Title: Emerging tools to study enteric neuromuscular function.

Short title: New tools for enteric neuroscience.

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Grant Support: BDG receives research support from the National Institutes of Health (NIH; R01DK103723), the Crohn’s and Colitis Foundation of America (CCFA; Senior Research Award) and the Department of Defense (DoD; GW150178).

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Disclosures: The author has no financial, professional or personal conflicts that are relevant to the manuscript.

Acknowledgements: The Author would like to thank Dr. John Wiley for the invitation to present this topic at the 2016 Federation of Neurogastroenterology and Motility Joint International Meeting and Dr. Nigel Bunnett for the opportunity to prepare the presentation as a mini-review.

Author Contributions: Manuscript written and edited by BDG.
Abstract

Investigating enteric neuromuscular function poses specific challenges that are not encountered in other systems. The gut has a complex cellular composition and methods to study diverse multicellular interactions during physiological gut functions have been limited. However, new technologies are emerging in optics, genetics and bioengineering that greatly expand the capabilities to study integrative functions in the gut. In this mini-review, I discuss several areas where the application of these technologies could benefit ongoing efforts to understand enteric neuromuscular function. I specifically focus on technologies that can be applied to study specific cellular networks and the mechanisms that link activity to function.

Introduction

The neural control of gut smooth muscle is a fundamental process that governs intestinal motility. Neural reflexes controlling motility are contained within the myenteric plexus of the enteric nervous system (ENS) and these intrinsic circuits are capable of coordinating basic gut functions in the absence of input from the brain or spinal cord. In this regard, the ENS can be viewed as the “sheriff” that enforces the "law of the intestine" described by Bayliss and Starling (5, 6). Some aspects of the neural control of gut motility are now well described such as the essential nature of the ENS and the physiology of the main populations of enteric neurons. What is much less clear is how homo- and heterotypic interactions between enteric neurons and the many other cell types involved form the basis of functional reflexes. For example, our understanding of the ENS at the network level is still relatively poor and the roles of key non-neuronal cells such as interstitial cells of Cajal (ICC), PDGFRα+ cells, enterochromaffin cells and enteric glia are still debated.

Addressing some of these outstanding questions requires the application of new technologies that will allow investigators to probe the functions of defined populations of cells in physiological settings. Fortunately, intense interest in functional networks in the brain has driven the development
of new techniques to study the activity of large ensembles of cells and methods to manipulate cellular function that exhibit high spatial and temporal resolution. Many of these technologies are transferrable to gastrointestinal research but their adoption has been relatively slow. The goal of this brief review is to introduce a handful of current technologies that would be useful in answering key questions in enteric neuroscience.

What do we know now?

The basic enteric reflex that controls intestinal motility is likely the most well studied aspect of the ENS to date. This has led to a detailed understanding of the neurochemical coding and electrophysiological properties of individual subtypes of enteric neurons in several species and readers are referred to several excellent books (12, 50) and reviews (11, 13, 47) for details. Based on the information from individual cells, one can construct a simple model of the enteric motility reflex that resembles a spinal reflex arc. According to this model, mucosal stimuli activate the processes of afferent neurons located in the myenteric plexus. Afferent neuron depolarization excites both ascending and descending interneurons that, in turn, activate excitatory motorneurons above, and inhibitory motorneurons below, the point of stimulation. This model is very useful to illustrate the basic concept of the motility reflex but we know now that the circuitry is actually much more complex and involves interactions with enterochromaffin cells at the level of the mucosa, processing within the plexus that involves multiple types of neurons and glia, and interactions with ICCs, PDGFRα+ cells and smooth muscle cells that form the SIP (Smooth muscle, ICC, PDGFRα+) syncytium (39).

What are the questions and what tools do we need to answer them?

In general, we still have a very poor understanding of how enteric neurons function together as a network and how they interact with other cell types. New tools are needed to both investigate questions regarding network activity as well as address outstanding debates in the field regarding the roles of EC cells, ICCs, PDGFRα+ cells and glia. Clarifying these issues will yield novel insight into
how the ENS process signals and subsequently modulates effector tissues. Understanding these processes is an essential first step in the development of more effective therapies for motility disorders. In the following sections, I will highlight a few key broad questions and describe some of the available tools that would help to address these issues.

**Who is active and when?**

One of the major hurdles in understanding ENS function at the network level has been the lack of appropriate techniques to record from large ensembles of cells. Single cell electrophysiological recordings have formed the fundamentals of neurogastroenterology (24, 33, 48, 49) and the importance of this technique cannot be overemphasized. Electrophysiology gives fine resolution information about individual excitable cells at a level of detail that is still far superior to other methods. The types of information that can be acquired by this technique include passive and active membrane characteristics, ionic properties, synaptic properties, and levels of excitability. However, single cell electrophysiological recordings are extremely low throughput, they are difficulty to learn, and they involve random impalement of neurons, interstitial cells or muscle. Likewise, using this technique to understand network dynamics is not feasible and new approaches are needed. The introduction of calcium and voltage indicator dyes partially alleviated this issue by allowing investigators to simultaneously record the activity of many cells within a ganglion and even across several ganglia (32, 44). However, these techniques are not without their shortcomings. Dye loading can be temperamental and bulk loading is not cell type specific. It also requires tissue dissection and is not reliable for *in vivo* recordings of network activity in the gut.

To circumvent these issues, the field is increasingly turning to genetically–encoded fluorescent indicators (**Figure 1**). As with other branches of optogenetics, their use has become widespread over the past two decades and their application for large–scale recordings of neural activity in the brain has been transformative for nervous system research. What makes this approach particularly useful is the ability to encode indicator expression in a cell type specific manner, and the fact that genetically encoded indicators typically have optical reporting properties that are superior to organic indicator
dyes (26). Several different variants of fluorescent protein indicators have been developed for the
detection of vesicle release (38), neurotransmitters (25), transmembrane voltage (23, 41) and
intracellular calcium (14, 26) and a variety of transgenic mouse lines and viral delivery systems are
readily available to drive expression. When combined with the appropriate microscopy technique,
optical indicators enable investigators to evaluate the activity of thousands, if not millions, of cells
simultaneously. For example, Yu Shin Kim and colleagues recently used mice that express a
genetically encoded calcium indicator (GECI) in sensory neurons to simultaneously monitor the
activity of over 1,600 neurons per dorsal root ganglion in vivo (21). Other groups have used similar
techniques to simultaneously record from several thousand neurons in the brains of awake, behaving
mice (40) and from all neurons within the brain of larval zebrafish (1).

Adopting these techniques to study functional cellular networks in the gut is relatively
straightforward and several groups have already demonstrated the feasibility of using genetically
encoded indicators to record the activity of enteric neurons (7, 17, 36), glia (7, 17, 30) and ICCs (3).
GECIs are by far the most widely used method to date in the gut. This is largely driven by the fact that
the GECI field is more mature compared to other genetically encoded indicators and because of the
physiological importance of calcium as a second messenger in excitable cells. Studies using GECIs
in the gut have taken advantage of several commercially available mouse lines that allow
investigators to express GECIs such as GCaMP3 (3, 7, 17) or GCaMP6 (30) in specific cells using a
Cre-loxP system. Current work with these models has been mostly limited to investigating cellular
responses in whole mount preparations of intestine and the results are very promising (3, 7, 17, 30).
Under these conditions, the specific cellular expression and large dynamic changes of the GECIs has
eliminated the need to dissect tissue and has allowed investigators to study cells in a more
physiological setting. Importantly, the success of these studies implies that similar techniques could
be used in combination with advanced microscopy to study the activity of large networks of cells in an
intact, or semi-intact organ. The ultimate goal would be to record cellular network activity in vivo and
new results suggest that this may be a possibility in the near future. For example, Rakhilin et al.
recently succeeded in recording neuronal responses *in vivo* by using an optical window into the abdominal cavity of mice expressing GCaMP3 in enteric neurons (36). Only small patches of the ENS were imaged in this study but its success is an important step towards observing functional networks *in vivo*. Interestingly, this study also employed graphene sensors along the serosal surface of the gut to record electrical activity in the ENS. How well these sensors specifically detect the electrical activity of neurons sandwiched between two electrically active smooth muscle coats is still debatable so additional validation of this method would be beneficial. A more feasible approach might again be to study the electrical activity of enteric neurons using genetically encoded voltage indicators. The development of new genetically encoded voltage indicators is still rapidly evolving and new mouse models are now available that permit the expression of advanced voltage indicators in a Cre-dependent manner (27). The superior cellular resolution and specificity that these model systems afford now make imaging with this class of indicators in intact tissue a real possibility.

**How does the activation of specific cell types contribute?**

Observing relevant forms of cellular activity with the methods above is a good starting point, but these observational methods must be complemented with tools to directly test the functional significance of the observed activity. These tools must be cell type specific, work through relevant mechanisms and be appropriate for use in intact organs or, more ideally, *in vivo*. Luckily, a powerful toolkit of genetically-encoded proteins has evolved in recent years that allows investigators to control the activity or functions of defined cells in their native environment. Collectively, these proteins are referred to as “actuators” for their ability to control cellular mechanisms. The most commonly used genetically-encoded actuators are ion channels, pumps or G-protein coupled receptors (GPCRs) (**Figure 2**) that allow investigators to excite or inhibit the activity of cells of interest (10, 37). However, other classes of cellular actuators are available to control diverse cellular functions such as protein trafficking (20), gene expression (20), protein interactions (45), protein conformation (35) and cell motility (51). Genetically-encoded actuators are broadly grouped according to the type of energy source used by the control signal. Thus, actuators driven by light are referred to as optogenetic, while
those driven by chemicals, magnetic fields or radio waves are referred to as chemogenetic, magnetogenetic and sonogenetic, respectively. However, the basic strategy with their use is the same: express an engineered receptor that signals through relevant endogenous transduction pathways, trigger its activation with biologically inert stimuli and observe the effect on a defined function. The ultimate choice of which protein to express is dictated by two major considerations: 1) how well the exogenous protein controls relevant endogenous signaling in the cell type of interest and 2) the feasibility of the activation strategy.

The field of optogenetics has gained great fame in the “age of light” for the ability to integrate optics and genetic engineering to measure or manipulate the activity of cells (8, 10). This is a powerful technique to control the activity of neurons, in particular, because of the capacity for fine temporal control on the order of milliseconds. Optogenetic actuators have great potential for studies of enteric neuromuscular function but their application has been limited to one published study thus far (42). In this study, Stamp et al (42) used neural stem cells expressing channelrhodopsin to show that these cells can be grafted into the adult intestine, develop into mature neurons and functionally innervate the gut smooth muscle. Studying functional innervation with optogenetics ex vivo by measuring excitatory and inhibitory junction potentials as was done in this study is an effective approach that could be applied to study multiple different cell types in the gut. However, translating these types of studies to in vivo work is much more challenging. Some of the hurdles that have limited widespread use of optogenetics in the gut include the limited number of cell type specific promoters and technical issues with delivering light to the cells of interest in vivo. Unlike the brain where fiber optic cables can be implanted and fixed in place to deliver light, the gut is a dynamically moving organ and implanting traditional fiber optics is not an option. One solution is to use implantable wireless optogenetic devices that could deliver light pulses to regions of the intestine (31). We recently piloted a variation of this technique and found that we could effectively control a small light source inside a synthetic fecal pellet (Figure 3). This approach may work well in vivo to selectively modulate the activity of cells in the superficial layers of the gut epithelium such as EC cells. However, it has not...
been validated for use stimulating cells in deeper layers of the gut wall and the light scattering properties of gut tissue will decrease the efficiency of short wavelength light used to gate the popular channelrhodopsins and halorhodopsins. Combining luminal devices that emit long wavelength light with red-shifted optogenetic channels may allow this technique to reach cells at the level of the myenteric plexus and smooth muscle coats but this is still theoretical.

Other classes of genetically-encoded actuators circumvent the issues of accessibility experienced by optogenetics and may be more easily adapted for \textit{in vivo} work in the intestine. For example, chemogenetics is widely used in both the brain and the periphery to control and investigate cell signaling (2, 37). Chemogenetic receptors are engineered so that they become unresponsive to native ligands and gain the affinity for specific small molecules that would otherwise be biologically inert. These attributes are very attractive because they allow precise cellular control with limited off–target effects. The main drawback of this technique is that it lacks fine temporal control \textit{in vivo} but modulating cellular activity with drugs is a very relevant approach for translational medicine. Chemogenetics is also a particularly attractive technique to investigate non-neuronal cells where the time course of signaling is slower and mainly driven by GPCRs. For example, we recently used a chemogenetic approach to selectively modulate the activity of enteric glial cells \textit{in vivo} and \textit{in vitro} to investigate their roles in intestinal motility (28) and secretomotor function (15). Similar strategies could easily be used to investigate the roles of other key populations of cells such as PDGFR\textalpha+ cells or EC cells. Importantly, chemogenetic receptors selective for different ligands can be used in tandem to control multiple cell populations at once or multiple signal transduction processes in the same cell type. For example, chemogenetic receptors such as hM3Dq, hM3Di, GsD and Rq(R165L) are specifically activated by the drug clozapine-n-oxide (CNO) while chemogenetic KORD receptors are controlled by salvinorin B (SALB) (43). However, investigators should be aware of the fact that biased agonism could affect the interpretation of data obtained with chemogenetic models. For example, some GPCRs are able to respond to different ligands by signaling through different pathways. Thus, a
small molecule may target one pathway, but miss a different biologically relevant pathway for that
given receptor.

Opto- and chemogenetics are extremely powerful tools but, as noted above, display several
limitations such as invasive methods of stimulation or slow kinetics. Of course, the ideal actuator for
work in the intestine would be one that could be controlled in a non-invasive manner and exhibit high
temporal and spatial precision. This may seem like a tall order but it is encouraging that these types
of technologies are beginning to come of age. Of particular interest are developments that are
occurring in genetically-encoded actuators that are controlled by sonic waves (sonogenetics) or
magnetic fields (magnetogenetics). For example, Ibsen and colleagues showed that they were able to
drive specific behaviors in *Caenorhabditis elegans* with low-pressure ultrasonic sound by expressing
the ultrasound-sensitive channel TRP-4 in certain neurons (18). Likewise, Wheeler and colleagues
recently synthesized a magnetically sensitive actuator by fusing ferritin to TRPV4 and were able to
genetically encode its expression in subpopulations of neurons (46). They went on to show that
neurons expressing TRPV4-ferritin could be remotely activated by magnetic fields *in vivo* and drive
behaviors in mice and zebrafish. Neither technique has been applied to the intestine yet but both are
extremely well suited for this application.

**What are the important cellular mechanisms?**

Assuming one has now used the techniques above to observe cellular activity associated with a
function and then selectively triggered cellular activity to drive the function, we are now able to begin
asking questions regarding the mechanisms that link activity to function. The most straightforward
way to do this is to use one of a growing number of techniques to silence specific cellular
mechanisms. The most classic approach is with *Cre-loxP* genetics where genes of interest are
deleted in specific cells. However, many cells compensate to the constitutive deletion of genes very
well so the field is increasingly turning to inducible mouse models that are based on either the tet-
operon/repressor bi-transgenic system or the estrogen receptor (ER) ligand-binding domain fused to
*Cre*. Inducible transgenes such as *CreERT* allow for the induction of recombination in specific cells at
defined time points by administering a drug; tamoxifen in this case. However, the potential effects of
the drug should not be taken lightly as some models use drugs that modify gut physiology. For
example, tet-operon/repressor bi transgenic systems (Tet-On, Tet-Off) utilize the antibiotic
doxycycline to regulate transgene expression and antibiotics have major effects on the gut
microbiota. In the future, investigators may consider turning to new photoactivatable Cre approaches
that could eliminate the need for drugs and restrict Cre activation to the gut (45). The main benefit of
inducible transgenic systems is that they avoid many of the complications that arise with traditional
knockout models during development and allow more direct studies of functional mechanisms in adult
animals. An excellent example of how this technology can be applied to the gut is the work of Klein
and colleagues (22) who used inducible Cre-loxP systems to specifically study the role of ICCs in
inhibitory neuromuscular transmission in the gut. The results from this study provided clear evidence
that ICCs are critically involved in mediating the nitrergic component of inhibitory neuromuscular
transmission in adult animals. Likewise, we have used similar strategies to selectively ablate
connexin-43 hemichannels from enteric glial cells in adult animals to investigate the role of this glial
signaling mechanism in motility (29), secretomotor function (15) and inflammatory processes (9).

Transgenic models historically have been the mainstay method to address mechanisms in
intact systems. However, transgenics are not without their drawbacks that include being largely
limited to mouse models, being cost and time intensive, displaying variable effectiveness and often
experiencing undesirable side effects of transgene expression. Some of these issues can now be
avoided by using new, more flexible, methods of genome editing such as the CRISPR (clustered
regularly interspersed short palindromic repeats)-Cas9 system (4, 16, 19). Since its conception in
2012 (19), this method has revolutionized genomic engineering and has made genome editing
possible in basically all living organisms. This system takes advantage of a bacterial adaptive immune
defense mechanism that involves directing an endonuclease (Cas9) to specific DNA sites with a
single guide RNA molecule, creating DNA cleavage and subsequently inserting mutations using error-
prone DNA repair mechanisms (16). This technology is now widely used and the methods are
commercially available. Genomic editing with CRISPR-Cas9 has massive potential to address mechanisms in gut physiology and pathophysiology in diverse model systems. However, the field is still developing and many questions remain about the specificity and efficacy of this technology (34). Despite these issues, CRISPR-Cas9 will likely be an extremely powerful tool to expand mechanistic studies in the intestine.

Conclusions
Expanding the diversity of techniques used to investigate enteric neuromuscular function has the potential to provide new insight into the mechanisms that control gut motility. The techniques discussed above represent only a small, but important, subset of the cutting edge approaches that are now becoming available. These techniques are widely used in neuroscience and have proven to be powerful tools to study functional networks and cellular mechanisms. Many of these techniques are well suited for work in the intestine. However, others will require adaptation to meet the specific needs of the unique environment of the gut. Combining several different technologies at once has even more potential experimental power. These are exciting prospects that will likely play a key role in generating a sophisticated, integrative understanding of enteric neuromuscular function.

References

11


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**Figure Legends**

**Figure 1.** The progression of methods to study large networks of cells. (a) Traditional electrophysiological recordings provide fine detail information about single cells. (b) Optical imaging dyes can be used to study the activity of cells in one, or several ganglia in isolated tissue preparations. (c) Optogenetic reporters can be expressed in specific cells and used to study network activity in intact tissue or *in vivo*. Images at top in c are representative of recordings from mice expressing the optogenetic reporter GCaMP5g (grayscale) and tdTomato (red) in enteric glial cells (from ref (30)). Only one myenteric ganglion is shown here for clarity but the same technique could be used to study large areas of the ENS during physiological functions.

**Figure 2.** Schematic of various (a) chemogenetic, (b) optogenetic, (c) sonogenetic and radiogenetic approaches to control cellular activity and their signaling properties. (a) The four chemogenetic receptors shown are modified GPCRs that signal through canonical and non-canonical (arrestin) GPCR pathways. Receptors specifically activated by clozapine-n-oxide include hM3Dq, GsD and Rq(R165L), all modified M3 muscarinic receptors, and hM4Di, a modified M4 receptor. Not shown is the chemogenetic receptor KORD. KORD is a modified k-opioid receptor that is selectively activated by salvinorin B and signals through GPCR pathways. (b) Three examples of light-gated channels and receptors. Channelrhodopsin (ChR) is a light-gated cation channel that is used to drive cellular excitability. Halorhodopsin (HR) is a light-gated ion pump that is selective for chloride ions and is often used to silence excitable cells. OptoXRs are a family of light-gated GPCRs that couple to
various intracellular signal transduction cascades. (c) Two examples of channels gated by sound waves (sonogenetics) or magnetic fields. TRP-4 is a mechanosensitive cation channel that is activated by low pressure ultrasonic sound. Magneto is a fusion protein between TRPV4 cation channels and ferritin. Magnetic fields physically pull this cation channel open.

Figure 3. Example of a low cost, wireless device to power a light-emitting fecal pellet. (a) The device consists of a power supply and a resonant cavity where electromagnetic energy is supplied to the LED within the animal. (b) The synthetic fecal pellet containing the small LED is powered by resonance energy transfer and emits light when placed into the electromagnetic field. This device was conceived and developed by David Fried.
a. Electrophysiology

b. Optical indicator dyes

c. Optogenetics

- iermo
- Baseline
- Peak
- Overlay