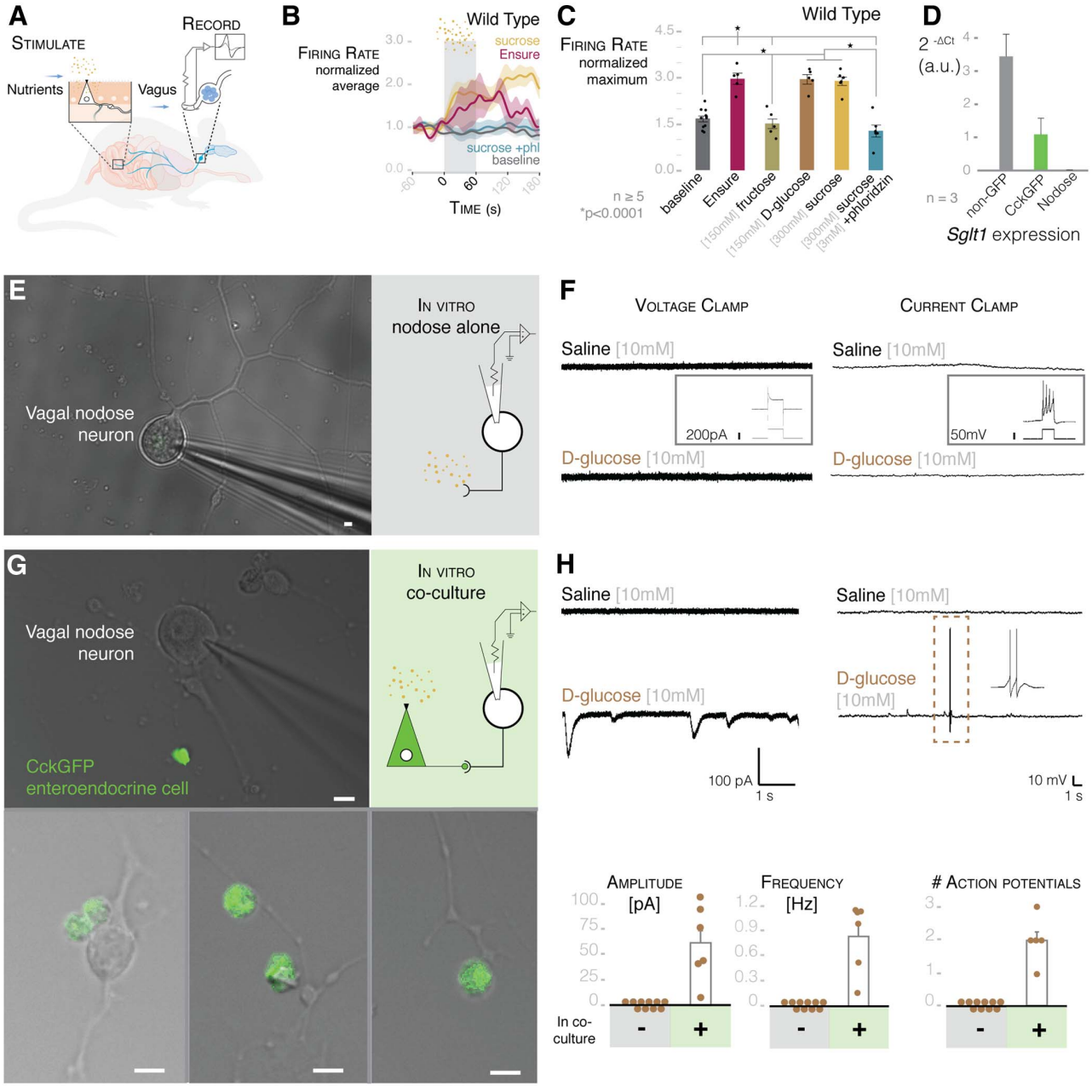

Fig. 3. Enteroendocrine cells transduce glucose stimuli onto vagal neurons. (**A**) Model of intestinal intraluminal perfusion and vagal nerve electrophysiology. (**B**) Normalized traces for baseline, Ensure, 300 mM sucrose, and 300 mM sucrose with 3 mM phloridzin (phl) in wild-type mice. Gray bar indicates treatment period; shading indicates SEM. (**C**) Ensure, 300 mM sucrose, and 150 mM D-glucose stimulate vagal firing rate, which is abolished by SGLT1-blocker phloridzin [$n \geq 5$ mice; $^*P < 0.0001$, analysis of variance (ANOVA) with post hoc Tukey's HSD test; error bars indicate SEM]. (**D**) Intestinal epithelial cells express *Sglt1*, but nodose neurons do not ($n = 3$ mice, >10,000 cells per cell type per mouse; data are presented as mean ± SEM). (**E**) Nodose neurons cultured alone for electrophysiology (widefield microscopy image on left, model on right). (**F**) Nodose neurons do not respond to 10 mM glucose in voltage-clamp (left trace) or current-clamp (right trace) mode. Insets show that neurons respond to voltage or current pulse, indicating viability. (**G**) Nodose neurons cocultured with GFP-positive enteroendocrine cells for electrophysiology (image on left, model on right). Innervated enteroendocrine cells are shown at the bottom. (**H**) In coculture, glucose evoked EPSCs (top left) and action potentials (top right) in connected neurons (scale of current or voltage and time are shown below the traces). Dashed-line box indicates action potentials expanded in right inset. Quantification of EPSC amplitude and frequency (bottom left and center; $n = 21$ neurons alone; $n = 6$ neurons connected to enteroendocrine cells) and action potentials (bottom right; $n = 21$ alone; $n = 5$ neurons connected to enteroendocrine cells) in GFP-negative (–) and -positive (+) cells. All scale bars, 10 μm.

TvA receptor. Therefore, EnvA-ΔG-rabies-GFP only infects cells that express the TvA receptor. In the PyyCRE_rabG-TvA mouse, PYY-enteroendocrine cells express the TvA receptor, and an enema of EnvA-ΔG-rabies-GFP infects enteroendocrine cells exclusively. Then, it spreads to synaptically connected neurons. Of a total of nine mice, five had visible infection of nerve fibers in the colon (Fig. 2D), and two of those five had visible infection in the vagal nodose (Fig. 2E and movie S1; confirmed in vitro in fig. S4). Labeled fibers were also observed in the dorsal root ganglia of four out of the five infected mice (fig. S5). No infection of nerves was observed in littermate controls that lack CRE recombinase ($n = 5$ mice).

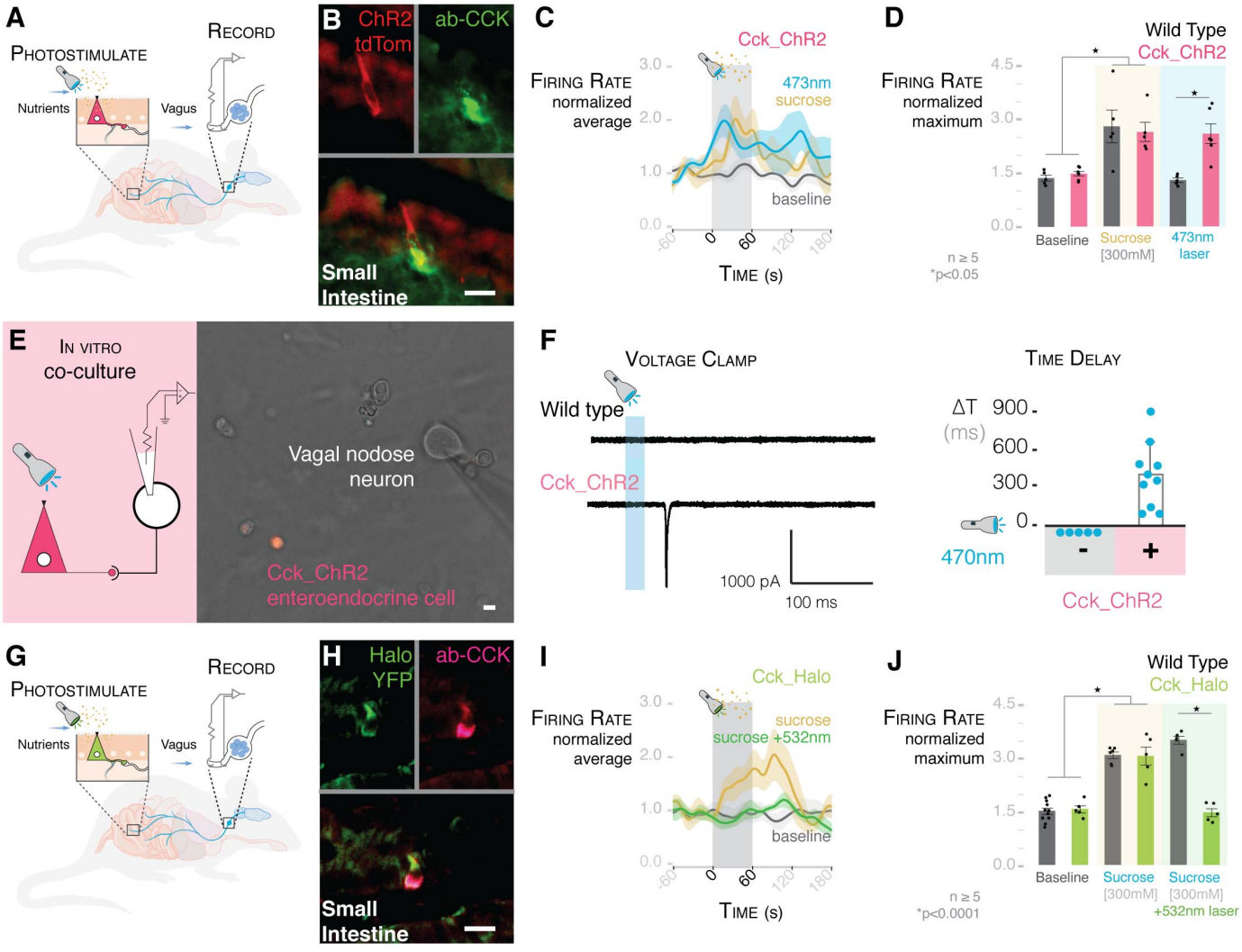

Fig. 4. Millisecond transduction from enteroendocrine cells to vagal neurons. (**A**) Model of intraluminal photostimulation and vagal electrophysiology. (**B**) In CckCRE_ChR2-tdTomato mice, intestinal enteroendocrine cells express ChR2. (**C**) Normalized traces for 473-nm intraluminal laser, 300 mM sucrose, and baseline in CckCRE_ChR2 mice. Shading indicates SEM. (**D**) 473-nm intraluminal laser stimulates vagal firing rate in CckCRE_ChR2, but not wild-type, mice ($n \geq 5$ mice; $^*P < 0.05$, ANOVA with post hoc Tukey's HSD test; error bars indicate SEM). (**E**) Patch-clamp electrophysiology of neurons (model on left) in coculture with CckCRE_ChR2 cells (image on right). (**F**) In coculture, 473-nm photostimulation evoked EPSCs (trace on left) in connected nodose neurons (quantification on right) ($n = 9$ neurons connected to enteroendocrine cells; –, neurons alone; +, neurons cocultured with enteroendocrine cells; ΔT, time between stimulus and onset of EPSCs). Scale of current and time is shown below the trace. (**G**) Model of intraluminal photoinhibition and vagal electrophysiology. (**H**) In CckCRE_Halo-YFP mice, intestinal enteroendocrine cells express halorhodopsin (eNpHR3.0). (**I**) Normalized traces for baseline, 300 mM sucrose, and 300 mM sucrose with 532-nm intraluminal laser. Shading indicates SEM. (**J**) In CckCRE_Halo, but not wild-type, mice, a 532-nm intraluminal laser abolishes the effect of sucrose on vagal firing rate ($n \geq 5$ mice per group; $^*P < 0.0001$, ANOVA with post hoc Tukey's HSD test; error bars indicate SEM). All scale bars, 10 μm.

Delivering the virus by oral gavage into CckCRE_rabG-TvA mice yielded similar results (fig. S5). In these mice, labeled vagal nodose neurons projected upstream into the nucleus tractus solitarius of the brainstem (Fig. 2F). Monosynaptic rabies tracing shows a neural circuit linking the small intestine or colon lumen to the brainstem in one synapse.

A gut-brain neural circuit in a dish

In coculture, vagal nodose neurons clearly extended axons to enteroendocrine cells of intestinal organoids (fig. S4A and movie S2). We traced this neural circuit in vitro using EnvA-ΔG-rabies-GFP to confirm that synapses are formed. To ensure that only infected neurons spread EnvA-ΔG-rabies-GFP, nodose neurons were incubated with virus before coculture with organoids. In control experiments, EnvA-ΔG-rabies-GFP did not infect wild-type nodose neurons (fig. S4B). However, EnvA-ΔG-rabies-GFP infected vagal nodose neurons that express the TvA receptor (Phox2bCRE_rabG-TvA). Forty-eight to 72 hours after coculture, the virus spread onto enteroendocrine cells in intestinal organoids, demonstrating synaptic connection in vitro (fig. S4C).

Transduction of a sense from gut to brain

We tested the function of this neuroepithelial circuit using luminal stimuli and whole-nerve electrophysiology. The initial stimulus used was Ensure—a whole-nutrient solution. Luminal Ensure stimulated an increase in vagal firing rate (Fig. 3, A to C). Next, we focused on a distinctive nutrient, sugar. When ingested, sugar is sensed in the duodenum, but it is unclear whether this stimulus is sensed by the vagus directly or transduced via enteroendocrine cells (*16*). In wild-type mice, perfusing the sugar sucrose (100 to 300 mM) significantly increased vagal firing rate

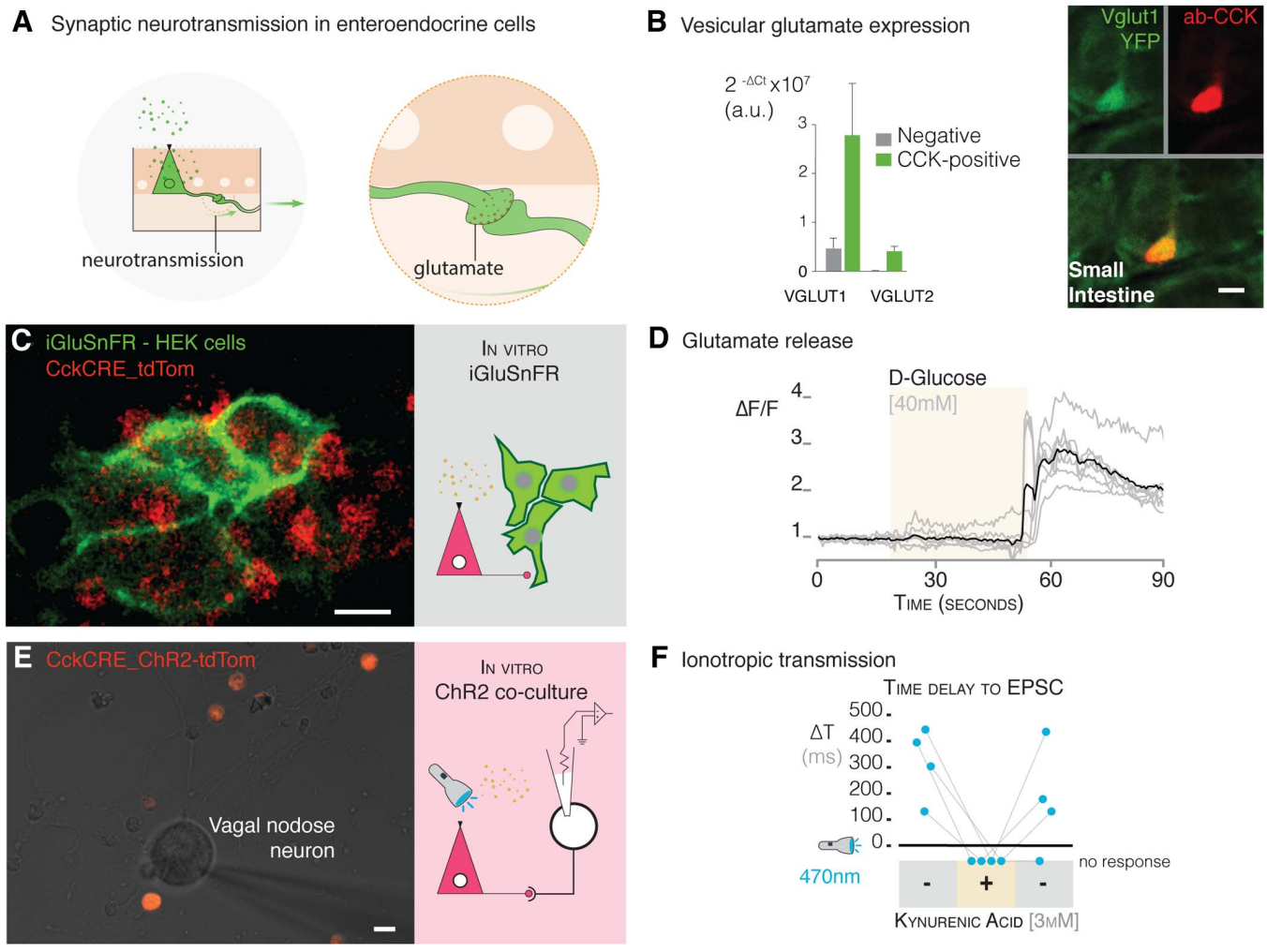

Fig. 5. Glutamate is used as a neurotransmitter between enteroendocrine cells and neurons. (**A**) Model of synaptic neurotransmission in enteroendocrine cells. (**B**) Enteroendocrine cells express the vesicular glutamate genes encoding VGLUT1 and 2 (*Slc17a7* and *Slc17a6*) (quantification by qPCR on left, confocal microscopy images on right). (**C**) CckCRE_tdTomato enteroendocrine cells were cocultured with HEK cells that express the glutamate sniffer protein, iGluSnFR (multiphoton microscopy image on left, model on right). (**D**) A stimulus of 40 mM D-glucose administered during the time period indicated by the beige shading elicits a response in iGluSnFR-HEK cells (n = 3 cultures; individual cell, gray trace; average of all cells, black trace). ΔF/F, difference in fluorescence intensity between resting state and after stimulus. (**E**) Coculture with neurons and CckCRE_ChR2 cells (multiphoton microscopy image on left) for electrophysiology of neurons and microperfusion of the glutamate-receptor blocker kynurenic acid (model on right). (**F**) In coculture, 473-nm photostimulation evoked EPSCs in connected nodose neurons, these currents were abolished, and no response was observed with the addition (+) of 3 mM kynurenic acid. The response was recovered after the drug was washed off (indicated by second "–" condition on right) (n = 4 neurons connected to enteroendocrine cells). All scale bars, 10 μm.

over baseline (Fig. 3, B and C, and fig. S6). D-Glucose (150 mM), but not fructose (150 mM), had the same effect. No effect was observed when the vagus was severed (fig. S7), when hyperosmolar phosphate-buffered saline was perfused (700 mosmol), or when sucrose was applied intraperitoneally (300 mM) (fig. S8). The vagal response was abolished when sucrose was perfused with phloridzin, a blocker of the electrogenic glucose transporter SGLT1 (*17*) (Fig. 3, B and C). A transcription profile showed that, unlike vagal nodose neurons, CCK-enteroendocrine cells express *Sglt1*, suggesting that the stimulus is transduced by the epithelial cells (Fig. 3D).

Evidence gathered on dissociated colonic enteroendocrine cells, and the enteroendocrine-like cell line STC1, has shown that enteroendocrine cells sense glucose (*18*). We therefore packaged a rabies virus to carry the calcium reporter GCaMP6s (ΔG-rabies-GCaMP6s) and used it to infect enteroendocrine cells in intestinal organoids. When presented with D-glucose (10 mM), calcium transients were elicited in CCK-enteroendocrine cells (56.0 ± 20.0% of the KCl control response; n = 3 cells) (fig. S2, B to D). One previous report found that rat nodose neurons respond to glucose (*19*). However, in contrast with enteroendocrine cells, vagal neurons are unlikely to face steep changes in glucose concentrations because they do not contact the intestinal lumen (*20*). We therefore measured calcium transients in dissociated nodose neurons and found that D-glucose (10 mM) did not elicit a response (fig. S9, A and B) (n = 246 cells pooled from three mice).

To discard the possibility that only nodose neurons innervating the intestine may sense glucose, we retrotraced them by injecting Fast Blue dye into the duodenum (fig. S9C). In Fast Blue–labeled vagal neurons, no calcium response was observed in the presence of D-glucose (20 mM) (fig. S9C). Furthermore, neither excitatory currents nor action potentials were observed in the presence of a D-glucose (10 to 20 mM) stimulus using patch-clamp electrophysiology (Fig. 3, E and F). Current injection demonstrated that these cultured nodose neurons were functionally viable (inset of Fig. 3F).

We then cocultured vagal nodose neurons with intestinal enteroendocrine cells (*10*). After 48 to 72 hours, there were visible connections between neurons and enteroendocrine cells (Fig. 3G). Coculturing did not alter the resting membrane potential, the current, or the spike threshold of the vagal nodose neurons. However, a D-glucose (10 mM) stimulus now evoked excitatory postsynaptic currents (EPSCs) and action potentials in those neurons connected to enteroendocrine cells (Fig. 3H). In voltage-clamp mode, the average current of the EPSCs was 61.65 ± 15.21 pA, and the average frequency was 0.86 ± 0.17 Hz (n = 6 neurons connected to enteroendocrine cells). In current-clamp mode, this in vitro connection was sufficient to elicit action potentials in the connected neurons (average of 2 ± 0.32 action potentials, n = 5 neurons connected to enteroendocrine cells).

Synaptic speed and specificity

Two recent reports have shown that hypothalamic neurons controlling food intake are inhibited by nutrients within seconds of the nutrients entering the duodenum (*21*, *22*). Therefore, it is likely that enteroendocrine cells transduce sensory signals from nutrients at a much faster rate than previously thought possible. To test the speed of transduction, we bred a mouse (strain CckCRE_ChR2-tdTomato) in which enteroendocrine cells express channelrhodopsin 2 (ChR2) —an excitatory light-gated ion channel activated by 473-nm light (Fig. 4, A and B). A 473-nm stimulus applied to these cells elicited excitatory currents and significantly reduced food intake by the mice, showing functional expression of the channel (fig. S10) (see methods).

Vagal firing rate is significantly increased when a 473-nm laser stimulus is applied to the duodenal lumen of CckCRE_ChR2 mice. No response was observed in wild-type controls (Fig. 4, C and D; for laser-activation controls, see fig. S11). The firing rate increased rapidly after laser stimulation, reaching its peak, on average, in 72.7 ± 20.9 s (fig. S12). In vitro, vagal nodose neurons cultured alone did not respond to photostimulation. To determine the precise transduction speed, we cocultured them with CckCRE_ChR2 enteroendocrine cells (Fig. 4E). In vagal nodose neurons connected to enteroendocrine cells, a 470-nm photostimulus elicited EPSCs within 60 to 800 ms (n = 9 pairs) (Fig. 4F).

To test the specificity of transduction, we bred a mouse (CckCRE_Halo-YFP) in which intestinal enteroendocrine cells express the light-inhibitory channel eNpHR3.0 (halorhodopsin)—an inhibitory light-gated ion channel activated by 532-nm light (Fig. 4, G and H)—and yellow fluorescent protein (YFP). In these mice, luminal sucrose (300 mM) elicited a vagal response; however, when a 532-nm laser stimulus was presented along with the sucrose, vagal activity was abolished (Fig. 4, I and J; for laser activation controls, see fig. S13). In control wild-type mice, a 532-nm laser stimulus failed to attenuate the sucrose response. These data revealed that enteroendocrine cells are necessary and sufficient to transduce a glucose stimulus onto vagal neurons within milliseconds.

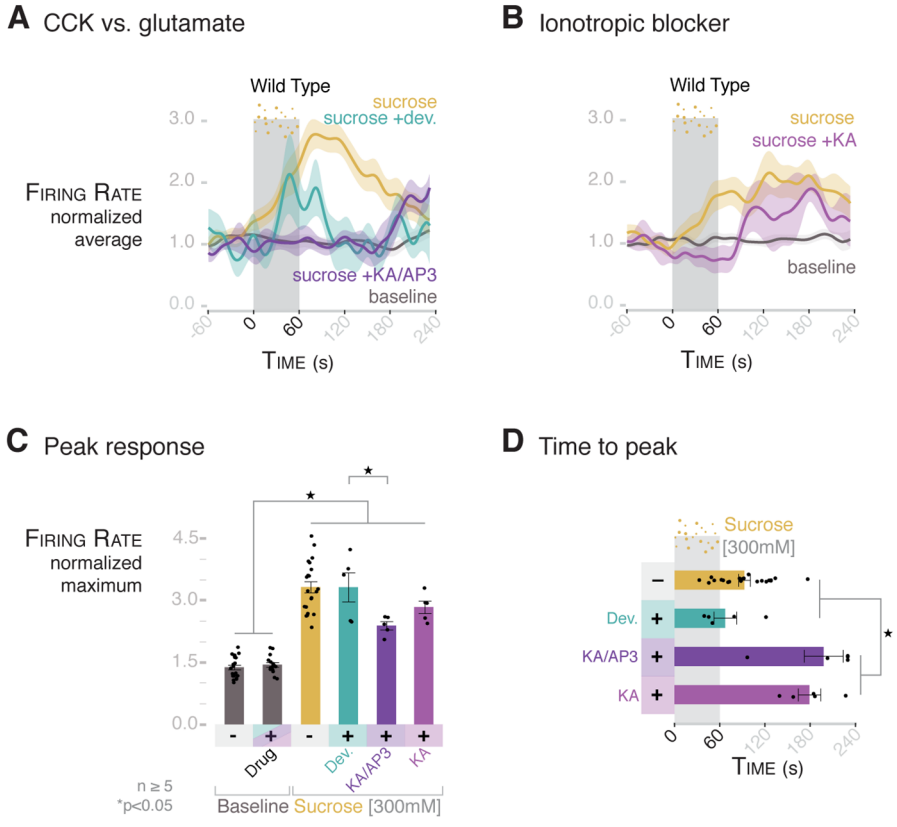

Fig. 6. The rapid vagal response to sucrose is dependent on glutamate, whereas CCK contributes to the prolonged response. (**A**) Normalized traces for baseline, 300 mM sucrose, 300 mM sucrose after treatment with 2 mg/kg devazepide, and 300 mM sucrose after treatment with glutamate inhibitor cocktail KA/AP3 [150 μg/kg kynurenic acid (KA) with 1 mg/kg DL-2-amino-3-phosphonoproprionic acid (AP-3)] in wild-type mice. Shading indicates SEM. (**B**) Normalized traces for baseline, 300 mM sucrose, and 300 mM sucrose after treatment with 150 μg/kg KA in wild-type mice. Shading indicates SEM. (**C**) KA/AP3 attenuates the maximum normalized vagal firing rate in response to sucrose, whereas devazepide and KA alone do not. (**D**) KA/AP3 and KA alone prolong the time to peak from an average of 92.8 s to 198 and 179 s, respectively. Devazepide (2 mg/kg) does not significantly change the time to peak (mean = 67.1 s). For (C) and (D), $n \geq 5$ mice per group; *$P < 0.05$, ANOVA with post hoc Tukey's HSD test; error bars indicate SEM.

The neurotransmitter

The possibility exists that innervated enteroendocrine cells could use a classic neurotransmitter to transduce the above-described sensory signals. Other sensory epithelial transducers—including photoreceptors (*23*), auditory hair cells (*24*), Merkel cells (*25*), and olfactory receptor cells (*26*)—use vesicular glutamate as a neurotransmitter. Thus, we hypothesized that enteroendocrine cells use glutamate as a neurotransmitter as well. We found that intestinal enteroendocrine cells express significant quantities of the transcript for the vesicular glutamate transporter 1 protein (VGLUT1) (Fig. 5, A and B). In a transgenic Vglut1CRE_YFP mouse, fluorescence was observed in distinct intestinal epithelial cells that resemble enteroendocrine cells, and almost 4 in 10 of those fluorescent cells costained for CCK (38.80 ± 2.53% SEM, 100 cells per mouse, n = 3 mice). Moreover, vagal nodose neurons express at least eight glutamate receptors (fig. S14).

To test whether enteroendocrine cells release glutamate, we used the sniffer protein iGluSnFR. This membrane-bound protein fluoresces green in the presence of glutamate (*27*). Transfected iGluSnFR–HEK (human embryonic kidney) cells did not respond to a D-glucose (40 mM) stimulus but did respond to glutamate (100 μM) (fig. S15). We then cocultured iGluSnFR-HEK cells with Tomato-expressing enteroendocrine cells (CckCRE_tdTomato) (Fig. 5C). This time, when presented with a D-glucose stimulus (40 mM), iGluSnFR-HEK cells fluoresced green (n = 3 cultures; Fig. 5D), indicating that enteroendocrine cells release glutamate. Then, we cocultured CckCRE_ChR2 enteroendocrine cells with vagal neurons to determine if glutamate serves as a neurotransmitter in this synapse. In connected neurons, a 470-nm stimulus elicited EPSCs that were abolished by adding kynurenic acid (3 mM), an ionotropic glutamate-receptor

blocker (Fig. 5, E and F). The response was recovered once the blocker was washed away (n = 4 neurons connected to enteroendocrine cells) (Fig. 5F).

Hormone versus neurotransmitter

In a transgenic mouse in which VGLUT1-enteroendocrine cells express ChR2 (Vglut1CRE_ChR2-YFP), a luminal laser stimulus of 473-nm significantly increased vagal firing rate (fig. S16). The amplitude and timing of the peak response was comparable to the CckCRE_ChR2 experiments (figs. S12 and S16). The same laser stimulus applied to the subdiaphragmatic or cervical vagus did not alter firing rate (fig. S17). However, the response was abolished when the 473-nm laser was presented along with a cocktail of glutamate-receptor blockers [metabotropic blocker AP-3 (1 mg per kg of body weight) with ionotropic blocker kynurenic acid (150 μg/kg)] (fig. S16). These data revealed a type of enteroendocrine cell that uses glutamate to drive vagal firing.

Next, we compared the respective contributions of CCK and glutamate to vagal firing. The peak vagal firing rate elicited by a sucrose stimulus was not affected when the CCK-A receptor was blocked with devazepide (2 mg/kg) (Fig. 6, A and C). In control experiments, the same dose of devazepide fully blocked the vagal response to luminal CCK (fig. S18). Although the peak response and time to peak were not altered by devazepide, the length of the response was attenuated after 120 s (Fig. 6, A, C, and D; and figs. S18 and S19), suggesting that it takes minutes for released CCK to stimulate vagal firing. By contrast, blocking both ionotropic and metabotropic glutamate receptors attenuated the speed, peak, and magnitude of the vagal response to sucrose (Fig. 6, C and D, and fig. S19). Indeed, the first 60 s of the vagal response to sucrose was suppressed by the ionotropic blocker kynurenic acid alone (Fig. 6B and fig. S20), delaying the time to peak to around 180 s (Fig. 6D and fig. S18C). These data revealed that synaptic glutamate is used by an epithelial sensor cell in the gut to rapidly transduce luminal stimuli to the central nervous system.

The neuropod cells

In recent years, enteroendocrine cells have emerged as sensors of mechanical, chemical, and bacterial signals in the gastrointestinal tract (*2*, *3*). However, their transducer properties have been obscured by their name. By synapsing with the vagus, these sensor cells provide a neuroepithelial circuit for fast sensory transduction. As such, we see the need for a new name to refer to gut sensory epithelial cells that synapse with nerves. We refer to these cells as neuropod cells. We hypothesize that the gut-brain neural circuit formed by neuropod cells and vagal nodose neurons could lead to the following possibilities: (i) rapid computation of stimuli to distinguish their physical (e.g., volume) versus chemical (e.g., calorie) properties; (ii) precise sensory representation of specific gastrointestinal regions; (iii) localized plasticity encoded within the neural circuit; and (iv) timely vagal efferent feedback to modulate gastrointestinal sensory function. Like other sensory transducers, neuropod cells use synaptic signals to help the brain make sense of the food we eat.

Materials and methods summary

Animals

Mouse care and experiments were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee at Duke University Medical Center under the protocol A009-16-01. Mice were housed in the Duke University animal facilities, where they were kept on a 12-hour light-dark cycle. They received food and water ad libitum. The specific strains can be found in the supplemental methods.

Rabies production and tracing

G-deleted rabies virus production was performed in house as described in Wickersham *et. al* (*28*). For colon monosynaptic tracing, P1 mice were given an enema of EnvA-ΔG-rabies-GFP (5.9×10^9 ffu/ml). For small intestine monosynaptic tracing, P1 mice were given a gavage of ΔG-rabies-GFP (9.8×10^8 ffu/ml). Mice were sacrificed 7 days after exposure at P8. Harvested tissue was fixed in 4% PFA then treated with serial sucrose solutions. Ganglia were whole-mount imaged with a multiphoton microscopy system (Bruker Ultima IV with a Chameleon Vision II tunable laser). All other tissue was frozen in OCT blocks and sectioned for immunohistochemistry.

Organoid culture

Organoids were cultured using a protocol adapted from Sato *et al.* 2009 (*29*). Isolated crypts were resuspended in Matrigel (Corning #356231) and plated 50 μl per well in a 24-well plate in organoid media. Organoid media contains 1x Glutamax, 10 10mM HEPES, 200 U/ml Penicillin-Streptomycin, 1× N2 supplement, 1× B27 supplement, 0.25 ng/ml EGF, 50 ng/ml Noggin, and 100 ng/ml r-Spondin in Advanced DMEM/f12.

Enteroendocrine cell and nodose neuron coculture

Enteroendocrine cells of CckGFP and CckCRE_ChR2-tdTomato small intestines were isolated as previously described in Bohórquez *et al.* (*10*). Enteroendocrine cells were sorted into organoid culture media (listed above) plus 10 ng/ml NGF. Sorted cells were plated on 1% Matrigel coated 12-mm coverslips at a concentration of ~5000 to 10,000 enteroendocrine cells per coverslip. Nodose neurons were dissected and incubated with Liberase (Roche) digestion enzyme. Neurons in media were plated evenly on up to eight coverslips with enteroendocrine cells. Patch-clamp electrophysiology was performed 2 to 5 days after plating.

Immunohistochemistry

Immunohistochemistry was performed as previously described in Bohórquez *et al.* (*10*). Primary antibodies: Rb-Anti-PYY [DVB3] (1:1000); Rb-Anti-CCK (1:1000; courtesy of Rodger Liddle or Phoenix Pharmaceuticals H-069-04); Gt-Anti-PSD95 (1:500; Santa Cruz Biotechnology: sc-6926); Rb-Anti-Syn1 (1:500; Cell Signaling Technology: 5297S); Ck-Anti-GFP (1:500; Abcam: ab13970]. Secondary antibodies from Jackson Immuno-Reseach: Dk-Anti-Rb-488 (1:250); Dk-Anti-Rb-Cy3 (1:250); Dk-Anti-Gt-Cy5 (1:250); and Dk-Anti-Ck-488 (1:250). Imaging was done on a Zeiss 880 Airyscan inverted confocal microscope. Data are presented as the mean percentage ± SEM.

Real-time quantitative PCR

RNA from CckGFP-positive and -negative epithelial cells was extracted based on the manufacturer's protocol using the RNeasy Micro Plus Kit (Qiagen #74034). Then cDNA was produced per manufacturer's protocol using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems #4368814). TaqMan probes used are listed in supplemental materials. Real-time qPCR was run on a StepOnePlus System (Thermo Fischer), using TaqMan Fast Universal PCR Master Mix (Applied Biosystems #4352042) according to the manufacturer's protocol. Transcription rate was determined as $2^{-\Delta Ct}$, or compared as fold-change over GFP negative epithelial cells using $2^{-\Delta\Delta Ct}$. All values are reported as mean ± SEM.

Electrophysiology

Enteroendocrine cells and nodose neurons were cocultured as described above. Recordings were carried out at room temperature using a MultiClamp 700B amplifier (Axon Instruments), digitized using a Digidata 1550A (Axon Instruments) interface, and pClamp software (Axon Instruments) for data acquisition. Recordings were made using borosilicate glass pipettes pulled to ~3.5 MΩ resistance. Extracellular solution contained (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 2 MgCl2, 10 HEPES, pH 7.4 (300 to 305 mosmol). For voltage-clamp recordings, intracellular solution contained (in mM): 140 CsF, 10 NaCl, 0.1 CaCl2, 2 MgCl2, 1.1 EGTA, 10 HEPES, 10 sucrose (pH 7.25, 290 to 295 mosmol). For current-clamp recordings, intracellular solution contained (in mM): 140 KCl, 0.5 EGTA, 5 HEPES, 3 Mg-ATP, 10 sucrose (pH 7.25, 290 to 295 mosmol). Data are presented as the mean ± SEM, and significance was determined using a two-tailed Student's t test.

iGluSnFR-HEK cell and enteroendocrine cell coculture and imaging

CckCRE_tdTomato enteroendocrine cells were isolated as described above. Isolated cells were mixed with iGluSnFR-HEK cells at a ratio of 10:1, then plated on 1% Matrigel coated coverslips. Control iGluSnFR-HEK cells were plated alone. Cells were incubated for 12 to 18 hours before imaging. Coverslips were imaged using a multiphoton microscopy system (Bruker Ultima IV with a Chameleon Vision II tunable laser). Imaging series were analyzed using Fiji (it's just ImageJ), and cell traces were plotted with Excel.

Vagus nerve recording

Wild-type control (n = 5 to 9), CckCRE_ChR2-tdTomato (n = 6), CckCRE_Halo-YFP (n = 5), and Vglut1CRE_ChR2-YFP (n = 6) mice were used for vagal recordings. The cervical vagus was exposed in anesthetized mice and two platinum iridium wires (Medwire by Sigmund Cohn Corp) were looped around the vagus nerve for recording. A 20-gauge gavage needle was surgically inserted through the stomach wall and into the duodenum. Saline and stimulant tubes were connected to the gavage needle. For optogenetic experiments, a fiber optic cable (FT020, ThorLabs) was threaded through the gavage needle into the lumen of the duodenum. A perfusion exit incision was made 10 cm distal to the pyloric sphincter. During each recording, PBS was constantly perfused through the duodenum using a peristaltic pump (Cole-Parmer) at the lowest setting for a flow rate of ~400 µl PBS per minute. For stimulus delivery, see extended methods in supplemental materials. Data acquisition: A differential amplifier and bandpass filter (1000× gain, 300-Hz to 5-kHz bandpass filter; A-M Systems LLC) was used and the signal was processed using a data acquisition board and software (20-kHz sampling rate; Signal Express, National Instruments Corp). The raw data was analyzed using a spike sorting algorithm (MATLAB by MathWorks). Spikes were detected using simple threshold detection based on RMS noise. The firing rate was calculated using a Gaussian kernel smoothing algorithm (200-ms time scale). Statistical Methods: Stimulation response was quantified as the maximum firing rate after stimulation (stimulant conditions) or during recording (baseline). Time to peak was calculated as time from start of stimulus to maximum firing rate. Area under the curve was calculated as area under the curve for the entire 6-min recording. Maximum firing rate, time to peak, and area under curve are analyzed across genotype, stimulation condition, and their interaction term by ANOVA, followed by Tukey HSD post hoc testing (JMP by SAS Institute).

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/361/6408/eaat5236/suppl/DC1
Materials and Methods
Figs. S1 to 20
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Movies S1 and S2
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A gut-brain neural circuit for nutrient sensory transduction

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Dissecting the gut-brain axis

It is generally believed that cells in the gut transduce sensory information through the paracrine action of hormones. Kaelberer *et al.* found that, in addition to the well-described classical paracrine transduction, enteroendocrine cells also form fast, excitatory synapses with vagal afferents (see the Perspective by Hoffman and Lumpkin). This more direct circuit for gut-brain signaling uses glutamate as a neurotransmitter. Thus, sensory cues that stimulate the gut could potentially be manipulated to influence specific brain functions and behavior, including those linked to food choices.

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Leading Edge
Perspective

Cell

Microbiome: Focus on Causation and Mechanism

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There is tremendous enthusiasm for the microbiome in academia and industry. This Perspective argues that in order to realize its potential, the field needs to focus on establishing causation and molecular mechanism with an emphasis on phenotypes that are large in magnitude, easy to measure, and unambiguously driven by the microbiota.

The microbiome research community is producing exciting results at a rapid clip. Many of these early findings are correlative and associative, but phenomenology often precedes mechanism in a new area; by uncovering important phenotypes impacted by the microbiome, these studies illustrate why we should delve deeper. Indeed, there is broad agreement that the microbiome is ready to mature into a mechanism-based discipline, and a small but growing number of studies have pioneered the quest for molecular understanding in this space.

In industry, a raft of early entrants is seeking to develop therapies based on tantalizing observations in the areas of infectious disease, cancer immunotherapy, and immune modulation (Atarashi et al., 2013; Gopalakrishnan et al., 2018; Matson et al., 2018; Routy et al., 2018; van Nood et al., 2013). However, high-profile failures (Ratner, 2016) have led to hand wringing about the feasibility of drug discovery and development in a murky space with few hard landmarks.

Although there is an abundance of enthusiasm for the microbiome, the approaches most likely to yield impactful molecular mechanisms are still topics of intense debate. The central argument of this Perspective is that academic and industrial efforts should focus on causality and mechanism with an eye toward phenotypes that are large in magnitude, easy to measure, and unambiguously driven by the microbiota. I examine two areas ripe for mechanistic inquiry, small-molecule production and immune modulation by the microbiome, and then consider two topics critical to studying and exploiting these phenotypes: the role of individual bacterial species and molecules in complex mixtures and synthetic ecology as a tool for studying and developing therapeutics from the microbiome.

Pursue Causation and Mechanism

Many microbiota-host interaction studies share a common format: they start with a phenotype (often linked to disease) and seek to understand the microbial taxa or genes responsible for it. This is akin to forward genetics, with three important distinctions (Figure 1): the phenotype and genotype are in different organisms (host and microbe, respectively), the primary technique is differential sequence analysis rather than genetics, and the outcome is usually a bacterial taxon associated with a phenotype rather than a gene responsible for the phenotype.

The forward genetic approach has had notable successes, which can be grouped into three categories (referenced examples throughout are meant to be illustrative, not exhaustive): (1) comparative sequence analyses of disease cohorts that result in taxonomic associations (Gevers et al., 2014; Qin et al., 2012), (2) studies that show a phenotype can be transferred by microbiome transplant in germ-free mice (Gopalakrishnan et al., 2018; Matson et al., 2018; Ridaura et al., 2013; Routy et al., 2018; Thaiss et al., 2016), and (3) efforts that narrow down to one or a few species that modulate a host phenotype (Atarashi et al., 2013; Buffie et al., 2015; Buffington et al., 2016; Surana and Kasper, 2017). Two challenges stand out: first, given the large number of strains in a comparative sequence analysis, the process of narrowing down species that are causally linked to a phenotype can suffer from the statistical problems of cherry picking. Given the practical challenges of testing every possible strain, strains that are merely correlated are often assumed to be causative. Second, even when a causative strain is identified, it remains difficult to establish the mechanistic link to phenotype.

In contrast, an approach analogous to reverse genetics has been used less often in microbiome studies. Two related experimental formats are most common: (1) colonizing mice with communities that differ only in the presence of a single strain and comparing the outcome (Buffie et al., 2015; Faith et al., 2015; Romano et al., 2017) or (2) colonizing mice with wild-type versus mutant versions of a strain (Cullen et al., 2015; Dodd et al., 2017; Round et al., 2011). Although it can suffer from the problem of cherry picking, reverse genetics has the advantage that it is a simpler entry point to establish causality and enable studies of mechanism. More complex model microbiomes, improvements in genetic tools for prominent gut bacterial strains, methods to cultivate previously uncultured bacterial strains (Browne et al., 2016; Lagier et al., 2016), new technologies that facilitate colonization in the background of a complex community (Shepherd et al., 2018), and better animal models of microbiome-related disease will strengthen both forward and reverse approaches in addressing causality and mechanism more directly.

Future technologies—e.g., the microbiome equivalents of whole-genome knockout and CRISPR screens—will blur the distinction between forward and reverse genetics. The important thing is not the difference between these approaches but, for a given situation, whether they can reveal causation and molecular mechanism. In the remainder of this section, we consider two areas in which inroads into causality and mechanism seem

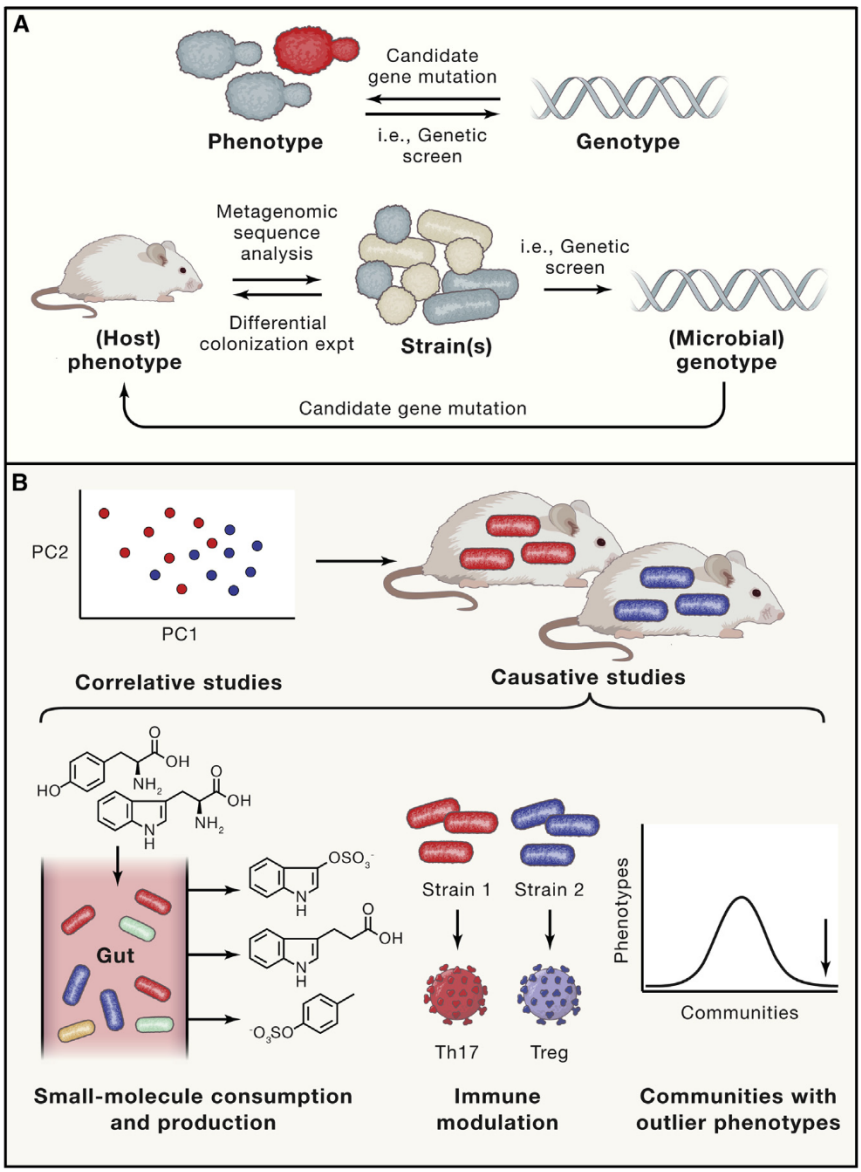

Figure 1. Challenges and Approaches to Decipher Mechanism in Microbiome Research

(A) In classical genetics, the phenotype and genotype are in the same organism. Genetic studies in the microbiome have two important distinctions: the phenotype and genotype are in different organisms (host and microbe, respectively), and the process of determining which microbial species in a complex mixture are responsible for a phenotype is a formidable challenge.

(B) Two areas in which studies of causality and mechanism have gained momentum are small molecule consumption/production and immune modulation. Communities with outlier phenotypes are an important vista for understanding and translating microbiome function.

likely: (1) production of small molecules and (2) modulation of the host immune response. Readers are referred to recent literature in a third area ripe for mechanistic investigation: colonization resistance against pathogens (Pamer, 2016; Panigrahi et al., 2017). We then raise the speculative possibility of outlier phenotypes with large effect sizes that, like rare human genetic variants, could hold great therapeutic promise.

Small-Molecule Production and Consumption

Given the prominent role of small molecules in mediating signaling interactions, the fact that the gut microbiota produce a pool of molecules tens of millimolar in concentration is of particular interest. One familiar example is the short-chain fatty acids (SCFAs) that accumulate at 150–600 mM/day (Wilson, 2005) and signal through G protein-coupled receptors 41 and 43 (GPR41 and GPR43) to modulate the host immune and metabolic systems (Ulven, 2012). Another is trimethylamine N-oxide (TMAO), which derives from gut microbial metabolism of choline and carnitine and is present at 2–6 μM in plasma (Tang et al., 2013). It is proposed to be not just a marker of cardiovascular disease but itself proatherogenic (Brown and Hazen, 2014) and prothrombotic (Zhu et al., 2016) in mouse models of disease. Although SCFAs and TMAO are heavily studied, the microbiota produce many other small molecules at concentrations comparable to those achieved by drugs used in the clinic. Aromatic amino acid metabolites are common and include the tryptophan metabolite indole, which is converted in the liver to indoxyl sulfate (IS) (10–130 mg/day) (Patel et al., 2012); the tyrosine metabolite p-cresol, which the host transforms into p-cresol sulfate (PCS) (20–230 mg/day) (Patel et al., 2012); and the phenylalanine metabolite phenylacetic acid, which the host conjugates to glutamine to form phenylacetylglutamine (PAG) (4.5–70 μm/mM creatinine) (Bouatra et al., 2013). TMAO, IS, and PCS accumulate in patients with renal failure (Sirich et al., 2013; Tang et al., 2015), suggesting that a microbiota-targeted therapy to eliminate their production could be a new therapeutic opportunity in renal disease.

An important source of small molecules is common dietary input, which can be converted by gut bacterial species into a variety of metabolic end products with different biological activities. For example, tryptophan can be converted to IS, indolepropionic acid, indoleacetic acid, and tryptamine. The levels of these metabolites vary widely among individuals, presumably reflecting differences in metabolic gene content among their gut communities. Thus, given a common diet, divergent microbiota can generate a metabolic output that can vary widely among individuals. This paradigm raises questions that are relevant to basic research and industry alike: which molecules are beneficial, and which others deleterious? What is the optimal gut

metabolic output, and how will it vary based on disease susceptibility? Can robust, resilient gut communities be designed to produce (and not produce) specific sets of molecules?

Modulation of the Host Immune Response

Several studies have demonstrated that the microbiota directly modulates the host immune response. Two key observations suggest that immune modulation by the microbiota might be far more specific than had previously been recognized. First, Littman and coworkers showed that the T_H17 cells induced in response to segmented filamentous bacterium (SFB), a well-studied mouse symbiont, express a T cell receptor (TCR) specific for an SFB antigen (Yang et al., 2014). Although it was previously known that SFB induces T_H17 cells, it is striking that a gut colonist—without breaching the intestinal epithelium—can "program" a population of immune cells that are specific to it. This paradigm has been extended by recent work on *Helicobacter hepaticus* (*Hh*), which induces *Hh*-specific regulatory T cells (Xu et al., 2018). Belkaid and coworkers have further demonstrated the generality of this result by showing that the $CD8^+$ T cell response to strains of the skin commensal *Staphylococcus epidermidis* not only is specific, but also lasts for months, indicating that the "programming" effect is potent and persistent (Naik et al., 2015).

This unanticipated degree of specificity in the mucosal immune response to the commensal microbiota has far-reaching implications and raises several key questions. (1) How many gut commensals induce their own corresponding immune cell population? Can a designed or engineered gut consortium serve as a tool for programming the host T cell response? (2) Does this phenomenon explain the profound influence of the microbiome on the efficacy of anti-PD1 therapy in melanoma and other cancers? (Gopalakrishnan et al., 2018; Matson et al., 2018; Routy et al., 2018) (3) What are the presumptive microbiota-derived or -induced molecules that determine T cell fate—and the cell types and signaling pathways through which they act? (4) If commensal-induced T cells express a TCR specific for a bacterial antigen, how do they—by cross-reactivity, proximity, or otherwise—modulate the immune response against host tissues? And can this antigen-specific response be re-directed by expressing host antigens in bacterial strains?

One goal, perhaps realizable in the near term, would be to use commensals or commensal-derived molecules as the basis of a new class of immunotherapies. One great advantage of this approach would be its selectivity, which could lead to a cleaner side-effect profile than systemic immunotherapies. For example, fine control over the local T cell response could enable an enhanced $CD8^+$ T cell response to potentiate checkpoint blockade for melanoma or colorectal adenocarcinoma without the risks of systemic immune stimulation. Likewise, local Treg stimulation could suppress immune activity in the small and large intestines (for inflammatory bowel disease) or skin (for psoriasis) without the downsides of general immune suppression. One could even imagine engineering a Treg-inducing bacterial strain to express a host autoantigen to reverse autoimmune disease.

Outlier Microbiome Phenotypes

One of the most direct benefits of DNA sequencing to human medicine has been to identify genetic outliers who harbor a rare mutation that confers protection against disease. For example, nonsense mutations in the proprotein convertase PCSK9 lower LDL-C and are protective against cardiovascular disease (Cohen et al., 2005), loss of function of $Na_v1.7$ confers insensitivity to pain (Cox et al., 2006), and CCR5 deficiency protects against HIV infection (Liu et al., 1996; Samson et al., 1996). All three proteins have become validated drug targets, and new therapeutics targeting each are now approved or in clinical trials.

Might the same concept apply to the microbiome? Unlike genome-wide and microbiome-wide association studies (Gilbert et al., 2016), which often reveal common variants with a small effect size, the goal would be to look for rare communities (harboring unusual bacterial species or common species in unusual ratios) with large, protective effects against a disease of interest. For example, do there exist rare individuals whose gut bacteria are exceptionally efficient at harvesting calories and therefore protect against metabolic disease? Might there be people with risk alleles for Crohn's disease who are protected against disease by a gut community that suppresses inflammation or prevents a bad actor from blooming? How about hospital workers who, despite constant exposure to *Staphylococcus aureus*, are not colonized due to a rare, protective skin community? Phenotypes like these would be a great setting in which to explore mechanism, and the transplantability of the microbiome could make it possible to endow millions of people with a rare disease-preventive gut or skin community.

Individual Organisms and Molecules Can Have a Big Impact

The sheer complexity of a host-associated community—hundreds of microbial species, thousands of molecules—raises the question of how much difference a single organism or molecule can make. Are most microbiota-related phenomena the result of dozens of microbes or molecules acting in concert, irreducible to the effects of individual actors?

Early evidence suggests otherwise. The specific T cell responses to SFB and *S. epidermidis* are examples of how a single bacterial species can exert a clear effect on the host, but several others are worth noting. *Faecalibacterium prausnitzii* (Miquel et al., 2013) and *Parabacteroides distasonis* (Kverka et al., 2011) have been linked to the suppression of intestinal inflammation, specific strains of *Lactobacillus* alter behavior and cognition in mice (Bravo et al., 2011; Buffington et al., 2016), and *Bacteroides fragilis* has been shown, *inter alia*, to counter the neurodevelopmental and gut barrier defects of mice in the maternal immune activation model of autism spectrum disorder (Hsiao et al., 2013). Though not an individual organism, a mixture of 17 anaerobic Firmicutes potently induces regulatory T cells (Atarashi et al., 2013).

Although these interactions are not yet understood at the level of molecular mechanism, individual molecules from the microbiota are known to have a signal that rises well above background. In addition to the well-documented examples of SCFAs and TMAO (see above), polysaccharide A (Mazmanian et al., 2008) and α-galactosylceramide from *B. fragilis* (An et al., 2014) are potent immune modulators that act through TLR2 and CD1d, respectively; heat-stable enterotoxin, from which linaclotide is derived, induces gastrointestinal (GI) motility via guanylate cyclase 2C (Schulz et al., 1990); and secondary bile acids,

including deoxycholic acid and ursodeoxycholic acid, have a wide range of effects on host metabolic and immune activity, as well as colon carcinogenesis (Wahlström et al., 2016). Moreover, individual host factors such as RegIIIγ and nitrate exert large effects on the composition and function of the gut community (Vaishnava et al., 2011; Winter et al., 2013). Given that the hunt for microbiota-derived metabolites of consequence is still in its infancy (Sharon et al., 2014), these examples are likely to be the first of many.

To place these findings in context, cell and developmental biologists have long understood that individual molecules can exert phenotypes that rise above the background of a complex pool. Mammals are complex mixtures of thousands of chemicals, yet signaling molecules such as serotonin (Canli and Lesch, 2007), oxytocin (Kosfeld et al., 2005), sphingosine-1-phosphate (Spiegel and Milstien, 2003), and estradiol (Balthazart and Ball, 2006) have potent, unambiguous activities, both at the level of cells and physiology. There is every reason to think that the paradigm of dominant activity for individual molecules will hold for an important subset of molecules from the microbiota. If so, it will be far easier than currently anticipated to understand the mechanisms of microbe-host interactions and exploit them therapeutically.

Develop the Basic Science Underlying Synthetic Ecology

Developments in academic and industrial microbiome research are creating a need for a mechanistic understanding of microbial communities. In academia, although germ-free mouse monocolonization experiments have yielded tremendous mechanistic insights into microbe-host interactions, experiments with very large, defined communities that approach native-like complexity would capture a great deal of biology missing in the simpler format (e.g., the effects of a pool of molecules tens of millimolar in concentration, perhaps tens to hundreds of distinct immune modulatory events). Experiments in which one strain (or, in the extreme, one gene from one strain) is removed and an effect on the host is observed are more likely to stand the test of time.

On the industry side, the striking efficacy of fecal microbiota transplant (FMT) in recurrent *Clostridium difficile* infection (van Nood et al., 2013), combined with a surprisingly low rate of acute adverse events (for exceptions, see Alang and Kelly, 2015 and Quera et al., 2014) and promising early data on engraftment (Smillie et al., 2018), has spurred efforts to test FMT in other indications. However, there are two key challenges in generalizing fecal transplant, both of which suggest important limitations of feces as a source of material for community transplants. (1) Its supply is inherently limited and difficult to scale. The need for safety, reproducibility, and ease of manufacture will favor a transition from donor feces to bacterial communities of defined composition. (2) A "lead" fecal sample cannot be optimized in the same way that medicinal chemistry or protein engineering can improve a small molecule or biologic lead. A defined community would enable the kind of tinkering that is critically important in drug discovery.

The academic and industrial challenges can both be addressed by developing new methods for constructing and interrogating synthetic communities that are defined but consist of hundreds of strains, approaching the level of complexity of a native fecal sample. Although there are many practical challenges to overcome, early work in the plant microbiome community has begun demonstrating the value of this approach (Bai et al., 2015). Such a system could open the door to studying an array of fundamental basic science questions: how many meaningful strain-strain interactions exist in a community of hundreds of strains? What constitutes a niche, and how do strains map to niches? What are the molecular correlates of stability, and how does a community reconfigure in response to a perturbation? Most importantly, it would enable landmark studies of causation and mechanism in the microbiome, bringing a near atomistic level of control to a system that could benefit tremendously from more reductionist inquiry.

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DECLARATION OF INTERESTS

The author is on the board of directors of Achaogen, on the scientific advisory boards of NGM Biopharmaceuticals and Indigo Agriculture, and a co-founder of Revolution Medicines.

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