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Marcello Costa *Editors*

The Enteric Nervous System

30 Years Later

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Introduction to the ENS II 2014, 30 Years Later

Nearly 30 years ago a number of scientists working on the Enteric Nervous System (ENS) gathered at Flinders to discuss the advances and future of their research. A photograph (see below, Fig. 1) captured the friendly and stimulating meeting attended by most of the major players in what was to become the new discipline of 'neurogastroenterology'.

This first meeting was small, but very successful, and involved all of the leading researchers in the field of enteric neuroscience from across the world. The reason for the meeting's success was that its entire purpose focused on discussing methodological strategies and unresolved issues in the field.

At that time we had just started at Flinders a golden decade for the two organisers John Furness and Marcello Costa, foundation lecturers at the newly established School of Medicine at Flinders, John in anatomy and Marcello in physiology. At that meeting they intended to look ahead for the next 10 years.

We are now looking back at more than 30 years, during which a second and a third generation of researchers in the field emerged, and many of the original researchers are still active, and some have come to this meeting held again in Adelaide (see Fig. 1).

Indeed many of the second and third generation researchers have accepted the invitation and contributed to the 2-day meeting in Adelaide. An extended audience of selected active researchers in the ENS attended (see Fig. 2).

The main Australasian Neuroscience Society (ANS) meeting was held in Adelaide 30 years later, providing a perfect opportunity to recreate a similar follow-up ENS meeting. As such the ENS II 2014 meeting aimed to identify how far we have come in the field of enteric neuroscience, where the future is heading and what technological advances have been made to address current and future unresolved questions.

The local committee consisted of Marcello Costa, Simon Brookes, Nick Spencer, Stuart Brierley and Phil Dinning, whilst Marcello Costa and John Furness, the organisers of the 1983 meeting, were the 'patrons' of this meeting.

Fig. 1 Attendees of the original ENS meeting held in 1983 at Flinders University, Adelaide, Australia

Fig. 2 Attendees of the ENS II meeting held at the National Wine Centre, University of Adelaide, Australia

Fig. 3 Speakers and chairs of the ENS II meeting

From around the world 30 speakers were invited to give talks to revisit the original expectations, the advances made since and the future directions on ENS research. This included three generations of investigators from seven different countries (see Fig. 3).

This group included 'historical' speakers, those who significantly contributed to the advances on the ENS over the past two decades (some of whom attended the first meeting) and the new generation that will continue to contribute to advancing our understanding of the ENS.

This publication represents the majority of the proceedings of the conference on The Enteric Nervous System II 2014, a meeting held on February 1st to 2nd at the National Wine Centre of Australia, Adelaide. It was an official satellite meeting of the 34th Annual Meeting of ANS, also held in Adelaide.

We thank all those who accepted the invitation to come, many from far away, and recreate a friendly yet rigorous atmosphere.

Some colleagues could not attend but did send greetings, and their messages are included in these proceedings (including letters from Joe Szurszewski and Michael Schemann). We would like to thank our local organising committee and the sponsors that made this conference possible.

Adelaide, SA, Australia

Stuart Brierley
Marcello Costa

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Chapter 1
Memories and Promises of the Enteric Nervous System and Its Functions

Marcello Costa

This is a very personal reminiscence of the long period of Enteric Nervous System research in which I have been involved. I started to work on the gut in the early 60s really because in Turin when I arrived from Argentina, where my family migrated temporarily after the WWII, nobody was seriously working on the brain. In Anatomy they were studying the neural "intramural plexuses" and that for me was close enough to the nervous system. I grew up in the mountains near Turin near the French border where our ex-family house still bears our name. I joined the Department of Anatomy as an intern student and I was privileged to sit at a desk where a previous generation of young scientists, who studied under the professor of Anatomy A. Levi, the founder of the methods for culturing neural tissue. They were Salvador Luria, Renato Dulbecco and Rita Levi-Montalcini, who, after migrating to the USA, were each were given the Noble prize.

Together with my supervisor Giorgio Gabella we adapted to the gut newly developed techniques of the fluorescence visualization of amines in tissues with formaldehyde vapors, and constructed a special freeze dry machine to prepare tissues. As I was most commonly full of grease and oils from the vacuum pumps I decided to avoid freeze drying machines and adapted the fluorescence technique to the now widely used 'whole mount preparations' of the layers of the gut.

Unbeknown to me at the time, far away in Melbourne Australia two young researchers Geoffrey Burnstock and Mollie Holman, students of Edith Bulbring the founder of smooth muscle electrophysiology in Oxford, had established advanced multidisciplinary laboratory in the two main universities.

The decade of the 1960s saw important developments including electromyographic bases of the motility of the human and experimental animal gut (by Ed Daniel, Yves Ruckhebusch and others), the histochemical visualization of adrenergic

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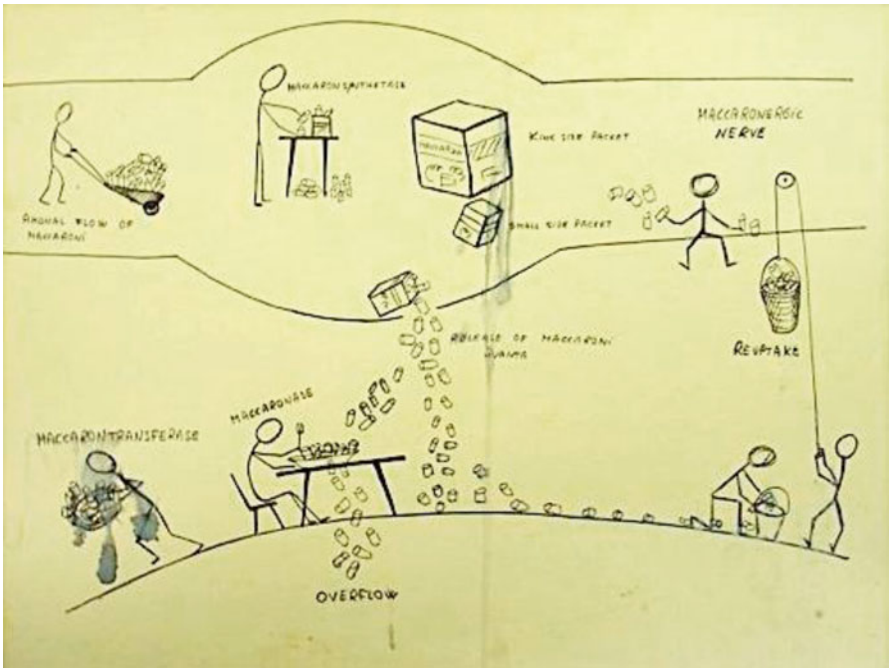

Fig. 1.1 This is the original version of the allegedly new transmitter macaroni in "maccaronergic nerves" to balance the proposal by Geoff Burnstock of the "purinergic nerves". These proved correct while the former regrettably were forgotten

nerves (by Norberg in Sweden and us in Turin), the discovery of inhibitory non adrenergic-non cholinergic neurons (NANCs) by Geoff Burnstock and younger students Graeme Campbell and Max Bennett, and the full description of the MMCX (by Joe Szurzewski and Charles Code).

I was invited to the Department of Zoology in Melbourne by the young professor Geoff Burnstock I met the year before on the step of a building in Venice in 1969. Migrated to Australia with my young wife Daniela, I encountered a remarkably free and stimulating research environment Geoff had created.

The 1970s saw the full development of the "purinergic hypothesis" of the inhibitory NANC transmitter proposed by Geoff Burnstock and since increasingly supported by many, despite of our youthful, somewhat arrogant, critical refusal to accept it. Testimony of this ironic approach to serious science is my cartoon of the "maccaronergic nerves" hypothesis to be juxtaposed to the "purinergic nerves" hypothesis (Fig. 1.1).

Geoff has been very generous with his pupils and John and I started to work from the very beginning together. In fact as I set foot in the department the same day I arrived, John challenged an observation of some adrenergic neurons I found in the guinea-pig proximal by fluorescence histochemistry back in Turin. The challenge was resolved in that same afternoon and I was for once right (Costa and Furness 1971). This marked a long-term collaboration and for the following 17 years we were together part of the advancing front of ENS research.

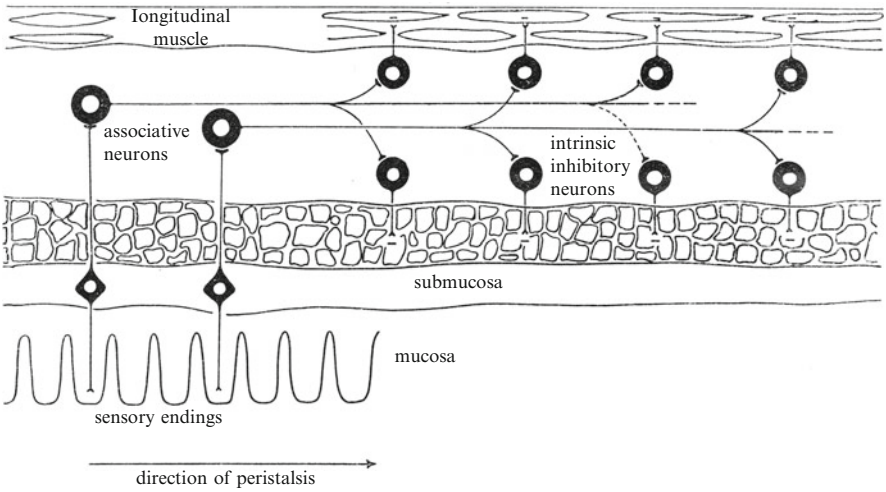

Fig. 1.2 Diagram of the projections and basic connections of the enteric inhibitory motor neurons to provide the descending inhibitory pathway underlying the functional polarity of the intestine

The 1970s saw the full development of the purinergic hypothesis of the inhibitory NANC transmitter. We joined investigations on the nature of mysterious enteric neurotransmitters. More importantly we were probably the first to suggest that the enteric inhibitory neurons of the purinergic hypothesis are essential part of the enteric circuits that from the esophagus to the anus ensure a suitable relaxation of the intestinal muscles ahead of the advancing boluses (Furness and Costa 1973; Fig. 1.2).

The first intestinal motility meeting in Banff Canada in 1973 game me for the first time a glimpse to other exciting research and extraordinary individuals. There I met Ladd Prosser, scientific grandfather to many in the USA, and the younger Alan North and David Hirst. I presented there our initial evidence for descending inhibition to the anal sphincter (Costa and Furness 1974).

In that decade suggestions that amine 5HT (serotonin or enteramine) and the peptide Substance P could be NANC excitatory enteric transmitters emerged. We found ourselves in the middle of the arguments (Costa and Furness 1979) that only subsided the following decade when experiments clarified the complex nature of multiple neural transmission.

I learned the joys of organ bath pharmacology in an earlier visit to the Department of Pharmacology of Antonio Crema in Pisa when I was still a medical student in the 60s. Antonio Crema had been also a Ph.D. student of Edith Bulbring in Oxford with Mollie Holman and Geoff Burnstock. In Melbourne Zoology John and I used organ bath experiments to explore the role of polarised enteric reflex pathways (Costa and Furness 1976) and revealed some of the features of the simple process of propulsion of natural pellets in the guinea-pig. Little I realized then that this work was to continue to the present, keeping me and other still in wonder of the remarkable 'simplexity' of this natural process even humans share with guinea-pigs.

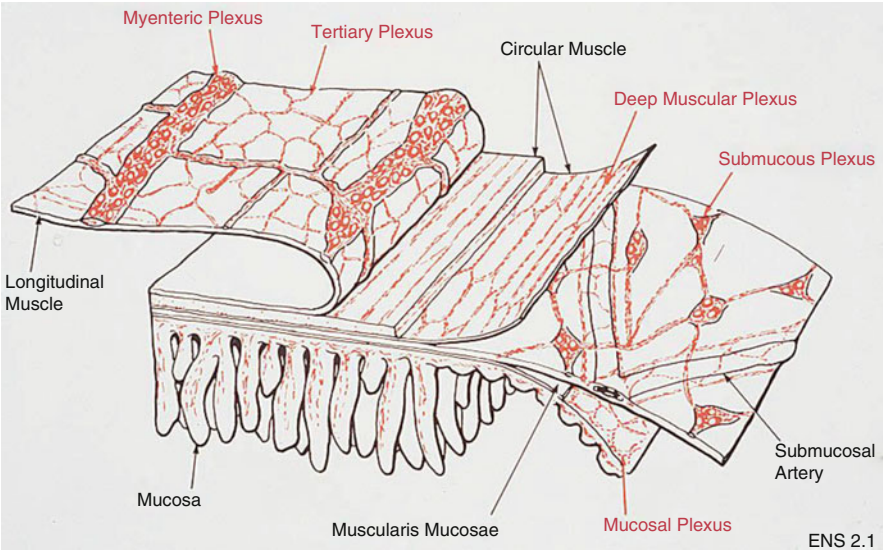

Fig. 1.3 Diagram of the general structure of the intestinal layers with the enteric plexuses

In parallel to our whole organ studies, others such as David Hirst, Alan North and Jack Wood were successfully attempting the first intracellular recordings of enteric neurons.

John and I moved in 1974–1975 to the newly established medical school at Flinders where we started with small foundation funding and develop further histochemical techniques. That was the initial boom of the neuropeptides with the work of Thomas Hockfelt in Stockholm who demonstrated that somatostatin was present in some sympathetic neurons.

The field of neuropeptides in enteric neurons blossomed, in parallel with our development of whole-mount immunohistochemistry of the intact enteric plexuses (Costa et al. 1980). I developed this method while on a short sabbatical at the University of Pavia in the Department of Histopathology of Enrico Solcia.

We could begin to draw enteric nervous system diagrams (Fig. 1.3 from Furness and Costa 1987) to include the growing number of potential types of nerve cells according to their morphology, histochemistry, connectivity and function. When this image became widely used, and most often without acknowledgment, we realized that the ENS had become really well established!

The 1980s started with a summary of the enteric nervous system that immediately become a citation classic (Furness and Costa 1980) and marked a decade of extensively mapping presence and distribution of neuropeptides in the enteric neurons in our and other laboratories (Costa and Furness 1982). In parallel we developed microsurgical techniques to interrupt selectively enteric pathways and unravel the wiring of the enteric neural circuits (Costa et al. 1981). Combining

lesions, histochemistry and electrophysiology was achieved with the crucial input of a then young Joel Bornstein and became a powerful strategy valid to this day (Bornstein et al. 1986).

Studies at the EM in our laboratory began to reveal the details of the synaptology of enteric neurons. Using combinations of all these methods we established that the enteric motor neurons to the muscle layers involved in intestinal motility are located in the myenteric ganglia and that they contained either SP or VIP (Wilson et al. 1987; Llewellyn-Smith et al. 1988). This was the first evidence that there are only two classes of motor neurons to the circular muscle, excitatory and inhibitory and thus if more than one substance was used as neurotransmitter by them they had to co-exist and the neurons had to use multiple transmitters (Costa et al. 1986a, b).

The concept of chemical coding emerged from these studies and consisted in the simple expectation that every functional class of neurons in any part of the nervous system is characterized by containing several specific chemicals some of which could act as multiple neurotransmitters (Furness et al. 1989). This concept has been consistently validated since then and has become part of modern neuroscience.

The ideas of multiple transmission and chemical coding were instrumental in clarifying much of the controversies around the nature of the NANC inhibitory transmission in the gut. In parallel with the purinergic hypothesis of Burnstock, VIP was shown by us to be present in the inhibitory motor neurons and proposed by others to be the unknown transmitter. The advent of NO as an inhibitory transmitter of the same neurons (Brookes et al. 1991) cemented the multiple transmission idea.

Initial links between cellular, organ and in vivo experiments of intestinal motility were made then by demonstrating that also in the guinea-pig the same fundamental mechanisms present in other species occur including slow waves, suspected to be absent from this species, and migrating motor complexes (Galligan et al. 1985).

The original proposal by Michal Gershon that 5HT is a neurotransmitter in the ENS, while not convincing to us, was supported by our own work providing a surprise to the audience at a meeting in Cincinnati where I showed the first image of 5HT immunoreactive enteric neurons to a very relieved Michael Gershon. I was lucky and privileged to be the key-note speaker at the retirement of Edith Bulbring at meeting in Banff in 1986. She was coauthor with Gershon of the original ideas of the role of serotonin in the gut in the 1960s. Of course I still think that the importance of 5HT as transmitter has been greatly exaggerated and remains still obscure.

Toward the end of the 1980s John Furness and I parted laboratories and he moved to Melbourne. I was very lucky to attract a young researcher from England, Simon Brookes. He introduced the new approach of retrograde labeling of enteric neurons by applying markers to targets and visualizing the enteric neurons of origin in organ culture of intact segments of intestine (Brookes and Costa 1990). With him we mapped systematically enteric neural projections and combined with targeted electrophysiological recordings represented a new strategy (Costa and Brookes 1994). The previous colleagues John Furness and Joel Bornstein in Melbourne continued and independently expanded similar studies and the 1990s generated much of the present knowledge of the enteric circuitries (Bornstein et al. 2004).

With Marcello Tonini from Pavia, a pupil of Antonio Crema, we developed partitioned organ baths to segregate the site of action of drugs to interfere with enteric pathways linking pharmacology to neuroanatomy of enteric circuits (Tonini and Costa 1990). We established that the old concept that peristalsis is a reflex does not reflects the true more complex nature of the movements of the intestine. Movements more akin to adaptable motor behavior than rigid stereotyped reflexes (Tonini et al. 1996). This other Marcello died prematurely only a few years ago.

Even the complexities of the human enteric neural circuits became amenable to be unraveled using similar multidisciplinary approaches in the unique Flinders setting of basic research laboratories and surgical theatres and gastroenterology labs within meters from each other and the unsurpassed good collaboration between clinical and basic researchers (e.g. Porter et al. 2002).

By the mid 1990s it was thus possible to reach a simplification of the enteric circuits by identifying and quantifying the classes of enteric neurons based on their chemical coding, projections and functions (Costa et al. 1996). Since then this summary has been perfected but not substantially changed.

In parallel the laboratories of Kent Sanders and Jan Huizinga investigating the mechanisms underlying the ongoing myogenic activity, proved that the ICCs act as pacemakers for the smooth muscle contractions. The interplay between the enteric neural circuits with the ICC pacemaker cell system began to be explored and provides powerful conceptual tools to explain the rich repertoire of gastrointestinal motility.

By the early 2000 three groups independently develop a new approach to extend the limited ability to record movements from a few point along the intestine based on the original graphic representation invented by Ludwig in the mid 1800s who provided the most powerful tool of smoked drum recording of physiological parameters, marking the birth of modern physiology and even modern science. The new approach replaced mechanical transducers to record movements with image analysis of video recording taking advantage of computing power and developing new software. One such novel approach, was developed by Grant Hennig while doing his Ph.D. in the laboratory and together we developed the method to constructing spatio-temporal maps of changes of gut wall diameters and lengths (Hennig et al. 1999). Similar methods developed independently also by others, enable for the first time to reveal the actual nature of the motor patterns that were only described by visual inspection or by very local recording sites. The idea of regarding behavior of any physiological system as a four dimensional structure promises to merge issues of form and function, shapes and movements, anatomy and physiology under one common conceptual frame.

In the past several years the opportunity arose to apply these new strategies of representing physiological events as spatiotemporal maps to clinical problems. From the initial attempt to make similar spatiotemporal maps with manometry recording of human gut from John Dent, a close colleague at Flinders and beyond (Andrews et al. 2001), to the very recent development of optical pressure probes for high-resolution manometry developed by John Arkwright and applied extensively to humans by Phil Dinning (Dinning et al. 2010). They have been attracted to Flinders by the promise of combining indeed spatiotemporal recording methods for

movements and forces responsible for these. In parallel a renewed period of research on intestinal motility at Flinders was triggered by the arrival of Nick Spencer and the ongoing collaboration and support by Simon Brookes at the Head of Physiology, fostered my return to the laboratory. Neurogastroenterology at Flinders had a long history and the Centre of Neuroscience, the first in Australia, still represents an ideal environment for intellectual and experimental cooperation. Together with these younger but senior colleagues and mentors, we have successfully developed combinations of measurements to identify the mechanically active or passive states of the intestinal muscle and thus revealing the spatio-temporal distribution of the neural activity behind (Costa et al. 2013). The idea that intestinal motility is the result of ongoing interactions between enteric neural circuits and the mechanical events as part of a neuromechanical loop provides a new and plausible conceptual model (Dinning et al. 2014).

What has become apparent since the first meeting of 1983, is that the explosion of potential transmitters substances and their multiple receptor systems, has yet to lead to a suitable understanding of intestinal functions. The actual role of most neuropeptides in enteric neurons and in the neuroendocrine mucosal cells is still unclear. Our knowledge of enteric neural activity with movements, secretion and circulation is still limited as is that of the neural traffic to and from the CNS. The mechanisms of interaction between the ever-changing luminal contents, including the barely known influence of the microbiome, the variety of food and potential toxins, are at their infancy.

It is often said quite correctly that when old investigators state that some things are impossible they are most likely wrong, but when they say that something is possible they are mostly right.

I am confident that, despite these complexities and difficulties, at the beginning of this second decade of the new millennium, we will see the emergence of new techniques that will bridge the gap between studies of human and animal motility. The development of new quantitative measurements of intestinal movements I have summarized, may well become part of these new approaches and will enable future investigators to distinguish and identify the underlying neural and myogenic mechanisms and their interactions in health and disease.

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Chapter 2
A Personal Perspective on the Development of Our Understanding of the Myogenic Control Mechanisms of Gut Motor Function

Jan D. Huizinga

Myogenic control mechanisms play a role in all motor activities of the gut. Myogenic control systems are defined here as control systems that are intrinsic to the smooth muscle cells and/or interstitial cells of Cajal (ICC) and that can operate without an essential contribution of the intrinsic (ENS) and extrinsic nervous systems. In vivo however, the ENS and the myogenic control systems always work in cooperation. Although myogenic control plays a role in every gut organ, this review focuses on the peristaltic and segmentation activity of the small intestine. It provides some historical perspectives and some discussion on the development of our understanding of the cooperative nature of the myogenic and neurogenic control mechanisms. It highlights how some influential papers inadvertently provided hindrance to full understanding, it discusses how the guinea pig model has hampered acceptance of myogenic control systems and it provides some background into the genesis of our understanding of control mechanisms involving ICC.

The Dominance of the "Law of the Intestine"

Nothing has hampered the acceptance of myogenic control mechanisms more than the formulation of the "law of the intestine" by Bayliss and Starling. The likely reason is the elegant descriptions of the evidence for it and the attractive nature of such a simple and effective, easily understood mechanism. This despite the fact that Bayliss and Starling prominently described myogenic control systems as well. In Bayliss and Starling's seminal paper from 1889 where the "law of the intestine"

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was formulated, the prominent existence of myogenic contractions was firmly established: “In the first place, we have the rhythmic pendular movements produced by simultaneous contractions of circular and longitudinal coats, and entirely myogenic in origin” (Bayliss and Starling 1899). These contractions were observed in the dog intestine occurring at 12/min, they were observed to propagate in both directions and have multiple origins along the intestine. The interesting thing is how the “law of the intestine” theory could have survived for so long as the stated dominant mechanism of peristalsis despite the concerns expressed from the early days. It was obvious already from Bayliss and Starlings’ work and confirmed by numerous authors in subsequent years that the law could often not be demonstrated in certain animal models or under certain conditions and that the weakest part was the inhibition of the musculature in front of the bolus, which was often not demonstrable. Nevertheless, the law survived and in the years that followed the notion of myogenic control somehow became less popular leading to exasperated statements from the Mayo Clinic gastroenterologist Alvarez. Alvarez wrote in 1922: “There are few statements in physiology upon which opinion is more unanimous than the one to the effect that the rhythmic contractions of the bowel are neurogenic, and due to impulses coming from Auerbach’s plexus. We find that statement in almost all of the textbooks and articles which we have consulted in the last eight years” (Alvarez 1922). Then he wrote: “Such unanimity is rather surprising, but it is still more surprising when we learn that practically all the research work done on the subject points clearly in the opposite direction”. This was followed by a discussion of the literature on this topic. At the end of his paper, Alvarez concluded: “… it should be pointed out that the question of the neurogenic or myogenic origin of the contractions is a more or less academic one because under normal conditions the muscle and nerve fibers are intimately associated and are designed to work together.” This sentiment was repeated in 2006 by Marcello Costa in his publication “All together now, from pacemakers to peristalsis” (Costa 2006). There has never been any doubt about the fact that the “law” can be demonstrated; the discussion should be to what extent are (most) normal peristaltic contractions governed by this law, by other neural programs and/or by myogenic controls systems. Statements on myogenic control have found their way in the literature at regular intervals, although neural control systems have always dominated the discussions on motor control. In 1986, Code and colleagues (Code et al. 1968) wrote: “the plexuses appear to program the motor action of the small bowel using the slow waves to consummate the program”.

The dominance of the ENS control of motility of the intestine is not without logic since we cannot survive without it (Huizinga et al. 2001) whereas the loss of the dominant pacemaker cells that generate the slow wave activity in animal models (Huizinga et al. 1995) does not lead to a dramatic failure of transit or absorption since alternative ways of propulsive contractile activity obviously develop.

The Incorporation of Interstitial Cells of Cajal in the Myogenic Control System

An important moment in the history of ICC physiology was the 9th International Symposium on Gastrointestinal Motility held in 1983 in Aix-en-Provence where Lars Thuneberg showed data on methylene blue mediated destruction of ICC-MP in the intestine and the loss of slow waves as a consequence. Thuneberg also showed video's of ICC in culture. For many of us, it was the first time that ICC came on our radar screen, and for many of us it was the start of incorporating ICC in our research protocols. But it took another 10 years to find convincing proof in the literature. Only a few months apart, two papers appeared, one at the end of 1994 and the other at the beginning of 1995 that provided evidence for and immediately firmly cemented a key role of interstitial cells of Cajal in the myogenic control system (Ward et al. 1994; Huizinga et al. 1995). It became clear that not smooth muscle cells but specialized pacemaker cells, the interstitial cells of Cajal were the generator of the omnipresent slow wave activity. This is how my laboratory got to write one of those papers: I have known Stephan (Steve) Collins for 30 years and in all these years of collaboration he has come to my office only once to show me a research paper he thought might be of interest to me, but once turned out to be enough. It was Maeda's paper describing how c-Kit antibodies could eliminate a certain cell type that would change the rhythmicity of intestinal contractions (Maeda et al. 1992). This was in 1993 and I can still see him standing in the doorway, casually giving me the paper he got from one of his post docs. I sat down at my desk and starting reading. After 1 h I could not contain my excitement and started walking up and down the hallway. Clearly this paper contained the gateway to proving or disproving the role of ICC in gut motility. I went back to Steve and told him that I wanted to work on this and how we should collaborate, but he told me that it was not his thing and I could go ahead. But the obstacles seemed large. The paper described how c-Kit antibodies could eliminate c-Kit positive cells from the intestine and at the same time affect the rhythmicity of the intestinal contractions. In the summer of 1993 I met Lars Thuneberg and Hanne Mikkelsen at the 14th International Symposium on Gastrointestinal Motility in Muskoka, Ontario and we discussed the project in Lars' hotel room and planned the immunohistochemistry and electron microscopy. It so happened that Mary Perdue occupied the office next to mine and she was working on WWv mice that lacked the c-Kit receptor (Perdue et al. 1991). She was interested in the role of mast cells in inflammation and I realized that the WWv mouse would be perfect for this study. But I knew little about the c-Kit receptor so I went to one of the experts, Bernstein at the University of Toronto. We decided to investigate the potential absence of ICC in the WWv mice using in situ hybridization. I remember one meeting at the University of Toronto. Thuneberg had come over to work with us on this project from Copenhagen. We wanted to show him the results

of the in situ hybridization that showed c-Kit positive ICC in control mice and no staining in WWv mice. When the figures were past on to him, he declared that we were mistaken, these brightly stained cells could not possibly be ICC because the size of the cells suggested them to be in an entirely different domain. Anxiety crept into the room and all eyes were on the calibration bars of the figures. Michael Kluppel, who had done the study, ran back to the lab to get the lab book. When the proper size of the cells was established, everything fell into place and we knew that we had proven the absence of ICC in the WWv mice. Lars went back to Copenhagen to confirm the in situ hybridization experiments with immunohistochemistry and in the mean time, John Malysz in my lab worked hard on getting electrical recordings from the control mice. It took many months to get reliable recording but John persisted and the result was one of the most exiting and convincing figures in the literature where we not only showed that WWv mice do not have slow wave activity but we also showed that the WWv intestine musculature is capable of generating action potentials indicating a normal musculature and the capability of contractile activity. Some time after we had started the experiments on the ICC in the mouse intestine I became convinced that Kent Sanders was probably experimenting with the same ideas since I discovered then that he had proof read the Maeda paper as mentioned in the acknowledgment section (Maeda et al. 1992). There was no doubt in my mind that Kent would have been just as excited about the paper as I was and that therefore he most definitely was working on the topic as well. I figured that the theory proven or disproven by two independent labs would be the best outcome. Just before we were ready to submit the paper to Nature, I met Casey VanBreemen and told him about my adventures with ICC. He insisted that I should talk about this at a conference he was organizing and I agreed. Kent Sanders was the chairman of that particular session and both he and I presented our data on the ICC back to back. Seldom have I experienced more excited anticipation than waiting for the talk from Kent about his ICC research. Our talks were remarkably similar, also he had decided to work with WWv mice instead of injecting c-Kit antibody.

A few years later, the final piece of evidence was reported, again communicated through two papers published a few months apart (Koh et al. 1998; Thomsen et al. 1998). If ICC were truly pacemaker cells, they ought to exhibit spontaneous rhythmic inward currents when isolated, and smooth muscle cells ought not to have that property. And indeed, ICC isolated from the myenteric plexus area of the mouse small intestine produce spontaneous inward currents with a reversal potential of +10 mV (Thomsen et al. 1998) or +17 mV (Koh et al. 1998). The most likely candidate for the channels producing the inward current was thought to be a nonselective cation channel at the time (Koh et al. 1998; Thomsen et al. 1998). Now this hypothesis has been superseded in favour of chloride channels (Wright et al. 2012; Gomez-Pinilla et al. 2009; Hwang et al. 2009).

The Role of the Guinea Pig in Our Thinking About Control Mechanisms

There can be no doubt about the important role the guinea pig model has played in the development of our understanding of the role of the ENS in motor control. The data derived from this model has been phenomenal and forms at this moment the fundamental basis of our understanding of the ENS (Costa and Brookes 2008; Furness 2006). It so happens that the guinea pig intestine may not show slow wave activity when an electrode is penetrated into the musculature under certain conditions and this has led to the myogenic control system taking a back seat and in most studies on guinea pig motility it has not been discussed. It is interesting to note that the influential Trendelenburg method, so frequently used in guinea pig peristaltic research, started with a 1917 paper from Trendelenburg in which he showed that the peristaltic activity of the guinea pig could occur without any neural influence (Trendelenburg 2006). Although peristaltic contractions could be inhibited by blockers of neural activity, it was clear to Trendelenburg that "conduction of peristalsis in the small intestine can also, like the propagation of peristalsis in the stomach, proceed without a nervous … conduction system". Furthermore, he showed in numerous figures the extreme rhythmicity of the peristaltic activity that occurred at the frequency of the slow wave activity as later shown by others (Donnelly et al. 2001; Smith 1989). The fact is that the guinea pig intestine exhibits robust slow wave activity but this activity is not omnipresent, it develops in response to a stimulus such as distention (Donnelly et al. 2001). There is no doubt in my mind that the rhythmic peristaltic activity of the guinea pig small intestine, already shown by Trendelenburg to be able to occur without neural influence is governed by slow wave activity generated by interstitial cells of Cajal (Komuro and Zhou 1996) with the ENS being the major excitatory force for smooth muscle depolarization. The guinea pig intestine can switch form peristalsis to segmentation with segmentation still occurring at the slow wave frequency (Gwynne and Bornstein 2007). But the tendency to deny a role for myogenic activity in the generation of such segmentation activity is strong (Gwynne and Bornstein 2007).

Expanding the Role of ICC in Control of Motility: The Segmentation Motor Pattern

Almost all motor patterns in gut organs have as primary function the mixing of content. Although classical peristalsis is equated with propulsion, and this is certainly the case in the esophagus, the predominant effect in all other organs is mixing and exposing the content optimally to the mucosal surface, because the propulsion ends somewhere and the content is moving back; only very rarely does propulsion end with

evacuation of content from the body (Huizinga and Chen 2014). The segmentation motor pattern is different from peristalsis in that it contains only stationary or very short distance propagating contractions and hence is considered a specialized motor pattern for mixing and absorption. The segmentation motor pattern was described and illustrated by Cannon in 1902 based on X-ray observations and shown to be extremely rhythmic (Cannon 1902). Walter Alvarez was the first to find that the frequency of rhythmic segmenting contractions occurred at the frequency of a myogenic pacemaker and that in various regions of the intestine the frequency decreased in the same way as the pacemaker frequency decreased (Alvarez 1914). In 1968, Code and colleagues (Code et al. 1968) also recognized the role of slow waves in segmentation; the slow waves were thought to go in and out of excitable regions of smooth muscle fibers. Although it is clear that segmentation and intestinal pacemaker activity have been associated in many discussions, experimental evidence as to how pacemaker activity would *create* segmentation was lacking. And therefore the attention shifted to the enteric nervous system to find a neural program that would change peristalsis into segmentation. One hypothesis states that cholinergic motor neurons acting on muscarinic receptors periodically activate the musculature and that inhibitory neurons surround this contraction to finalize the motor pattern (Gwynne and Bornstein 2007; Gwynne et al. 2004). Another study states that CCK and 5-HT are critical for the generation of segmentation (Ellis et al. 2013). Segmentation has also been suggested to result from a reduced degree of synchrony of AH neuron activity and by sustained inhibition by the after hyperpolarization (Ferens et al. 2007; Huizinga and Chen 2014). A few years ago, my own hypothesis was that a contraction could activate enteric sensory neurons which would result in activation of short nitrergic inhibitory neurons to transiently inhibit a slow wave driven propulsive contraction so that it periodically became annihilated causing rhythmic segmentation. Until I saw a perfect segmentation motor pattern in the presence of TTX (Huizinga et al. 2014). While on sabbatical at the laboratory of Jihong Chen at Wuhan University we experimented with decanoic acid that had been shown to induce segmentation (Gwynne et al. 2004). We discovered a remarkable feature: decanoic acid changed the electrical activity of the intestine from regular slow waves to a waxing and waning pattern. At first we hypothesized that if two pacemaker sites from the ICC-MP network were sending slow wave activity into the musculature at slightly different frequencies, waxing and waning might occur and possibly segmentation (Fig. 2.1). This was based on ideas and evidence presented by Diamant, Bortoff and Suzuki (Diamant and Bortoff 1969; Suzuki et al. 1986). Indeed, when two sine waves of slightly different frequency interact by addition, a waxing and waning pattern develops. But in that case, the maximum amplitude becomes twice the original amplitude. When we confirmed the induction of waxing and waning by intracellular recording of the electrical activity, no change in maximum slow wave amplitude occurred (Pawelka and Huizinga 2015). Suzuki deduced the existence of two prominent similar frequencies in the waxing and waning pattern based on FFT analysis but FFT might not be the best method to evaluate frequencies in a signal that changes markedly over time. When we explored Continuous Wavelet Transform analysis, a single dominant slow wave frequency was obvious together with a low frequency

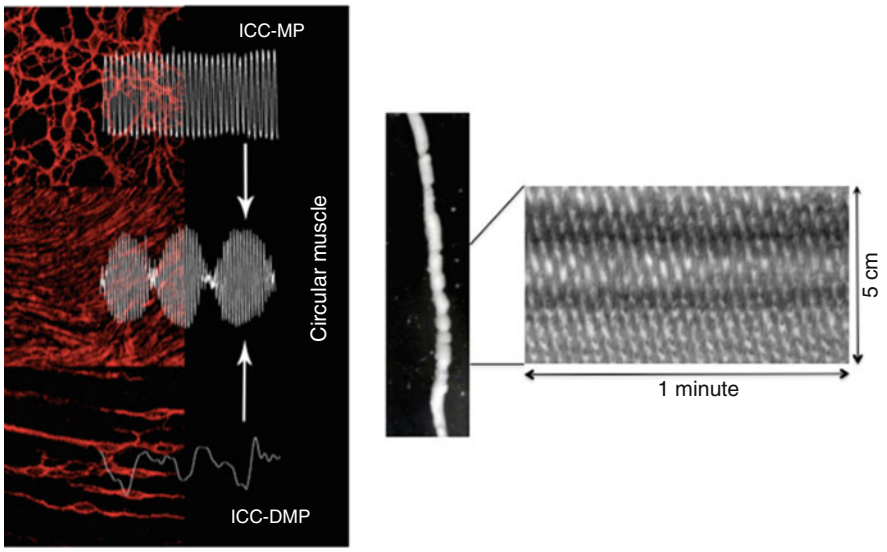

Fig. 2.1 ICC associated with the myenteric plexus (ICC-MP) generate slow wave activity. ICC associated with the deep muscular plexus (ICC-DMP) generate rhythmic transient depolarizations upon receiving specific stimuli such as decanoic acid or butyric acid, directly or indirectly. The two signals originating at opposite sides of the circular muscle layer can interact with each other by phase-amplitude coupling once propagated into the musculature. That is, the phase of the low frequency component modulates the amplitude of the high frequency component resulting in waxing and waning without increase in amplitude of the maximal slow wave. This electrical activity can orchestrate the checkered segmentation motor pattern. One frame of a video recording is shown showing multiple simultaneous circular muscle ring contractions, also evident in the spatio temporal map of the checkered segmentation motor pattern. Modified from Huizinga et al. (2014)

component. Calcium imaging gave us the hypothesis that the origin of the low frequency electrical signal was the network of ICC-DMP (Huizinga et al. 2014). Discussions with Bardakjian and McGinn at the University of Toronto led to an exploration of how the low and high frequency oscillations were interacting with each other within the musculature as coupled oscillators. Based on theories and methods of phase–amplitude coupling in the central nervous system (Tort et al. 2010) we obtained strong evidence that the waxing and waning electrical activity can be explained by the phase of the low frequency component modulating the amplitude of the high frequency component resulting in a waxing and waning motor pattern (Huizinga et al. 2014). Importantly, analysis of the checkered motor pattern of segmentation also provided evidence for phase–amplitude coupling underlying this motor pattern. The evidence that the pattern of segmentation can develop when a low and a high frequency myogenic pacemaker interact, does not exclude a role of the ENS in the development of segmentation in vivo. In most experimental conditions, the enteric nervous system will provide an essential stimulus for the motor activity to develop and hence a variety of nerve conduction blockers or neural receptor blockers will inhibit segmentation activity. Whether or not a motor pattern occurs in response

to nutrients or distention is often determined by the response of the ENS to the stimulus. This is also the case for segmentation, and several components of the ENS have been shown to be involved (Gwynne and Bornstein 2007). This neural activity then works in concert with the ICC pacemaker activities to generate the motor pattern of segmentation (Huizinga and Chen 2014).

In summary, myogenic control systems are present in the intestine and are part of the orchestration of most motor patterns. They provide the rhythmic propulsion within phase III of the migrating motor complexes (Hall et al. 1982) expressed during fasting at night, they provide rhythmicity and propulsion to peristaltic activity, in particular in the proximal intestine (Der-Silaphet et al. 1998), and they provide the basis for the segmentation motor pattern (Huizinga et al. 2014).

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Chapter 3
Enteric Inhibitory Neurotransmission, Starting Down Under

Kenton M. Sanders

Introduction and Historical Note

Gastrointestinal motility requires neural regulation to coordinate contractions of smooth muscle cells in the walls of the organs. Neurons providing motor regulation have cell bodies in the myenteric plexus and can provide both excitatory and inhibitory input to the gastrointestinal (GI) tract. These neurons are activated through sensory inputs and their input generates patterns of contractile activity, such as tonic contractions, peristalsis, and segmentation.

The notion of an inhibitory innervation of the gut arose when Geoff Burnstock's group at the University of Melbourne measured inhibitory responses in the taenia coli in the early 1960s (Burnstock et al. 1963; Bennett et al. 1966). This type of neural regulation is not common to the autonomic nervous system and therefore must be thought of as a very special capability of the enteric nervous system and in regulation of gut motor patterns. Enteric inhibitory neurotransmission became known as non-cholinergic, non-adrenergic (or NANC) neurotransmission because of its resistance to antagonists of norepinephrine and acetylcholine. In 1970 Professor Burnstock published evidence that ATP (or a closely related nucleotide) fulfilled the criteria as an inhibitory neurotransmitter (Burnstock et al. 1970). This highly cited paper demonstrated release of purines during transmural nerve stimulation and similarities between the responses to exogenous ATP and the enteric inhibitory neurotransmitter in several GI muscle preparations. Later apamin was found to block purinergic responses, providing evidence that small-conductance Ca^{2+}-activated K^+ (SK) channels are responsible for inhibitory junction potentials (IJPs) (Banks et al. 1979). A great deal of study was applied to the pharmacology of

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nucleotide receptors, and the molecular age allowed identification of several families of genes encoding a large number of purine and pyrimidine receptors. Like cholinergic receptors, one family of purine receptors mediated ionotropic effects (P2X) and another family mediated metabotropic effects (P2Y).

Enteric Inhibitory Motor Neurotransmitters

At the current time, the identity of ATP as the enteric inhibitory neurotransmitter has come into doubt, as the Eccles criteria for a neurotransmitter are better met by β-nicotinamide adenine dinucleotide (β-NAD) (Mutafova-Yambolieva et al. 2007; Hwang et al. 2011) or a metabolite ADP-ribose (ADPR) (Durnin et al. 2012). Stimulation of motor neurons in mouse or primate colon by agonists that activate ganglionic receptors causes release of ATP and β-NAD/ADPR, but only the release of β-NAD/ADPR was blocked by tetrodotoxin, suggesting that most of the ATP released comes from ganglionic sources, not from varicosities along projections of motor neurons that innervate the smooth muscle layers (Durnin et al. 2013).

There was a flurry of activity regarding the role of peptides as neurotransmitters in the GI tract during the 1980s. Several studies linked peptides, such as neurokinins and vasoactive intestinal polypeptide (VIP), to motor neurotransmission, and VIP (or related peptides) were initially considered the long sought-after non-cholinergic, non-adrenergic (NANC) inhibitory transmitter (Goyal et al. 1980). However, responses to nerve stimulation were considerably different than responses to VIP (Mackenzie and Burnstock 1980; Hills et al. 1983), and it is now difficult to picture how responses that are so clearly dependent upon NO, could have been reduced or blocked by systemic immunoneutralization of VIP. There were also studies using VIP fragments (e.g. $VIP_{10–28}$) to antagonize VIP receptors and peptidases (α-chymotrypsin) to break down peptides released into the interstitium and claims that antagonists of this sort could block NANC neurotransmission (Grider and Rivier 1990), but other investigators failed to show antagonism of NANC neurotransmission with VIP analogues or block of NANC responses by peptidases. Recent studies using $VIP^{-/-}$ mice have clearly shown a VIP-dependent component of motor neurotransmission, but VIP-dependent responses are generally induced by sustained, multi-sec stimulation and/or at higher stimulus frequencies than 5 Hz (Keef et al. 2013).

A new, non-conventional neurotransmitter emerged on the scene as an important mediator of NANC inhibitory neurotransmission in 1989–1990 (Gillespie et al. 1989; Bult et al. 1990). The evidence for nitric oxide (NO) as a NANC neurotransmitter was rather convincing from the first papers on this topic: a component of inhibitory neurotransmission was blocked by inhibitors of NO synthesis and by hemoglobin, an isoform of nitric oxide synthase (nNOS) was localized in nerve varicosities (where it co-localized with VIP), stimulation of intrinsic neurons caused release of NO, and NO elicited hyperpolarization and relaxation of GI muscles (Sanders and Ward 1992). The concept of NO as an enteric inhibitory neurotransmitter

was not without controversy, and proponents of VIP (and related peptides) continued to argue aggressively that the authentic neurotransmitter was VIP and NO was a secondary neuromodulator produced in post-junctional cells (Murthy et al. 1996) or as a secondary mediator released from neurons, but only after release of VIP (Mashimo and Goyal 1999). Eventually, the arguments against NO or involving the need for a peptide middleman to induce NO-dependent relaxation failed to explain the data, and studies with specific knockouts of NOS demonstrated that nNOS, expressed by motor neurons, was the source of this important component of enteric inhibitory responses (Dick et al. 2002).

Purines, Nitric Oxide and Peptides Elicit Different Parts of the Enteric Inhibitory Response

Enteric inhibitory responses are multi-facetted. Stimulation of motor neurons releases at least three types of neurotransmitter substances, and recordings of post-junctional changes in membrane potential and suppression of the spontaneous electrical activity of GI muscles, best, but not fully, enunciate these responses. The relative contribution of each class of enteric neurotransmitter to post-junctional responses, differs in various tissues of the GI tract and in different species. Responses are dependent upon stimulus frequency: single or a few pulses of transmural nerve stimulation elicit post-junctional responses that are mediated by purines and NO and are blocked by apamin, P2Y1 receptor antagonists and N^G-nitro-L-Arginine; stimulus frequencies of 5 Hz or more are typically required to elicit responses due to release of peptides. The kinetics of purinergic and nitrergic responses are markedly different. The purine response is a fast, large amplitude hyperpolarization response that can approach the K^+ equilibrium potential, but the response wanes within several hundred milliseconds. The nitrergic response develops more slowly, and responses to even a single stimulus can persist for a few seconds. The nitrergic hyperpolarization is smaller in amplitude than the purinergic response and may be due either to activation of K^+ channels (Koh et al. 2001) or suppression of an inward current, most likely suppression of a non-selective cation conductance or Ca^{2+}-activated Cl^- channels (Zhang and Paterson 2002; Hirst et al. 2004). The integrated response to purinergic and nitrergic neurotransmission is a two phase inhibitory junction potential, consisting of a fast IJP and a slow IJP. VIP and PACAP elicit responses that are much slower to develop and are partially mediated by activation of K_{ATP} channels (Keef et al. 2013).

Responses to specific components of the IJP can be dissected pharmacologically because relatively selective reagents are available to block the nitrergic and purinergic components (via block of NO synthesis, NO receptors and purine receptors). Responses due to release of peptides are more difficult to delineate pharmacologically, but as above, $VIP^{-/-}$ mice are being used to clarify the nature of peptidergic responses (Keef et al. 2013). Present data suggest that the mouse is a relatively good experimental model for studies of neural regulation in the GI tract, however it is important to continue to check for species differences.

Obviously, motility is dependent upon contractile activity, so it is important to comment on the differences in contractile responses elicited by different classes of neurotransmitters. Purinergic motor responses appear to be mediated by an electrical mechanism (i.e. hyperpolarization and consequent reduction in smooth muscle excitability), but both nitrergic and petidergic responses may have, in addition to electrical mechanisms, important non-electrical mechanisms of action (e.g. Ca^{2+}-desensitization of the smooth muscle contractile apparatus). Ca^{2+} sensitization mechanisms in GI muscles have been studied largely by addition of excitatory or inhibitory neurotransmitter substances to organ baths and measuring the extent of protein phosphorylation in Ca^{2+} sensitization pathways or by testing the effects of Rho kinase antagonists. However, study of responses to exogenous compounds is not an accurate means of investigating the role of Ca^{2+} desensitization mechanisms in neurotransmitter responses, because neurotransmitters may not bind the same population of receptors as exogenous transmitter substances added to the organ bath (Bhetwal et al. 2013).

Post-junctional Cells that Mediate Enteric Neurotransmission

The classic concept of motor neurotransmission in the gut is that neurotransmitters released into the interstitium diffuse to receptors expressed by smooth muscle cells to elicit responses. In fact the receptive field for motor neurotransmitters is more complicated and composed of multiple types of cells that express receptors and transduction mechanisms for enteric motor neurotransmitters. At least two classes of interstitial cells (interstitial cells of Cajal (ICC) and PDGFRα^+ cells) have been shown to contribute to neural responses: ICC mediate at least a portion of the responses to NO and acetylcholine (ACh) and PDGFRα^+ cells mediate purinergic neurotransmission (Burns et al. 1996; Ward et al. 2000; Kurahashi et al. 2011). Interstitial cells convey electrical conductance changes to the smooth muscle cells via gap junctions. Electrical connectivity between these cells means that conductance changes in any of the coupled cells affects the excitability of the smooth muscle component. Together smooth muscle cells, ICC and PDGFRα^+ cells have been referred to as the SIP syncytium (Sanders et al. 2012), and this integrated syncytium of cells constitutes the receptive field for enteric neurotransmission.

Receptors for Enteric Inhibitory Neurotransmitters

We now know that enteric inhibitory neurotransmission is mediated by P2Y1 receptors (Gallego et al. 2006), and the fast IJPs associated with purinergic neurotransmission are absent in P2Y1$^{-/-}$ mice (Gallego et al. 2012; Hwang et al. 2012). PDGFRα^+ cells express P2Y1 to a far greater extent than other cells of the

SIP syncytium (Peri et al. 2013). PDGFRα$^+$ cells respond to P2Y1 agonists with activation of SK currents, possibly due to openings of SK3 channels that are also highly expressed in these cells (Klemm and Lang 2002; Iino and Nojyo 2009; Kurahashi et al. 2011; Peri et al. 2013). Ca^{2+} transients are elicited in PDGFRα$^+$ cells in response to purines, and this signaling is likely to couple P2Y1 receptors to activation of SK3 channels (Baker et al. 2013). Current data suggests that smooth muscle cells are unlikely to mediate purinergic hyperpolarization responses, because at physiological transmembrane potentials, smooth muscle cells generate small amplitude inward currents and depolarization in response to purines (Hwang et al. 2011; Kurahashi et al. 2011).

The receptor for NO is soluble guanylate cyclase (sGC), composed of α and β subunits (Groneberg et al. 2011). Several studies have shown that ODQ, an inhibitor of sGC, blocks the nitrergic component of enteric inhibitory neurotransmission (Franck et al. 1997; Hirst et al. 2004). sGC has been shown to be expressed robustly in ICC of the GI tract, and sGC expression has also been resolved in some types of PDGFRα$^+$ cells (Iino et al. 2009). Expression of sGC has not been resolved in SMCs by immunohistochemistry, suggesting that levels of sGC are comparatively low in these cells. Recent studies have employed cell-specific iCre expressing mice to knock down expression of sGC in ICC, SMC or in both cell types (Groneberg et al. 2013). These studies showed that knock-down of sGC in ICC or smooth muscle cells did not block nitrergic response, but when knock-down occurred in both types of cells, nitrergic responses were depressed. Thus, nitrergic responses may require activation of signaling molecules and effectors in multiple types of cells in the SIP syncytium.

Cellular Effectors that Mediate Enteric Inhibitory Responses

Many studies have identified apamin-sensitive K^+ channels as the main mediators of purinergic inhibitory input to GI muscles (Banks et al. 1979). As above, SK3 channels are highly expressed in PDGFRα$^+$ cells (Klemm and Lang 2002; Iino and Nojyo 2009; Kurahashi et al. 2011; Peri et al. 2013), however SMC also express SK channels and can respond to purines by activation of SK currents (Koh et al. 1997; Vogalis and Goyal 1997; Ro et al. 2001; Klemm and Lang 2002). It should be noted that the current density due to SK channels is far lower in SMC (Kurahashi et al. 2011), and as mentioned above, the net response of SMC at physiological membrane potentials is generation of inward, not outward currents.

There is controversy about the mechanism of NO action GI muscles. Initially, the hyperpolarization response to nitrergic stimulation suggested activation of a K^+ conductance (Dalziel et al. 1991; Thornbury et al. 1991), however others have suggested that suppression of an inward current may be the mechanism for hyperpolarization responses (Zhang and Paterson 2002; Hirst et al. 2004). The nature of the conductance responsible for the inward current may vary by anatomical location and

in different species. For example, responses in the guinea pig colon were unaffected by 9-AC, a chloride channel blocking drug, but in lower esophageal sphincter, responses were inhibited by another chloride channel blocking drug, niflumic acid. Contractile responses to nitrergic stimulation may also be influenced by Ca^{2+} uptake into stores and by Ca^{2+} desensitization mechanisms. To affect contractility, these responses would likely occur in SMC, however if nitrergic responses are linked to Ca^{2+}-activated Cl^- channels (CaCC), then responses involving Ca^{2+} sequestration and release from intracellular stores would be mainly focused upon ICC, because it is ICC that express CaCC in the SIP syncytium (Hwang et al. 2009).

Summary and Major Questions Remaining for Enteric Inhibitory Regulation of the GI Tract

There are still significant questions to be answered regarding enteric inhibitory neurotransmission. Progress has been made on the relative contributions of specific cells and pathways that are activated by enteric neurotransmitters from studies of clearly identified isolated cells of the SIP syncytium. Delineation of the cells, receptors and ion channels mediating enteric inhibitory responses will likely result from genetic studies in which genes can be knocked-down selectively in specific classes of cells. It will be important to assess the degree of knock-down in specific cellular populations to verify cell-specific loss-of-function, and as seen with the sub-resolution levels of sGC in SMC, protein levels undetected by immunohistochemical techniques may be effective in mediating neurotransmission. Which SIP syncytium cells mediate responses to neuropeptides; additional studies on isolated cells are needed to investigate this question. Study of how SIP cells develop and why they are lost or become dysfunctional in disease conditions might explain defective neural regulation in a variety of GI motor disorders. Of particular interest is how inflammatory factors affect the responsiveness of the SIP syncytium to neurotransmission. It is possible, for example, that inflammatory mediators might remodel the responses of the SIP syncytium, such that aberrant motor responses might be elicited by normal neural inputs. Very little is known about prejunctional regulation of neurotransmitter release from enteric motor neurons. Knowledge of prejunctional regulatory pathways might provide ideas about enhancing or depressing the level of regulation by enteric motor neurons and could develop as useful therapeutic tools. More studies to characterize enteric neural responses in human muscles are needed to incorporate and validate what is known from animal studies into our knowledge of the physiology and pathophysiology of human neurogastroenterology.

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Chapter 4
Spatio-Temporal Mapping and the Enteric Nervous System

Grant W. Hennig

Recording GI Motility

Measuring the motility of the gut has been a challenge since the first recordings of intraluminal pressure 145 years ago by Legros and Onimus in 1869 (Legros and Onimus 1869). The gut is a continuous tube about 9 m long in humans, making it virtually impossible to study in toto, although the continued development of scanning technology [e.g. MRI, see studies by Andreas Steingoetter's group (Sauter et al. 2012)] or gigantic organ baths [Nick Spencer's Lab (Kuizenga et al. 2015)] are bringing us closer to this ideal.

In such a long organ, there is always a trade-off between accuracy and resolution of recording methods. Muscle strip experiments using isotonic or isometric transducers have always enabled precise measurements of length and tension respectively, allowing characterization of many aspects of the motor output of the enteric nervous system. Recording contractions with multiple transducers (length/tension/pressure) in vivo or in isolated segments of gut allows some quantification of the propagation of contractions, such as the migrating motor complex [Terence Smith's Lab (Heredia et al. 2009)]. Sampling multiple sites at close distances increases the confidence in determining the propagation characteristics of contractions, but eventually one reaches a finite number of transducers one can attach to intact GI organs, and the attachment points may alter normal gut motility.

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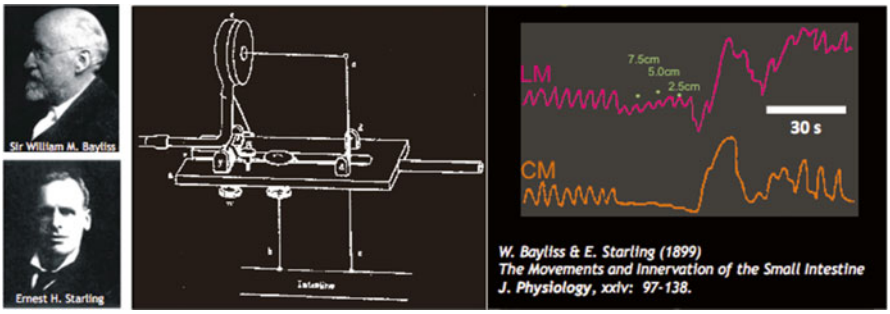
The "enterograph" devised by Bayliss and Starling (pictured) allowed simultaneous recording of longitudinal and circular motions of the gut via a system of levers (Bayliss and Starling 1899)

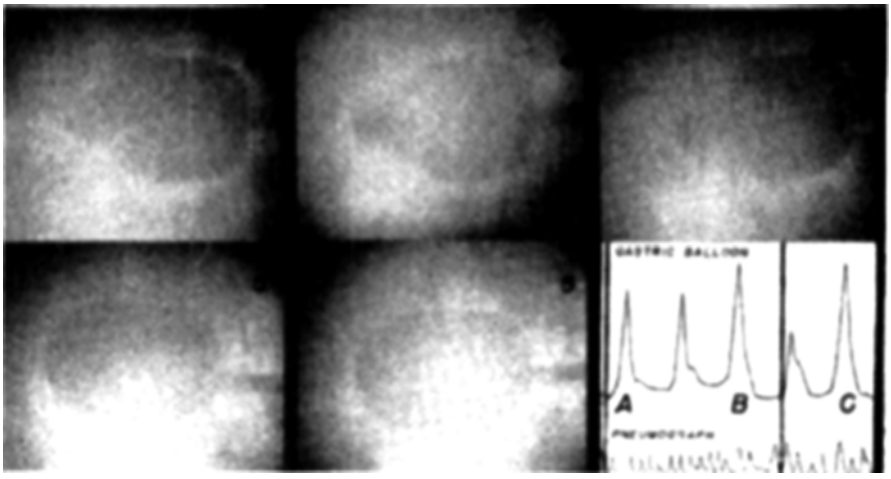


Movies

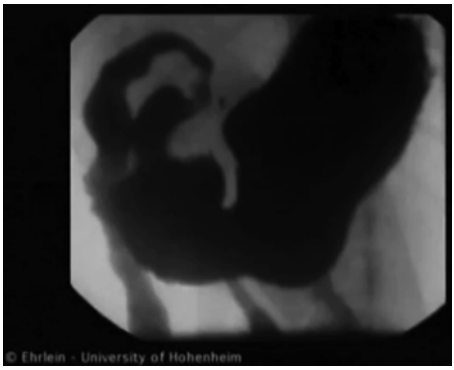
Interestingly, a method was in existence at least 4 years before the seminal study of the peristaltic "reflex" by Bayliss and Starling in 1899 (Bayliss and Starling 1899) that offered a way to remotely monitor thousands of points along the gut—the movie camera. A portable version (the "Cinématographe") invented by the Lumiere brothers in 1895 (http://www.earlycinema.com/pioneers/lumiere_bio.html) using 35 mm film could, in theory, resolve at least 1 million "pixels" even after accounting for poor optics and film. This technology was quickly adapted to record the then recently discovered X-rays (Roentgen) and used to great effect by Walter Cannon (1899–1911) to document the motions of the gastrointestinal tract. Cannon's descriptions and drawings provided the first vivid and compelling evidence of the wide repertoire of motor patterns the gut produces. Combining cineradiography and pressure transducers allowed the shape of GI organs to be correlated with the forces they were generating [see studies by Charles Code (Smith et al. 1957)]. Using a modernized version, Ehrlein's videofluoroscopy (Seigle and Ehrlein 1987) project in the 1980s to document of gut motility patterns throughout the GI tract remains one of the most lucid presentations of the complexity of gut motility.

The "Cinématographe". A portable 35 mm movie camera developed by the Lumiére Brothers in 1895 (http://www.earlycinema.com/pioneers/lumiere_bio.html)

Simultaneous "Cineradiogram and Kymogram" recordings of the stomach allowed the changes in shape to be correlated to intragastric pressure during antral peristalsis (Smith et al. 1957)



Videofluoroscopy allowed highly detailed movies of the shape of the stomach to be recorded in vivo. "Antral Peristalsis" from DVD: Ehrlein HJ "Gastrointestinal motility shown by Video-fluoroscopy", see Seigle and Ehrlein (1987)

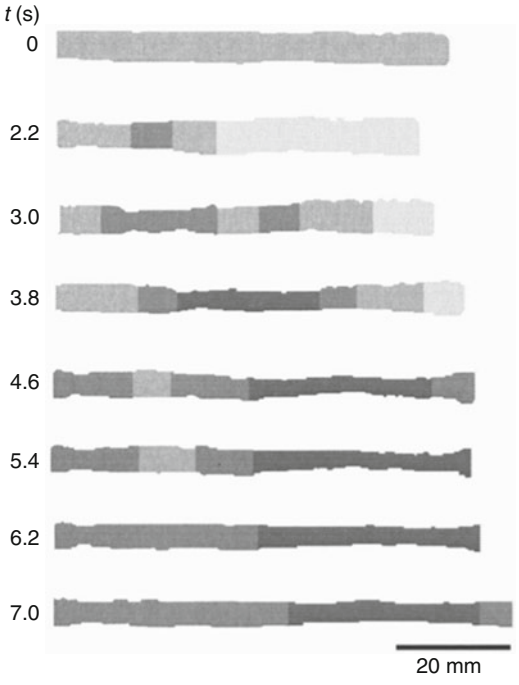


It is somewhat perplexing why the superior resolution of movie cameras did not gain wider acceptance in research labs around the world. While fluoroscopy is used clinically to qualitatively assess mainly esophageal and colonic motility, its use as quantitative tool to examine gut motility in research has been limited. Certainly the problems of using ionizing radiation limited the time of recordings, but perhaps a greater influence on its lack of adoption was the enormous effort needed to analyze the data. Developing and projecting the film, then tracing and measuring contractions took a considerable investment of time, concentration and effort—and could be quite daunting when faced with repeating the process for hundreds or thousands of frames. In contrast, gut motions recorded using a smoked drum could be analyzed in minutes.

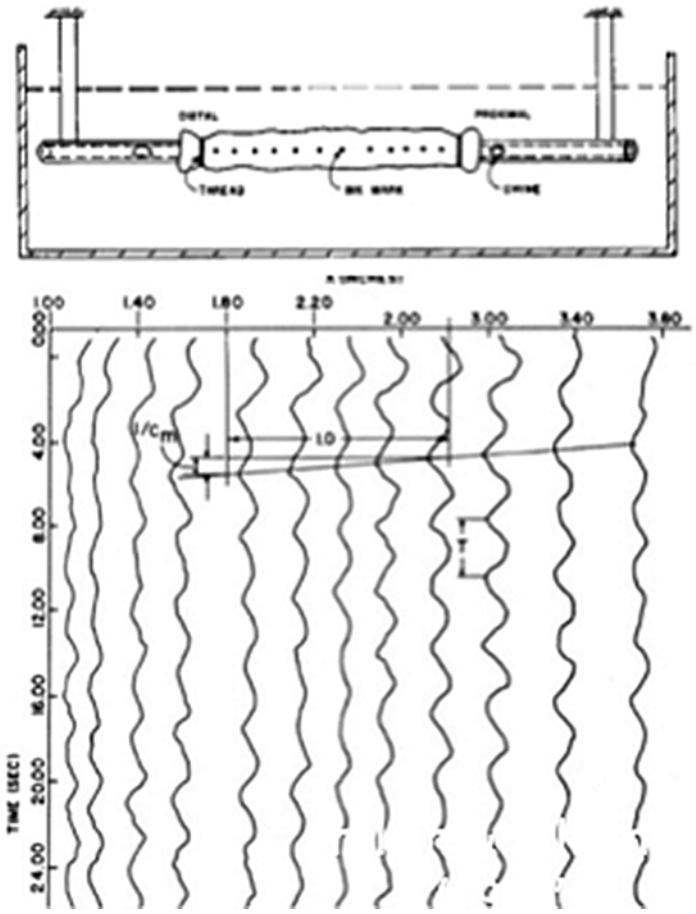
A pivotal period during which analysis of gut contractility from movies became more feasible was when framegrabber boards became available for mainstream computers to digitize video in the late 1980s (Commodore Amiga A1000). For example, Apple Computer's incorporation of Quicktime into their operating system, SuperMac framegrabber boards and free analysis software developed by the NIH (NIH Image; Wayne Rasband) simplified the process enormously so that measurements of wall motion measurements could be made relatively easily [Waterman/Costa Lab (Waterman and Costa 1994)]. However, the difficulty associated with analyzing the changes in the shape of GI organs as they contracted in movies remained. Representing contractions at single points along the gut like conventional isotonic timeplots was useful, but neglected much of the recorded informa-

tion for the sake of simplicity. Plotting multiple points over time gave a better sense of the spatio-temporal characteristics of contractions, such as longitudinal movement of markers in isolated segments of small intestine by Melville et al. in 1975 (Melville et al. 1975). The use of a "waterfall plot" in which time was plotted on the Y-axis allowed a more intuitive recognition of oral ↔ anal movements. Eventually though, there was also a finite limit on how many timeplots could be presented in a figure without considerable overlay of the traces. What was needed was a way to automate the extraction of movement data and a way to present it so that none of the data was obscured.

Manually color-coding diameter changes during peristalsis allowed easier visualization of contraction and relaxation during peristalsis (Waterman and Costa 1994)



Tracking the trajectories of markers on the surface of the intestine to resolve longitudinal contractions along the intestine (Melville et al. 1975)

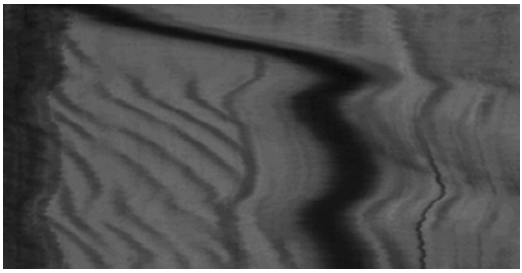


ST Map Inception

The idea of structures that could portray both space and time has been common throughout the twentieth century in physics (e.g. Einstein) and philosophy (e.g. Webb 1971), but had limited adoption in enteric/gastrointestinal research. Graphic representations of changes in diameter using color have been used to illustrate peristalsis (Waterman and Costa 1994), but still relied on presenting individual outlines of the gut. The development of spatio-temporal maps of contractile activity in the gut developed in two centers around the same time. Benard et al. (1997) developed a dedicated program and system to detect the edge of the gut and calculate

diameters automatically. In contrast, at Flinders University, the storing of diameter values in picture memory buffers to overcome the size limits of text buffers in NIH Image led to the accidental creation of ST Maps [Hennig/Costa Lab (Hennig et al. 1999)]. Both techniques used a simple technique in order to fit all the diameter information along the gut into a single image without overlapping traces: *conversion of diameter information into a color*.

The first ST Map generated in the Costa Lab (unpublished, G. W. Hennig circa 1997: guinea-pig distal colon, see Hennig et al. (1999))

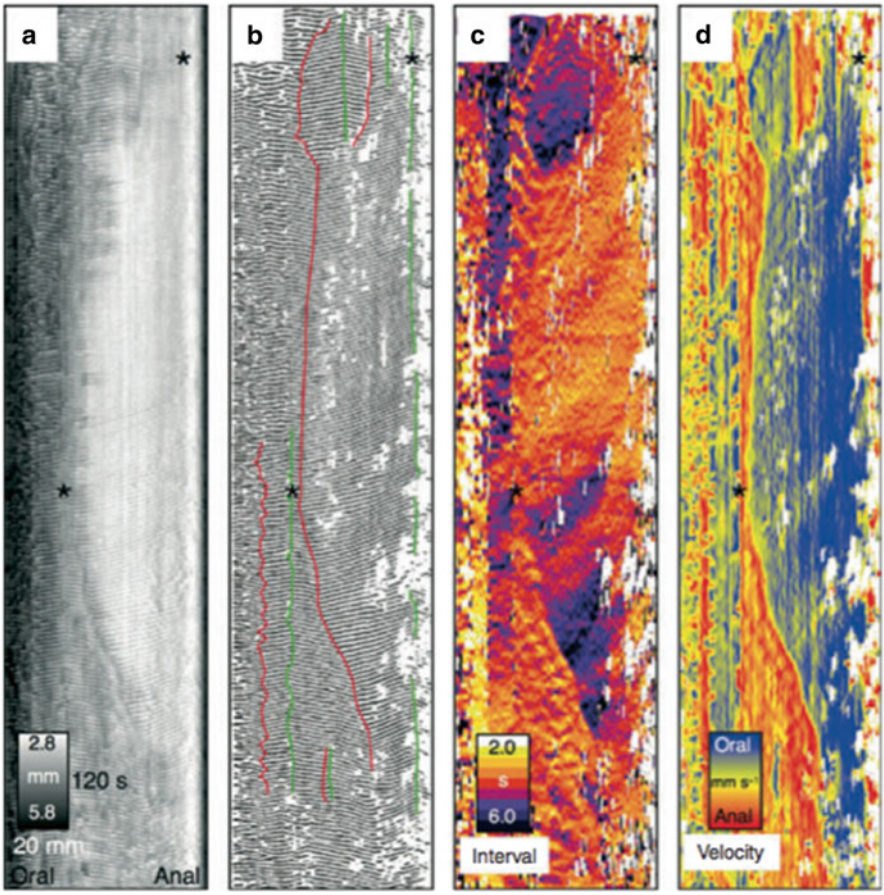


Spatio-temporal maps (ST maps, aka DMaps) brought a number of immediate advantages to the field of enteric neuroscience/gastroenterological research, such as (1) the ability to visualize the full complexity of motor patterns over long periods of time, (2) to appreciate and accurately measure the propagation characteristics of contractions and (3) ease of analysis. As ST maps are essentially an image, various image analysis techniques commonly used for photos work equally well on ST maps such as filtering (kernels), differentiation and edge detection. But perhaps the largest advantage of using movies and ST maps was that the technique was non-invasive and is likely to be a much better representation of physiological motor patterns compared to studies that used attached transducers. While not quite as sensitive as isotonic transducers in quantifying the amplitude of contractions, they are exceptional in detecting small changes in the *pattern* of contractions in large regions of the gut. They also shifted the emphasis of research to take into account both the changes in amplitude and *coordination* (e.g. propagation direction, coherence, spacing) of contractions.

Derived ST Maps

During long recordings in which many events are recorded, the compression of time in ST Maps makes interpretation of individual events difficult. To remedy this issue, particular motion parameters can be calculated within each ST Map and used to create *derived* ST Maps. Color is usually used to encode motion parameters such as interval (frequency), velocity, direction [Hennig/Costa Lab (Hennig et al. 2010)] and cross correlation [e.g. de Loubens/Lentle (de Loubens et al. 2013)].

Frequency (interval) and velocity maps derived from diameter maps (Hennig et al. 2010)

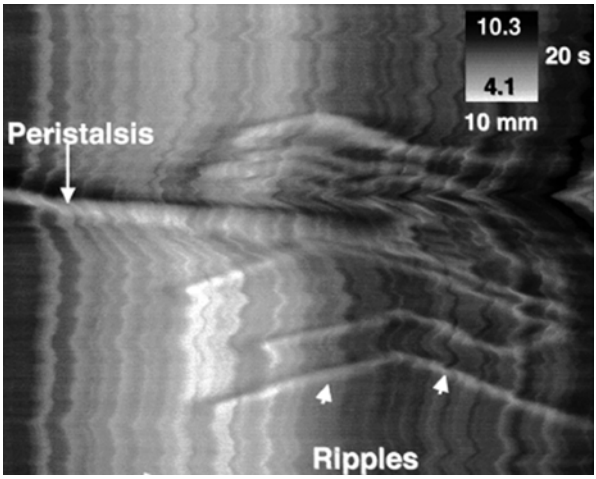


ST Map Adoption and Development

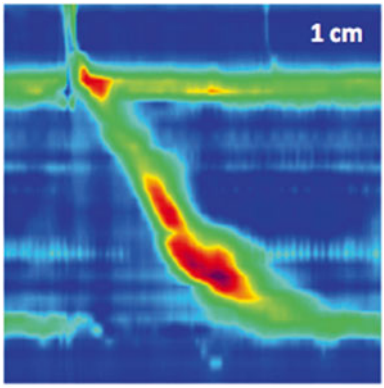
ST maps have been adopted widely in G.I. clinical diagnosis and research since the late 1990s. As the technique is relatively simple, most laboratories have developed their own hardware/software systems suitable to answer the questions they are most interested in. ST maps have revealed patterns of motility that have not been documented previously, such as 'ripples' in the colon [D'Antona/Costa Lab (D'Antona et al. 2001)]. They are commonly used to diagnose esophageal motility using ST maps of pressure recorded using manometry catheters [see Brasseur/Dinning/Smout (De Schepper et al. 2014)]. The research labs using ST Maps are too numerous to mention individually here, but have been used to document development of the gut (Young), mixing behaviors (Huizinga/Bercik/Bornstein/Hennig), in vivo motility (Furness) and fish motility patterns (Olsson/Rich) to name a few. ST maps have also been useful, not

only to map patterns of contraction in the gut, but also patterns of enteric neuron firing and ICC and smooth muscle activity (Smith/Hennig/Sanders Labs).

Shallow, propagating, myogenic "ripples" occurring in tandem with neurally-mediated waves of peristalsis (D'Antona et al. 2001)

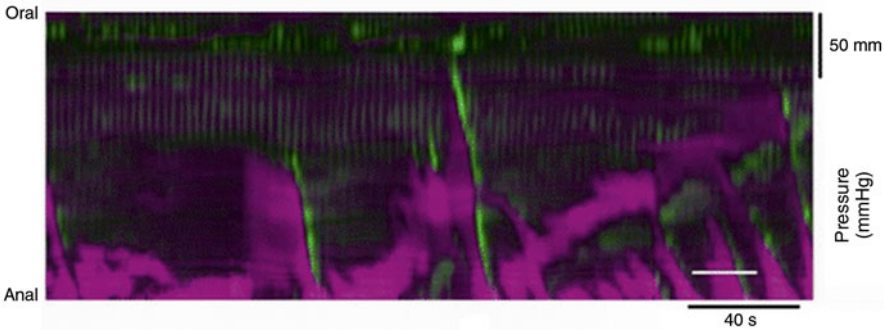


Example of an ST Map calculated from multipoint manometry during an esophageal swallow (De Schepper et al. 2014)



ST Maps are continually being developed and modified to better describe and analyze motility patterns. Combining ST Maps with other measures of gastrointestinal performance (e.g. video + pressure transducers; Costa et al. (2013)/Spencer Lab) allows for more detailed information about how gut contractions move contents. Other groups have developed ST Maps to better describe the phase of contractions [see Lentle (de Loubens et al. 2013)]. Weems (1982) summarized that *flow* throughout the gut was the most important parameter to measure, and recent combinations of ST Maps of video and pressure may begin to realize this ultimate goal.

Example of an ST Map displaying both diameter (purple) and pressure (green) changes along the intestine (Costa et al. 2013)



Limitations

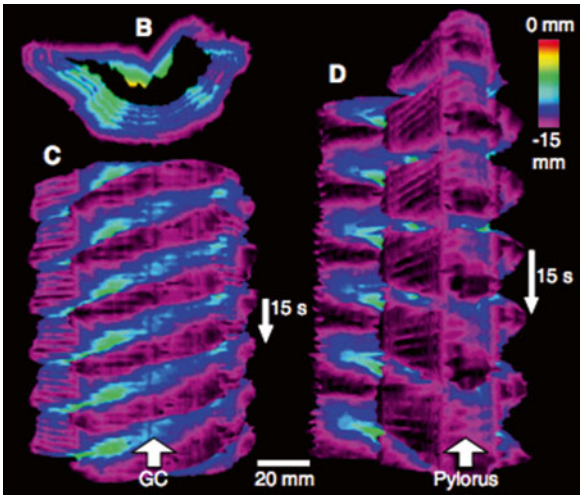
During the initial development of ST maps for use in GI research, the largest flaw of this technique was not fully realized. The small intestine can be prepared so that it remains fairly linear in an organ bath, and given that most contractions of the small intestine indent the gut wall a similar amount around the circumference of the organ, there was no issue in using diameter to represent the overall constriction around the organ. Using ST map techniques on the curved stomach required some modifications (Berthoud/Costa Lab), or in tissues where contractions did not cause the same degree of indentation around the circumference (Hennig/Costa Lab). *However, the problem remains that traditional ST Maps cannot distinguish where around the circumference contractions are occurring*. Most of these problems arose due to the sacrifice of spatial information around the circumference into a single value. Until information around the circumference could be retained, the use of ST maps to capture contractility patterns would continue to be limited.

The Solution: Spatio-Temporal Objects

Ever since the first ST maps were constructed, researchers have attempted to preserve information around the circumference of the gut. Some inelegant solutions used color to map circumferential position, but in the end, any solution needed to leverage an additional spatial dimension (i.e. 3D). Rendering contractions calculated from each frame of a movie as a 3D translucent cube allowed better visualization of the coordination of circumferential contractions, however at the time

(Hennig/Costa Lab, late 1990s) such rendering were extremely taxing to computers due to the sheer amount of data that could be captured using video. It wasn't until the rapid evolution of graphic cards starting 2005–2007 for PC gaming that enough computing power was available to seriously develop methods to utilize 3D representations spatio-temporal data. A number of options now exist to preserve circumferential information, but perhaps the least taxing for common computing equipment found in most GI research labs is the use of edge detection, and triangle mesh creation of outline of organs. This offers a number of advantages over traditional ST maps as: (1) contractions can be followed and quantified on curved surfaces, (2) the amplitude of contractions at multiple points around the circumference can be quantified and (3) different derived parameters can be converted to colors and incorporated into ST objects to allow more comprehensive visualization of motility patterns [e.g. instantaneous velocity. Hennig Lab (Kholmovski et al. 2014)].

Example of an ST Object calculated from an in vivo MRI recording of antral peristalsis in humans (Kholmovski et al. 2014)



Conclusion

Appreciating GI motor patterns in their full complexity gives us better insights into how the ENS can generate and modulate gut contractility. The use of ST maps has broadened our understanding of ENS control of gut motility and changed the way we conceptualize GI behavior.

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Chapter 5
Development of Neural Activity in the Enteric Nervous System: Similarities and Differences to Other Parts of the Nervous System

Marlene M. Hao

Introduction

All the neurons and glia of the enteric nervous system (ENS) arise from neural crest-derived cells that migrate into the gastrointestinal (GI) tract during development (Yntema and Hammond 1954; Le Douarin and Teillet 1973). Most of the ENS originates from vagal neural crest cells (NCCs), which arise from the caudal hindbrain region of the neural tube, adjacent to somites 1–7. In the developing mouse, vagal NCCs migrate into the developing oesophagus and stomach at embryonic day (E)9.5, enter the small intestine at E10.5, and colonise the developing GI tract in a rostral-to-caudal wave, reaching the anal end of the colon at E14.5 (Serbedzija et al. 1991; Kapur et al. 1992; Anderson et al. 2006). Recent evidence indicates that there is also trans-mesenteric migration of vagal NCCs, where some NCCs leave the small intestine and migrate directly across the mesentery into the colon (Nishiyama et al. 2012). Sacral NCCs also contribute to a small population of neurons and glia in the colon (Burns and Le Douarin 1998; Wang et al. 2011).

After they arrive in the gut, neuronal differentiation commences before the enteric neural crest-derived cells (ENCCs) have colonised the entire length of the GI tract. In the mouse, around 10–15 % of ENCCs in the small intestine express pan-neuronal markers at E10.5 (Baetge and Gershon 1989; Young et al. 1999), including Hu (Fairman et al. 1995), neuronal class III β-tubulin (Tuj) (Fairman et al. 1995; Conner et al. 2003), neurofilament-M (Payette et al. 1984), PGP9.5 (Young et al. 2002) and microtubule-associated protein 2 (MAP2) (Baetge et al. 1990). Unlike many regions of the developing central nervous system (CNS), there are no distinct proliferative or differentiation zones, and neuronal differentiation occurs

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throughout the ENCC-colonised regions, including at the migratory wavefront (Young et al. 1999; Conner et al. 2003). In addition, some ENCCs expressing pan-neuronal markers continue to proliferate (Teitelman et al. 1981; Young et al. 2005) and migrate (Hao et al. 2009).

For a cell to function as a neuron, the machinery involved in action potential firing and neurotransmission must be present. This includes a variety of voltage-gated ion channels, the most important of which are voltage-gated Na^+ channels (VGSCs), voltage-gated K^+ channels (VGPCs), and voltage-gated Ca^{2+} channels (VGCCs). Ion pumps and channels that set up the electrochemical gradient across the cell membrane to produce the membrane potential of neurons are also essential. Prior to the acquisition of their mature neuronal electrical properties, spontaneous activity is often observed in the developing nervous system, in particular, spontaneous changes in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) (Rosenberg and Spitzer 2011). This spontaneous activity is vital for the development and maturation of the nervous system, and it appears that the ion channels and receptors in developing neurons may be optimised for the generation of spontaneous activity (Moody and Bosma 2005; Spitzer 2006; Ben-Ari and Spitzer 2010). In this article, I will review evidence for the presence of spontaneous activity and electrical maturation in the developing ENS in comparison to similar events in other parts of the developing nervous system.

Neuronal Differentiation and Early Spontaneous Activity in Migratory NCCs

In the mouse, vagal NCCs do not express any neuronal markers en route to the foregut (Anderson et al. 2006), although differentiation takes place very shortly after they enter the GI tract at E9.5 (Baetge and Gershon 1989). Prior to entry into the hindgut, some sacral NCCs express pan-neuronal markers, however, these could be neurons of the developing pelvic ganglia, which also arise from the sacral NCC, rather than NCCs that are destined to migrate into the colon (Anderson et al. 2006). Studies of other parts of the peripheral nervous system such as the ciliary ganglion and dorsal root ganglia indicate that many cells are capable of firing action potentials as soon as they reach their final location (Bader et al. 1983a; Gottmann et al. 1988; Lechner et al. 2009). However, one important difference between these populations and the vagal NCCs is that reaching the foregut does not signify the end of migration for vagal NCCs, and subpopulations of vagal NCCs must continue to migrate caudally until the entire GI tract is colonised. So far, no studies have examined the electrical activity of vagal NCCs during their migration en route to the gut.

To examine the development of neural activity in migrating populations of NCCs, many studies have used neural tube explants in which the neural tube is excised from embryos prior to NCC migration, cultured and then recordings made from NCCs as they migrate away from the neural tube. For avian mesencephalic NCCs, which give rise to ciliary ganglia, cells with neuronal morphology expressing

neuronal markers appeared at 24 h in vitro, but they only exhibited voltage-dependent K^+ currents (Bader et al. 1983b; Bader et al. 1985; Arcangeli et al. 1997). Fast inactivating voltage-dependent inward Na^+ currents were first present at 36 h in vitro but were not prevalent until after 48 h in vitro, and voltage-dependent Ca^{2+} currents were also seen in some, but not all neurons at this age (Bader et al. 1983b). The order in which particular forms of activity develops is similar to that in developing enteric neurons (described below). Interestingly, an immature inward-rectifying K^+ current was recorded from avian mesencephalic NCCs, as well as NCCs from other levels of the neural tube (Arcangeli et al. 1997). This immature current resembled the I_{HERG} recorded from cardiac muscle cells, but its properties changed during maturation to resemble the more mature inward-rectifying K^+ current (I_{IRK}) after a longer period of in vitro culture and at older embryonic ages. This change appeared to be correlated with a hyperpolarisation of the membrane potential, and hence, the immature HERG-like current may help maintain the membrane potential at more depolarised levels to favour spontaneous activity (Arcangeli et al. 1997).

Voltage-dependent inward and outward currents resembling Na^+ and K^+ currents have been recorded from trunk NCCs cultured in vitro for 7–8 days (Howard et al. 1995). Of interest, these responses were observed in both neuronal and non-neuronal cells, as characterised by their morphology. The presence of these currents in migrating non-neuronal NCCs in vivo has not yet been investigated. The culture conditions are likely to play an important role in neuron differentiation in these in vitro studies, as media with less factors added appear to promote neuron differentiation more than serum-containing media (Boisseau and Simonneau 1989).

In more recent studies of migrating trunk NCC, spontaneous $[Ca^{2+}]_i$ transients were recorded from cells prior to neuronal differentiation, in NCCs that did not morphologically resemble neurons, including murine trunk NCCs migrating in vitro (Carey and Matsumoto 1999) and avian NCCs in explant slices (McKinney and Kulesa 2011). Intracellular Ca^{2+} stores were found to be the main source of cytosolic Ca^{2+} increases, regulated by the inositol triphosphate receptor (IP_3R) (Carey and Matsumoto 2000). The spontaneous $[Ca^{2+}]_i$ transients were shown to be important for differentiation (Carey and Matsumoto 1999) and the coalescence of cells into ganglia (McKinney and Kulesa 2011). In the GI tract, spontaneous $[Ca^{2+}]_i$ transients can be recorded from dissociated ENCCs that express pan-neuronal markers and those in which pan-neuronal markers cannot be detected (Hao et al. 2011). It is possible that non-neuronal ENCCs with $[Ca^{2+}]_i$ transients are cells that are committed to, or are in the process of, differentiating into neurons, however, this remains to be confirmed as they could also differentiate into glia.

Different mechanisms may underlie the mode of cytosolic Ca^{2+} increase in early enteric neurons in comparison to non-neuronal ENCCs, as electrically-evoked $[Ca^{2+}]_i$ transients in the developing ENS appears to be restricted to cells that express pan-neuronal markers (Hao et al. 2011). In the developing cerebral cortex, neurons are generated from proliferating precursors in the ventricular zone that then migrate towards the developing cortical plate. Cells in both the ventricular zone and cortical plate exhibit spontaneous $[Ca^{2+}]_i$ transients (Owens et al. 2000). However, the $[Ca^{2+}]_i$ transients in ventricular zone cells appear to be driven by intracellular Ca^{2+}

stores, whereas the $[Ca^{2+}]_i$ transients in cortical plate cells appear to be driven by extracellular Ca^{2+} entry (Owens and Kriegstein 1998).

Early Versus Mature Action Potentials

Action potentials and voltage-dependent Na^+ currents can be recorded from dissociated ENCCs with neuronal morphology at E11.5 and E12.5 (Hao et al. 2012). Along with dorsal root ganglion neurons and spinal neurons (Krieger and Sears 1988; Lechner et al. 2009), the ENS is one of the earliest parts of the nervous system to exhibit classical voltage-dependent Na^+ current-mediated action potentials.

Action potentials and spontaneous $[Ca^{2+}]_i$ transients are not the only forms of activity recorded from developing enteric neurons. Graded active potentials (GAPs) are active increases in membrane potential recorded from ENCCs with neuronal morphology in response to depolarisation, however, they are not all-or-nothing events (Hao et al. 2012). Instead, the amplitude of the responses are graded according to the magnitude of the depolarisation. Cells that exhibit GAPs could be immature neurons that have insufficient expression of VGSCs. The current(s) responsible for the change in membrane potential in GAPs are still to be identified; they are resistant to tetrodotoxin and lidocaine, two potent blockers of VGSCs (Hao et al. 2012). One possibility is that they are generated by voltage-dependent Ca^{2+} currents. Ca^{2+}-driven action potentials have been recorded in the spinal neurons of developing *Xenopus laevis* (Baccaglini and Spitzer 1977), and in addition, early spontaneous activity in developing mouse hindbrain neurons also includes spontaneous Ca^{2+} "spikes" (Gust et al. 2003; Moruzzi et al. 2009). However, these events, which last hundreds of milliseconds to seconds, are much longer in duration than the GAPs of enteric neurons, which are milliseconds to tens of milliseconds in duration. All ENCCs with neuronal morphology exhibited voltage-dependent K^+ currents (Hao et al. 2012). Not surprisingly, the action potentials of enteric neurons increase in amplitude and decrease in half-duration through embryonic and postnatal development, most likely due to increases in the expression, and hence the density, of voltage-gated ion channels, which has been described in other parts of the developing nervous system (McCobb et al. 1990; Mienville et al. 1994; Gao and Ziskind-Conhaim 1998; Rothe et al. 1999; Picken Bahrey and Moody 2003; Fry 2006).

In the adult ENS, there are two electrophysiologically distinct classes of neurons: AH and S neurons (Hirst et al. 1974; Bornstein et al. 1994; Furness 2006). AH neurons typically exhibit a "hump" on the repolarising phase of their action potential, a prominent slow after-hyperpolarising potential (sAHP) that follows action potential firing (Hirst et al. 1974; Bornstein et al. 1994), and also a hyperpolarisation-activated H-current that is active at rest (Galligan et al. 1990; Rugiero et al. 2002; Mao et al. 2006). S neurons typically exhibit fast excitatory postsynaptic potentials

(Nishi and North 1973; Hirst et al. 1974; Bornstein et al. 1994). These potentials and currents that distinguish different classes of enteric neurons are not prominent in embryonic enteric neurons, but are present by birth, although there is continued postnatal maturation (Foong et al. 2012; Hao et al. 2012).

The Development of Enteric Glia

Enteric glia in the mature ENS express GFAP (glial fibrillary acidic protein) (Jessen and Mirsky 1980), S100β and Sox10 (Hoff et al. 2008). In the mouse ENS, the development of enteric glia occurs later than that of enteric neurons. In the mouse, immunoreactivity for S100β can be detected at E14.5 (Young et al. 2003) and GFAP at E16.5 (Rothman et al. 1986). Prior to this, the precursors of enteric glia maintain *Sox10* expression, which is downregulated in neurons (Young et al. 2003). Immunoreactivity for brain-derived fatty acid binding protein (B-FABP), which is a selective marker of glial precursors and differentiated glia, is present in the GI tract at E11.5 (Young et al. 2003). In the chick gut, glial differentiation appears to occur earlier than in the mouse, as GFAP immunoreactivity is present close to the migratory wavefront (Conner et al. 2003).

Action potentials have long been considered the hallmark of a neuron, however, recent studies have shown that oligodendrocyte precursor cells in the CNS, can fire APs (Karadottir et al. 2008). In addition, many voltage-gated ion channels, including VGSCs, are expressed by many non-neuronal cell types (reviewed in Black and Waxman 2013). In enteric glia, patch-clamp recordings of myenteric glia from adult guinea pig have shown that the majority of cells exhibit passive currents only, due to coupling of cells by gap junctions (Maudlej and Hanani 1992; Hanani et al. 2000). When coupling was inhibited, voltage-dependent outward K^+ currents were observed in many cells. In a small number of cells, a tetrodotoxin-sensitive fast inward voltage-dependent Na^+ current was observed, however, it was much smaller in amplitude than the fast inward Na^+ currents recorded from myenteric neurons (Hanani et al. 2000). In addition, tetrodotoxin-sensitive Na^+ currents have also been recorded from glial cells isolated from the myenteric plexus after several weeks in culture (Broussard et al. 1993). However, the significance of these currents is unknown. There have been many studies investigating neuron-glial communication, which has been identified as an important component of ENS signaling (reviewed in Gulbransen and Sharkey 2012), although there are a number of technical challenges (Boesmans et al. 2013). Glial activity during ENS development has been examined in one study in vitro, where neurons and glia were taken from dissociated E13.5 mouse gut and activity recorded between 4 and 9 days in culture using calcium imaging (Gomes et al. 2009). Glia were found to respond to ATP released from enteric neurons in these cultures. The development of activity in enteric glial cells remains to be investigated further.

Conclusions and Future Challenges

The maturation of developing neurons involves changes in their electrical properties. For the ENS and most other parts of the developing nervous system, this means an initiation and increase in the expression of voltage-gated ion channels that are involved in action potential firing. However, neuronal precursors and progenitor cells are not completely lacking in electrical properties. Moreover, there is a growing body of evidence that the bioelectric state of a cell is important for determining its developmental behaviour, not only in the nervous system but also in other developing tissues and organs (Sundelacruz et al. 2009). For example, a more depolarised membrane potential in neuronal progenitors is essential for proliferation (Blackiston et al. 2009). These progenitor/precursor cells often exhibit spontaneous activity, which is very different from the activity of mature neurons, and also plays important roles in guiding nervous system development.

In the developing ENS, whilst the changes in the electrical properties of excitable action potential-firing neurons have been characterised, the electrical properties of non-action potential-firing cells remain to be investigated further. Spontaneous $[Ca^{2+}]_i$ transients and GAP activity has been recorded from developing enteric neurons, but the underlying mechanisms of these activities are still unknown. In addition, the development of activity in enteric glia remains to be examined.

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Chapter 6
ENS Development Research Since 1983: Great Strides but Many Remaining Challenges

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What Was Known About ENS Development in 1983?

The origin of the ENS was first discovered in 1954, when Yntema and Hammond surgically ablated defined levels of the neural crest in chick embryos and showed that neurons in the gut, lung and heart arise from vagal level (caudal hindbrain) neural crest cells (Yntema and Hammond 1954). Subsequent studies using chick-quail chimeras by Le Douarin and colleagues confirmed that vagal neural crest cells give rise to neurons along the entire length of the gut, and also revealed that sacral level neural crest cells give rise to some enteric neurons, mostly in the colon (Le Douarin and Teillet 1973).

By 1983, it was also known that although very few mature enteric neurons are catecholaminergic, many enteric neuron precursors transiently synthesize catecholamines (Teitelman et al. 1981). Moreover, some neurochemically-defined subpopulations of enteric neurons were shown to differentiate prior to birth or hatching (Epstein et al. 1980; Gershon et al. 1980; Rothman and Gershon 1982), and functional innervation of the gut muscle had also been demonstrated to occur prior to birth (Gershon and Thompson 1973).

In 1983, there were only three publications in the field of ENS development (Ciment and Weston 1983; Epstein et al. 1983; Gershon et al. 1980). Since then, the

The first enteric nervous system (ENS) conference, organized by Marcello Costa and John Furness, was held in Adelaide, Australia in 1983. In this article, we review what was known about the development of the ENS in 1983 and then summarize some of the major advances in the field since 1983.

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field has grown enormously, due largely to the power of genetically engineered mice, and cross-fertilization between human molecular geneticists studying patients with the ENS developmental disorder, Hirschsprung disease, and researchers using animals models including mice, chick and zebrafish.

Major Advances Since 1983

Due to space restraints, here we summarize just 10 of the major advances in the field since 1983. Other advances in ENS development research are discussed in more comprehensive reviews (Goldstein et al. 2013; Heanue and Pachnis 2007; Gershon 2010; Lake and Heuckeroth 2013; Laranjeira and Pachnis 2009; Obermayr et al. 2013; Sasselli et al. 2012).

Identification of the Major Signalling Pathways Involved in ENS Development

In 1983, none of the signalling pathways required for ENS development had been identified. In 1994, a group of studies published in *Nature* showed that mutations in the gene encoding the receptor tyrosine kinase, RET, were associated with Hirschsprung disease (Edery et al. 1994; Romeo et al. 1994), and mice lacking Ret did not have any neurons in the small and large intestines (Schuchardt et al. 1994). At this time, Ret was an orphan receptor. However, subsequent studies showed that mice lacking glial cell line-derived neurotrophic factor (GDNF) have a similar phenotype to mice lacking Ret (Moore et al. 1996; Pichel et al. 1996; Sanchez et al. 1996), and that GDNF signals through the Ret receptor (Durbec et al. 1996). GDNF is produced by the gut mesenchyme, while Ret is expressed by enteric neural crest-derived cells (ENCCs) (Heanue and Pachnis 2007). Research from Vassilis Pachnis' laboratory and others showed that Ret signalling plays an essential role in multiple processes in ENS development including proliferation, neuronal differentiation, survival and migration (Hearn et al. 1998; Heuckeroth et al. 1998; Taraviras et al. 1999; Chalazonitis et al. 1998; Young et al. 2001; Uesaka et al. 2007, 2008; Gianino et al. 2003).

Although Ret signalling is the most important pathway for ENS development, several other signalling pathways also play roles including endothelin-3 acting on endothelin B receptor (Hearn et al. 1998; Druckenbrod and Epstein 2009; Nagy and Goldstein 2006; Sidebotham et al. 2002), neurotrophin-3 acting at TrkC receptors (Chalazonitis 2004), sonic hedgehog, which is produced by epithelial cells and acts on the Patched receptor expressed by ENCCs (Fu et al. 2004; Sukegawa et al. 2000) and bone morphogenetic proteins (Chalazonitis et al. 2004, 2010; Fu et al. 2006).

Transcriptional Control of ENS Development

In the past 20 years, studies of mice with targeted or spontaneous mutations have identified over a dozen transcription factors that act as major intrinsic regulators of ENS development (Obermayr et al. 2013; Howard 2005). For example, mice lacking Phox2b (Pattyn et al. 1999), Sox10 (Southard-Smith et al. 1998), Foxd3 (Teng et al. 2008), Ascl1 (Blaugrund et al. 1996) or Pax3 (Lang et al. 2000) lack neurons from all or particular regions of the gastrointestinal tract. Several of these transcription factors are essential because they directly or indirectly regulate Ret expression (Pattyn et al. 1999). In many parts of the nervous system, the transcriptional control of the differentiation of different neuronal subtypes is now well understood (Goridis and Brunet 1999; Rohrer 2011; Ang 2006; Srinivasan et al. 2012). There are many different sub-types of enteric neurons (Costa et al. 1996), but little is still known about the transcription factors involved in the generation of different neuron subtypes during ENS development.

Identification of Migratory Pathways and Migratory Behaviour

Vagal ENCCs appear to follow the same pathway from the caudal hindbrain to the foregut that is later followed by the vagal nerve (Baetge and Gershon 1989; Anderson et al. 2006). After entering the foregut, vagal ENCCs had been assumed to migrate caudally to the anal end exclusively within the gut mesenchyme. However, it has been recently shown that during the time ENCCs are colonizing the midgut of embryonic mice, the mid- and hindguts are transiently closely apposed, and a sub-population of ENCCs takes a short cut across the mesentery into the colon without passing through the caecum (Nishiyama et al. 2012). It is likely that this transmesenteric migration of ENCCs also occurs in humans as the mid- and hindguts in fetal humans are also closely apposed when the midgut is being colonized by ENCCs (Nishiyama et al. 2012). Some sacral neural crest-derived cells enter the distal hindgut and contribute to the ENS, but they first migrate to the vicinity of the hindgut and undergo a waiting period of several days before entering the gut along the axons of extrinsic neurons (Burns and Le Douarin 1998; Wang et al. 2011).

There are some species and regional differences in the order in which the myenteric and submucosal regions are colonized by ENCCs (McKeown et al. 2001). In the small and large intestines of mice and humans, ENCCs first populate the myenteric region, and submucosal ganglia arise from a secondary, centripetal migration of ENCCs through the circular muscle layer several days later (McKeown et al. 2001; Payette et al. 1984; Wallace and Burns 2005). The centripetal migration of ENCCs is driven by the chemoattractant, netrin, which is expressed by the mucosa (Jiang et al. 2003).

Time-lapse imaging experiments using explants of embryonic gut have revealed that ENCCs migrate in chains with high cell-cell contact (Druckenbrod and Epstein

2007; Young et al. 2004). The migratory behaviour of individual ENCCs is very variable and shows considerable differences from cranial and trunk neural crest cells (Kulesa et al. 2010; Theveneau and Mayor 2012; Young et al. 2014). Many of these differences are likely to be due to the fact that some ENCCs must populate each gut region at the same time as other ENCCs advance caudally (Young et al. 2014).

ENCCs Act as a Community

It is now well established that the behaviour of ENCCs is influenced by interactions with neighbouring ENCCs (Kapur et al. 1995). For example, when only a subpopulation of ENCCs carries mutations in genes involved in proliferation or differentiation, the unaffected (wild-type) ENCCs change their rate of proliferation and neurogenesis to compensate (Lei and Howard 2011; Mundell et al. 2012). The mechanisms by which ENCCs communicate with each other to act as a community are still unknown.

Migration Is Influenced by Proliferation and Differentiation

Migration, proliferation and differentiation of ENCCs are usually studied as separate processes. However, a variety of studies have shown that both proliferation (cell number) and differentiation influence ENCC migration. For instance, studies using chick embryos showed that reducing the number of premigratory neural crest cells reduces the distance along the gut that ENCCs migrate (Yntema and Hammond 1954; Peters-van der Sanden et al. 1993; Barlow et al. 2008). Moreover, signals that promote neuronal differentiation retard ENCC migration (Hearn et al. 1998; Wu et al. 1999). Thus the colonization of the gut by ENCCs requires the tight coordination of ENCC migration, proliferation and differentiation; this also reinforces the concept that ENCCs must communicate with each other.

Different Enteric Neuron Subtypes Exit the Cell Cycle at Different Times

Before differentiating into a neuron, precursors undergo a final division, which is termed the birthdate. In many parts of the nervous system different neuron types have been shown to have different birthdates (McConnell 1989). An important study from Michael Gershon's laboratory, published in 1991, showed that some enteric neuron subtypes also differ in their birthdate, with 5-HT interneurons being born first (Pham et al. 1991). Subsequent studies using markers of different functional classes of enteric neurons showed that excitatory motor neurons are born last, with sensory neurons and inhibitory motor neurons born at intermediate stages

(Bergner et al. 2014). The age at which cell cycle exit occurs influences the effects of developmental cues and disturbances on the generation of different types of enteric neurons (Chalazonitis et al. 2008; Li et al. 2011; Wang et al. 2010). Moreover, premature or delayed generation of one neuron subtype is likely to influence the differentiation of other neuron subtypes.

The Maternal Environment Can Influence Prenatal ENS Development

Hirschsprung Disease is the congenital absence of enteric neurons from varying lengths of the distal bowel due to a failure of ENCCs to colonize the entire gut. Hirschsprung Disease was thought to be caused exclusively by genetic defects. The penetrance and severity of Hirschsprung disease is variable, and very recent studies from Robert Heuckeroth's laboratory have shown that non-genetic factors can influence the development of the ENS and Hirschsprung Disease in animal models; these factors include vitamin A deficiency and some medicinal drugs (Fu et al. 2010; Lake et al. 2013).

Neuronal Differentiation and Neural Activity Commences Early, and Neurotransmitters Influence Neuronal Differentiation

As ENCCs are populating the length of the gut, a sub-population starts to differentiate into neurons and express pan-neuronal markers (Baetge et al. 1990). For many years, it was unclear whether these early "neurons" behaved like neurons. Recent calcium imaging and patch clamp studies showed that the cells are electrically active and some can fire action potentials (Hao et al. 2011, 2012). The ENS is one of the first parts of the nervous system to exhibit mature forms of activity. This early activity in the ENS does not play a role in motility (see below), but spontaneous release of neurotransmitters by early born neurons appears to regulate the differentiation of later born neurons (Li et al. 2011). The mechanism by which this occurs in the developing ENS is unknown, but in the cerebral cortex it has been shown that some transcription factors involved in the generation of interneuron subtypes are induced by neuronal activity (Denaxa et al. 2012).

The First Motility Patterns Are Not Mediated by Neurons

Enteric neurons are essential for gut motility after birth. It is well established that human fetuses swallow amniotic fluid, which progresses along the bowel (McLain 1963). Studies using embryonic mice have shown that propagating contractions

commence around 1 week prior to birth; however, gut from mutant fetal mice lacking enteric neurons, or interstitial cells of Cajal (ICC), exhibit the same motility patterns as age-matched wild-type mice (Roberts et al. 2010). Moreover the movement of meconium through the gut of mouse fetuses lacking enteric neurons is not different from that in wild-type fetal gut (Anderson et al. 2004). Thus, neither neurons nor ICC are required for gut motility at most fetal stages, and neurally-mediated motility does not commence until just prior to birth in mice (Roberts et al. 2010). It appears that propagating gut contractions during most fetal ages in mice are myogenic.

Developmental Defects in Connectivity Result in Motility Defects Without Associated Changes in Neuron Number

A recent study showed that mice with mutations in the PCP (planar cell polarity) pathway have motility defects, but do not have any defects in myenteric neuron numbers or in the numbers of the major neuron subtypes; however, these mice had developmental defects in connectivity (Sasselli et al. 2013). This study is very important as it is likely that some infants with motility disorders have defects in enteric neuron connectivity, but currently there are no methods available to detect defects in connectivity in the human ENS.

Conclusions and Future Challenges

Research into ENS development is one of the most vibrant sub-fields of ENS research, and dramatic progress has been made in the past 20 years in our understanding of how enteric neurons develop from neural crest-derived cells. However, there are many future challenges including the identification of how ENCCs communicate, how different enteric neuron subtypes are generated including neurotransmitter specification and direction of axon projection, how synapses are formed between specific subtypes of enteric neurons, and how the environment affects the postnatal development of the ENS. It is also crucial to gain a better understanding of the etiology of pediatric motility disorders.

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Chapter 7
Extrinsic Sensory Innervation of the Gut: Structure and Function

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Extrinsic Afferents 30 Years Ago

It was known since the early 1800s that the dorsal roots largely contain sensory fibres, whereas ventral roots are primarily motor. In fact, some visceral afferents had been shown to project in the ventral roots in the 1970s (Ryall and Piercey 1970; Clifton et al. 1976; Coggeshall and Ito 1977). The first recordings of visceral afferent neurons were from vagal afferents to the stomach by Iggo and Paintal in the early 1950s (Paintal 1954; Iggo 1955). Their recordings identified a class of low-threshold, tension-sensitive afferents to the upper gut. A few years later, a distinct class of mucosal, chemosensitive vagal afferent fibres to the stomach was identified (Clarke and Davison 1978). This indicated that multiple functional classes of extrinsic visceral sensory fibres might exist, each encoding different types of mechanical and chemical stimuli. Early recordings from mesenteric nerves indicated that the spinal afferent innervation of the gut contained sensory units with properties that differed from vagal afferents (Bessou and Perl 1966). Many of the high threshold spinal fibres had branches associated with mesenteric arteries (Morrison 1973; Floyd and Morrison 1974). Further studies showed that these same fibres were responsive to hypoxia (Longhurst and Dittman 1987) and to a wide range of mediators released during damage and inflammation (Blackshaw and Gebhart 2002). Vagal

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and spinal afferent neurons were directly compared in the opossum oesophagus (Sengupta et al. 1992), showing clear differences in mechanosensitive responses, with many vagal afferents being saturating mechanoreceptors, while splanchnic afferents tended to have higher thresholds and a wider dynamic range (Sengupta 2000). A range of similar studies led to a widespread acceptance that spinal afferent pathways contain more neurons with nociceptor-like responses than vagal pathways (Berthoud et al. 2004; Beyak et al. 2006; Grundy et al. 2006; Brierley et al. 2012).

Classes of Visceral Afferents: The Last Three Decades

Studies in the last 30 years have added considerably to our understanding of the structure-function relationship of extrinsic sensory nerves to the gut. Molecular biological techniques have driven a revolution in understanding of the ion channels, receptors, second messenger systems and genetics of sensory neurons. However, this review will be restricted to a few key papers that have improved our understanding of structure-function relationships, specifically.

Vagal and Sacral Sensory Pathways

Anatomical studies in the early to mid 1990s, using tracers injected into the nodose ganglion, revealed both the morphology and extent of vagal afferent nerve endings in the gut wall (Berthoud et al. 1995, 1997; Fox et al. 2000). Systematic recordings showed that vagal mechanoreceptors are not all low threshold saturating fibres: there are also wide dynamic range endings too, at least in the oesophagus (Yu et al. 2005). The chemosensory afferents in vagus nerve have been shown to be activated by release of mediators from entero-endocrine cells (Blackshaw and Grundy 1990; Eastwood et al. 1998). Different classes of spinal afferents can be distinguished by sensitivity to distension, mucosal stroking and strong compression (Lynn and Blackshaw 1999). During this period, it was shown that there are differences in the spinal afferents that innervate the rectum (via sacral/pelvic pathways) compared to the colon (via splanchnic pathways). For example, a large population of low threshold mechanoreceptors innervates the rectum: these are much sparser in the colon and splanchnic pathways (Lynn et al. 2003). Systematic studies extended these findings, showing that there were significant differences in both mechanosensitivity and chemosensitivity (Brierley et al. 2004, 2005) of spinal afferents in pelvic and splanchnic pathways to the mouse large intestine. The upper gut and the rectum both receive prominent parasympathetic efferent innervation—from vagal and sacral pathways respectively. Similarly, both upper and lower gut are innervated by specialised afferents (from vagal and sacral ganglia) which include many low-threshold mechanoreceptors. These are strongly activated during normal physiology and presumably are responsible for vago-vagal

and sacral parasympathetic reflexes involved in gastric accommodation and defaecatory behaviours respectively.

Vascular Afferents

One specific class of spinal afferents is particularly significant: these are higher threshold sensory neurons that have endings closely associated with mesenteric blood vessels (Bessou and Perl 1966; Morrison 1973; Floyd and Morrison 1974). Immunohistochemical studies showed that these neurons (and many other nociceptor-like cells) have a distinct chemical coding, containing immunoreactivity for the neuropeptides CGRP and a tachykinin (Gibbins et al. 1985). This fitted nicely with long-established finding that sensory neurons can cause peripheral vasodilation (Bayliss 1901), via the release of CGRP (Kawasaki et al. 1988). Studies tracing the pathways of these "vascular afferents" showed that they are not restricted to mesenteric vessels—they also innervate intramural blood vessels, particularly in the submucosa (Song et al. 2009). Their endings on blood vessels are sensitive to distortion of the vessel (Humenick et al. 2015) and to distension of the gut wall; these neurons appear to function as medium-to-high threshold mechanonociceptors (Song et al. 2009). Furthermore, they often have multiple receptive fields, spread over several centimetres of bowel (Berthoud et al. 2001) with the same neuron innervating both intramural and extramural blood vessels (Song et al. 2009). This provides a firm anatomical foundation for the observation that large distensions of the bowel cause upstream vasodilation of mesenteric arteries via an axon reflex (Meehan and Kreulen 1992). These same vascular afferents are sensitive to a wide range of mediators released by inflammation and by cell damage, thus they function as sophisticated polymodal nociceptors, alerting the central nervous system about actual or potential damage to the gut wall, while simultaneously triggering a protective hyperaemia.

In many organs, including the gut, populations of sensory fibres exist that cannot be activated by conventional mechanical and/or chemical stimuli; these are so-called "silent afferents". In the gastrointestinal tract, application of mediators associated with damage and inflammation acutely cause sensitisation of many visceral sensory neurons (Su and Gebhart 1998). In some cases "silent afferents" then become mechanically sensitive (Feng and Gebhart 2011). Experimental colitis also induces chronic hypersensitivity of some classes of visceral afferents, which outlasts the period of inflammation. These include vascular afferents with "serosal or mesenteric" endings (Hughes et al. 2009). Specialised low threshold rectal afferents are not sensitised to the same degree (Lynn et al. 2008). There is also evidence that experimental inflammation chronically activates "silent afferents" at least some of which are mechanically-insensitive vascular ("serosal") afferents (Feng et al. 2012). Potentially, this may explain the hypersensitivity associated with inflammatory conditions of the bowel, since more nociceptors become capable of responding to mechanical stimuli and each nociceptor's response is exaggerated. Low-level

inflammatory mechanisms may occur in functional bowel disorders, including Irritable Bowel Syndrome (IBS) (Wahnschaffe et al. 2001; Tornblom et al. 2002; Barbara et al. 2004). Responses to inflammatory mediators are likely to be important in generating pain in these conditions.

Morphological Studies of Afferent Nerve Endings

Some of the work in our laboratory in the last 15 years has characterised structure-function relationships of extrinsic visceral afferent neurons. Using a combination of rapid anterograde tracing (Tassicker et al. 1999) and in vitro afferent recording, we have identified the structure of some visceral afferent nerve endings and transduction sites in the gut wall. Using these techniques, the low threshold vagal mechanosensors in the stomach and oesophagus were shown to correspond to "intraganglionic laminar endings" in the upper gut (Zagorodnyuk and Brookes 2000; Zagorodnyuk et al. 2001). Comparable low threshold mechanoreceptors were also described in the guinea pig rectum and shown to have similar flattened "intraganglionic laminar endings" in myenteric ganglia to those of vagal tension receptors (Lynn et al. 2003). Studies on the high threshold mechanonociceptors associated with mesenteric blood vessels characterised their endings as branching varicose axons on both extramural (mesenteric) arteries and on intramural arteries in the submucosa (Song et al. 2009). This study also showed that there are few sensory endings in either the serosal membrane or the mesenteric membranes (apart from those on blood vessels) indicating that the terms "serosal" afferent and "mesenteric afferent" are not anatomically accurate. We have also characterised the enteric viscerofugal neurons that project out the gut wall via the mesenteric nerves, where their action potentials can be recorded alongside extrinsic afferent fibres (Cervero and Sharkey 1988). They project to sympathetic ganglia (Kuramoto and Furness 1989; Messenger and Furness 1993) and, in the distal colorectum, to the spinal cord (Doerffler-Melly and Neuhuber 1988). Combining dye filling with recordings from mesenteric nerves, it was shown that action potentials of viscerofugal neurons can be recorded from mesenteric nerves (Hibberd et al. 2012b) and that the cell bodies of these neurons are mechanosensitive (Hibberd et al. 2012a). They also receive synaptic drive from other enteric neurons (Hibberd et al. 2014). Other classes of extrinsic afferents have also been characterised using these techniques, including mechanoreceptors innervating the internal anal sphincter (Lynn and Brookes 2011).

Overall, in the last 30 years, structural and functional studies of extrinsic sensory nerves that innervate the gastrointestinal tract have made considerable progress. Discrete classes of neurons that encode specific combinations of mechanical and chemical stimuli and transmit this information to the central nervous system. Whether these "labelled lines" of afferents synapse onto different classes of second order neurons in the spinal cord seems likely, but has not yet been systematically investigated. The presence of multiple classes of extrinsic sensory neurons undoubt-

edly complicates analysis of sensory signalling from the gut. However, it also raises the possibility that specific classes of afferents may be targeted by future therapeutics to modify common disorders of intestinal functions.

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Chapter 8
Ageing and Gastrointestinal Sensory Function

Christopher Keating and David Grundy

The gastrointestinal tract must balance two ostensibly conflicting tasks: absorption of nutrients and protection against potential harmful pathogenic or antigenic material. Critical for both is the ability to sense luminal content and the orchestration of secretory and motor activity appropriate for either digestion and absorption or dilution and elimination. In this respect the GI tract has a rich sensory innervation conveying to the CNS a wealth of information relating to the physical and chemical composition of gut contents. Much of this sensory information is processed below the level of consciousness, providing the basis for reflexes and eating behaviour. Gastrointestinal afferent neurons play a pivotal role in these processes. Vagal and spinal afferents are the pathways that convey sensory information from the gut to the central nervous system (CNS). Combining electrophysiological, morphological and molecular approaches has enabled the different populations of vagal and spinal afferents that innervate the bowel to be characterized. The sensory cell soma are psuedo-unipolar, with one axon branch projecting centrally to terminate in the nucleus tractus solitarius (nTS) for vagal afferents or in the dorsal horn for spinal afferents. Their peripheral axons terminate, usually as bare nerve endings, at various locations within the gut wall. Depending on their location, they can be classified as having serosal, mesenteric, muscular or mucosal receptive fields, and in some cases branched endings terminating in both muscle and mucosa (Brookes et al. 2013). These endings have different functional properties. Their sensitivity is determined by their location in the gut wall, their relationship with other cells and structures and the receptors and ion channels that they express on their nerve terminals.

Mechanosensitivity is a common feature of most, if not all gastrointestinal afferents, although some so-called silent afferents only develop mechanosensitivity when

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the surrounding tissue is damaged or inflamed (Feng et al. 2010). Mucosal deformation activates endings close to the mucosal epithelium. These endings also possess chemosensitive properties responding to chemicals either crossing the mucosal barrier or released from within the epithelium itself in response to luminal content. Physiological levels of distension are effective for stimulating endings in the muscle layers including intramuscular arrays and intraganglionic laminar endings that form baskets around myenteric ganglia (Zagorodnyuk et al. 2001). Serosal and mesenteric afferents are particularly sensitive to distortion as the bowel moves during filling or when the bowel contracts powerfully and are considered to be nociceptors transmitting pain signals from the bowel. In addition to direct activation of mechanosensitive afferent endings, mechanical stimulation can release mediators from non-neural cells that stimulate sensory endings indirectly, via ionotropic or metabotropic receptors. Afferent endings in the gut wall form close associations with other cell types including mast cells, epithelial cells, enteroendocrine cells, macrophages, ICCs, enteric neurons etc. All of these have the potential to mediate indirect mechanosensitivity.

An important element for understanding pain has been the recognition that sensory signaling is not fixed but is modulated by a variety of insults associated with injury, ischaemia and inflammation. Ion channels and receptors that determine excitability and sensitivity can be upregulated as part of a process, as well as changes in the synaptic mechanisms in the spinal cord, leading to hyperalgesia and allodynia (Brierley and Linden 2014).

Our recent efforts have focused on understanding the mechanisms that determine sensory signal generation in afferents supplying the bowel and we have used an in vivo model of transient inflammation, the *Trichinella spiralis* infected mouse, to understand the mechanisms that trigger and maintain visceral hypersensitivity following resolution of the inflammatory insult. Enteric infection with parasites has proven to be a powerful tool in which to study the interaction between the immune system and physiological processes of the gastrointestinal tract (Khan and Collins 2006; Palmer and Greenwood-Van Meerveld 2001), and the choice of *T. spiralis* as our model system is based upon a wealth of information generated by our lab and others describing:

- A model of transient inflammation with a natural end-point when the parasite is expelled from the gut. *T. spiralis* infection triggers a transient inflammatory response which resolves to leave long term sensory and motor dysfunction (Bercík et al. 2004; Keating et al. 2008).
- The well described time-course of immune response. This model has discrete phases with an inflammatory response that peaks at around 10 days post infection (PI) in which acute jejunal inflammation, rapid cell infiltration, mastocytosis, and altered villus histology are apparent (Bercík et al. 2004; Wheatcroft et al. 2005; Keating et al. 2011). At d 28 PI the gut is characterised by increased numbers of enterochromaffin cell (EC cells), mast cells and visceral hypersensitivity (Wheatcroft et al. 2005; Keating et al. 2008). Furthermore, visceral hypersensitivity and EC cell hyperplasia persist up to 56 d PI which makes this a valuable model for studying maintained gut dysfunction (Wheatcroft et al. 2005; Keating et al. 2008).

- A natural parasite of mice which means that infection in different strains or genetically modified mice can be used to explore the effects of an altered immune environment upon *T. spiralis* infection. In a recent study we have shown that hypersensitivity fails to develop in the P2X7 knockout mouse which has a blunted inflammatory response (Keating et al. 2011)
- Infection is associated with strong, measurable, behavioural responses which includes visceral hypersensitivity and altered gut motility. The visceral hypersensitivity associated with this model has two essential components. Firstly, altered levels of chemical mediators in the gut wall are associated with the post inflammatory phases and contribute towards the generation of visceral hypersensitivity via peripherally mediated mechanisms (Rong et al. 2009; Keating et al. 2008). Secondly, changes in sensory neuronal plasticity contribute towards an increased excitability of the sensory afferent neuron driven by alterations in ion channel expression and function (Bercík et al. 2004; Keating et al. 2008).

Since hypersensitivity is a hallmark of IBS and in many patients the onset of IBS is linked to a bout of gastroenteritis, it is likely that these mechanisms contribute to the development of pain and discomfort arising from the bowel in patients and which also contribute to altered bowel habits. This concept is supported by the observation that biopsies from patients with IBS exhibit an altered chemical milieu that can activate and sensitize neuronal firing in both the enteric and extrinsic innervation (Barbara et al. 2007; Buhner et al. 2014). Supernatants from these patients can serve as biomarkers of IBS and of hypersensitivity and the next decade should see the development of diagnostic tools and treatment options as well a paradigm shift in how we view the pathogenesis of IBS.

Our recent research efforts have included investigation of the ageing bowel because of the prevalence of constipation and incontinence in the elderly and the associated economic and societal impacts that include increased health care costs, morbidity and mortality (Vreeburg et al. 1997). One feature of ageing is impaired sensory perception, including a diminished sensory response to inflammatory evoked gastrointestinal injury (Moore and Clinch 2004). Since nociception is a key consequence of disease or tissue injury, triggering neurogenic inflammation and pain behaviour, its attenuation with age may be critical in the development of GI disorders. However, there is very limited information describing how ageing affects sensory signalling from the bowel. We have hypothesised that ageing attenuates sensory function mainly as a consequence of neurodegenerative changes in the gut wall and changes in sensory signalling pathways and have started to examine this in electrophysiological studies by comparing afferent sensitivity in young and aged mice. In so far unpublished studies we have found that ageing is associated with a progressive attenuation of sensory signalling in response to bowel distension. This was apparent for afferents supplying both proximal and distal regions of the gastrointestinal tract. Deficits were evident in different gastrointestinal afferent subpopulations conveying low and high threshold mechanosensory information and there was impairment in the ability of sensory neurons to sensitize in response to chemical mediators such as 5-HT. This alteration in serotonin signalling was paralleled by alterations in enterochromaffin cell number and in the expression of

the uptake transporter (SERT) and the synthetic enzymes TPH1 and TPH2. The mechanisms underlying these changes remain to be elucidated but oxidative stress is an obvious target. These initial studies have therefore highlighted pathways susceptible to ageing and flagged potential targets for future research into treatment strategies for age-related gastrointestinal sensory dysfunction.

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Chapter 9
Altered Ion Channel/Receptor Expression and Function in Extrinsic Sensory Neurons: The Cause of and Solution to Chronic Visceral Pain?

Stuart Brierley

Introduction

Neural control of gastrointestinal function is a highly integrated system, comprised of distinct populations of neurons, whose cell bodies are either intrinsic or extrinsic to the gut wall. Neural control involves interactions between; (1) local enteric reflexes within the gut wall; (2) reflexes that pass through prevertebral sympathetic ganglia and (3) reflexes that pass to and from the gut via the central nervous system (CNS) (Furness 2012). To add further intricacy the gastrointestinal tract is an incredibly complex signalling environment. Neurons are subjected mechanical events such as distension and contraction, whilst being inundated with a constantly changing milieu of endogenous mediators (Brierley and Linden 2014). Inflammation of the gut, either through abnormal immune responses or via gut infection has been consistently demonstrated to cause neuroplasticity and abnormal neuronal function. These profound effects result in disregulated neuronal signalling, abnormal secretion, motility and sensory signalling resulting in the development of diarrhea, constipation, discomfort and pain. The importance of this neuroplasticity is highlighted in a number of highly prevalent organic and functional gastrointestinal disorders. In organic disorders such as Inflammatory Bowel Disease (IBD), which includes Crohn's disease and Ulcerative Colitis, chronic uncontrolled inflammation of the

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intestinal mucosa is recognised as the pathogenesis of neuronal dysfunction and correspondingly the presentation of symptoms (Kaser et al. 2010; Nell et al. 2010). However, for functional bowel disorders such as Irritable Bowel Syndrome (IBS), where macroscopic mucosal damage is not evident, but symptoms of persistent abdominal pain, discomfort and abnormal bowel function are evident (Brierley and Linden 2014). The underlying source of this neuronal deregulation remained unclear, until the recent association with infectious gastroenteritis. Numerous clinical studies have attributed IBS symptom development to a preceding bout of gastroenteritis induced by pathogens such as *Campylobacter* (Spiller et al. 2000; Thabane et al. 2010), *Escherichia coli* (Thabane et al. 2010), *Salmonella* (Dunlop et al. 2003), *Giardia lamblia* (Hanevik et al. 2009). Whilst IBS is multi-factorial and several additional risk factors may also be required for development (Dinan et al. 2010; Ohman and Simren 2010), acute gastroenteritis can trigger IBS symptoms that persist for at least 8 years (Marshall et al. 2010). In the relatively short term setting of tissue damage, inflammation is a protective process which facilitates wound healing, however these clinical findings suggests that in these individuals the neuroplasticity induced by infection and inflammation, fails to reset back to normal long after healing of the intestinal tissue. As increasing effort has been directed towards determining the extent of neuroplasticity that is associated with gut disorders then the number of different models used to investigate it has also increased. Experimental models of gut inflammation have included administration of dextran sodium sulphate (DSS), chemical irritants such as mustard oil and acetic acid, infection with nematodes (e.g. *Trichinella spiralis*) or bacteria (e.g. *Citrobacter rodentium*), and haptens such as trinitrobenzene sulphonic acid (TNBS). However, the time course and nature of the resultant inflammation is different between these models and is defined by the different categories of immune cells involved in the response (Antalis et al. 2007; Fasano and Shea-Donohue 2005). For example TNBS combines with endogenous proteins and antigens to evoke a transmural Th1-mediated inflammation, whilst DSS is more dependent on innate immunity and is restricted to the mucosa. Furthermore, zymosan which unlike the models described above, does not induce an increase in myeloperoxidase (MPO) activity at any time after intracolonic treatment, does result in a brief monocyte-based inflammation (Feng et al. 2012a). However, despite these differences an increasing amount of data suggests neuroplasticity can occur inextrinsic sensory afferent neurons, their peripheral and central projections and their resultant communication with the CNS. These changes are likely to further alter communication along the brain-gut axis, and have a profound effect on resultant neuronal plasticity. Given this complexity and that understanding of many of these interactions remain in their infancy; this review will focus on the resultant effects of gut inflammation on neuronal function in the gut-brain pathway and the persistent long term neuroplasticity than remains following resolution of inflammation (Fig. 9.1). Where apparent this review will also highlight the channels, receptors and mediators involved in this process.

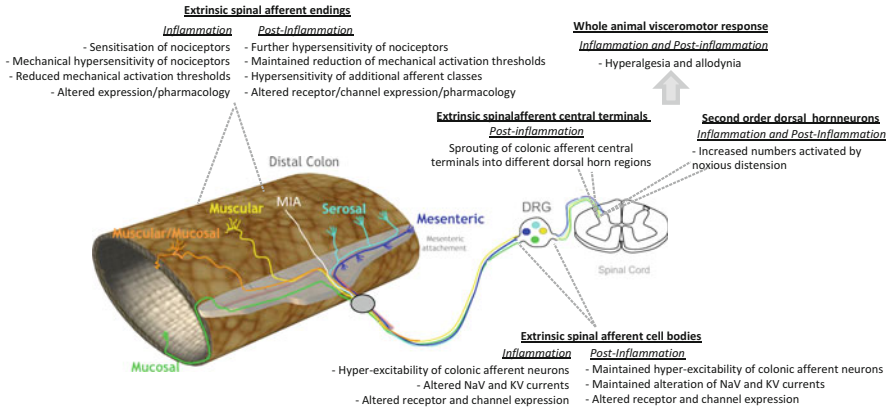

Fig. 9.1 Neuroplasticity in extrinsic sensory afferent pathways during and following resolution of gut inflammation. During inflammation nociceptive sensory afferent endings are hypersensitive, are activated at lower stimulus intensities and displayed enhanced mechanical responsiveness, whilst their cell bodies in the DRG also display hyperexcitability. This translates to increased activation of dorsal horn neurons in the spinal cord and in whole animal studies enhanced pain responses to colorectal distension. Many of these changes are still present or are even enhanced following resolution of inflammation. Nociceptive sensory afferent endings now display even greater mechanical hypersensitivity, and their cell bodies in the DRG remain hyper-excitability. An increased density of colonic afferent central afferent terminals is now evident, as is sprouting of these terminals into different regions of the dorsal horn of the spinal cord. This plasticity results in greater numbers of second order dorsal horn neurons in the spinal cord being activated in response to noxious colorectal distension. There is evidence of enhanced pain responses to colorectal distension, which can be dependent upon the experimental model used and influenced by the severity of the initial insult

Extrinsic Sensory Afferent Pathways Innervating the Gastrointestinal Tract

Distinct from the enteric and sympathetic nervous systems are the extrinsic sensory innervations of the gastrointestinal tract. These pathways have become one of the most intensely studied areas of neuro-gastroenterology, as they are the first step in generating sensations. In particular they are responsible for signaling nociceptive stimuli from the gut, and ultimately the conscious perception of pain. Therefore, identifying the afferents, mediators and mechanisms involved in this process is crucial in understanding the mechanisms of neuroplasticity underlying inflammatory and chronic visceral pain. Most studies have focused on the innervations of the small intestine, colon and rectum, as these regions are associated with the symptoms of IBD and IBS. The complexity of this intact system means that many of its individual components; afferent endings in the gut wall, cell bodies in the DRG, activation of pathways in the spinal cord and the overall pain-related behavior to gut distension (visceromotor response to colorectal distension) have been studied

independently, either in vitro or in vivo. These studies indicate that mechanisms underlying inflammatory and chronic post-inflammatory visceral pain are varied, but originate from changes in the periphery (Barbara et al. 2002, 2004, 2007; Liebregts et al. 2007; Spiller and Garsed 2009). The peripheral endings of particular afferent subtypes feed into nociceptive pathways within the spinal cord and pain sensing regions in the brain. Whilst pain is an emotive process, the threshold of nociceptors has to be high enough not to interfere with normal physiology, but low enough that it can be evoked before marked tissue damage occurs (Costigan et al. 2009). In order to achieve this function nociceptive nerve endings express a variety of ion channels and receptors, which regulate neuronal excitability and transduce mechanical or chemical stimuli (Beyak 2010; Beyak and Vanner 2005; Blackshaw et al. 2010; Brierley 2010; Brierley and Kelber 2011). To add further complexity there are several schools of thought regarding the types of afferent that contribute to nociceptive signaling and therefore inflammatory and chronic pain. These subtypes include low- and high-threshold afferents, and mechanically insensitive 'silent' afferents. However, by definition nociceptors selectively respond to noxious or potentially tissue damaging stimuli and can be sensitized, or increase their excitability in response to tissue insult or inflammation.

Neuroplasticity in Extrinsic Sensory Afferent Pathways Innervating the Gut

It is clear that experimentally induced inflammation or infection causes afferent hypersensitivity, neuronal hyper-excitability and correspondingly hyperalgesia and allodynia in whole animal models. Consistent findings of neuroplasticity have been most apparent when studying isolated neuronal cell bodies across different regions of gut and across different experimental models. Most studies utilizing inflammatory (TNBS), nematode (*T. Spiralis, Nippostrongylus brasiliensis*) or bacterial models (*Citrobacter rodentium*) show that neurons innervating the stomach (Dang et al. 2004; Gebhart et al. 2002; Bielefeldt et al. 2002a, b), small intestine (Hillsley et al. 2006; Moore et al. 2002; Stewart et al. 2003; Keating et al. 2008) and the colon (Beyak et al. 2004; Ibeakanma et al. 2009; King et al. 2009) display pronounced hyper-excitability after the initial insult. This hyper-excitability is characterized by a decreased threshold for activation, increased firing rate, increases in TTX-resistant Na_V currents and suppression of K_V, I_A and I_K channels. Recent reports indicate a crucial role for $Na_V1.8$ in colonic innervating DRG neurons, with its expression differentially regulated across different time points during colitis (King et al. 2009). Furthermore, *Nippostrongylus brasiliensis* induced jejunal neuronal hyper-excitability is lost in $Na_V1.8$-/- mice, but not $Na_V1.9$-/- mice (Hillsley et al. 2006). Longer term neuroplasticity is also evident as K_V, I_A and I_K currents are reduced in colonic innervating DRG neurons 10 days post-*Citrobacter rodentium* infection, whilst suppression of K_V and I_A currents contributes to neuronal hyper-excitability 30 days post-infection (Ibeakanma et al. 2009).

Neuroplasticity of peripheral sensory afferent endings is also evident across a range of different experimental models; however different afferent subtypes, different neuronal pathways and time courses are involved in this process. For example, following *T. spiralis* infection both low- and high-threshold jejunal afferents initially display significant *reductions* in mechanosensitivity at 14 days post-infection. However, at 28 and 56 days post-infection pronounced mechanical hypersensitivity is now evident (Keating et al. 2008). The development of this longer term mechanical hypersensitivity is dependent upon a $P2X_7$ receptor-dependent increase in immune cell IL-1β expression and release. Notably these $P2X_7R$ -/- animals display a clear attenuation of the innate inflammatory response and no post-infectious mechanical hypersensitivity at any time point (Keating et al. 2011).

DSS-induced colonic inflammation does not induced afferent mechanical hypersensitivity (Coldwell et al. 2007), or short or long term hyperalgesia in response to colorectal distension (Larsson et al. 2006). However, DSS treated animals display increased visceral sensitivity to capsaicin and 5-HT (Larsson et al. 2006; Eijkelkamp et al. 2007). By contrast, TNBS induced colitis causes high-threshold nociceptors to become mechanically sensitized, have reduced activation thresholds, and display hypersensitive responses in inflammatory and post-inflammatory states (Hughes et al. 2009a, b). This hypersensitivity is particularly apparent in splanchnic afferents with high mechanical activation thresholds, which is partially mediated by TRPA1 (Brierley et al. 2009). A potential contributing factor is also a reduction in the mechanosensitive K2P channels TREK-1 and TREK-2, as these hyperpolarizing K^+ channels are significantly reduced in splanchnic and pelvic colonic DRG neurons during TNBS inflammation (La and Gebhart 2011). The extent of this mechanical hypersensitivity in high threshold afferents is greater following recovery from overt tissue damage (28 days post-TNBS) (Hughes et al. 2009a, b). This hypersensitivity translates to an increased density and sprouting of colonic afferent central terminals in the thoracolumbar spinal cord and an increased number of activated DH neurons in the spinal cord in response to noxious colorectal distension (Harrington et al. 2012). In contrast, the same investigators have shown TNBS induced mechanical hypersensitivity is not evident during inflammation in afferents with low-thresholds (mucosal, muscular and muscular/mucosal). However, pelvic high-threshold and mucosal afferents only become hypersensitive post-inflammation (Hughes et al. 2009a, b). Other studies have shown transient, absent or inconsistent effects of TNBS-induced inflammation on low-threshold distension-sensitive afferents (De Schepper et al. 2008a; Lynn et al. 2008; Sengupta et al. 1999; Feng et al. 2012b) and transient hypersensitivity during in vivo colorectal distension studies (Lamb et al. 2005). The apparent discrepancy of these findings with TNBS may relate to the severity of mucosal inflammation, which is a predictor for alterations of visceral sensory function in rodents (Adam et al. 2006) and in humans. However, acute zymosan treatment, which recruits a different immune response, does lead to low-threshold sensitive afferents displaying short and long term hypersensitivity (Feng et al. 2012a, b), which is partially dependent on TRPV1 (Jones et al. 2007), ASIC3 (Jones et al. 2007) and P2X receptors (Shinoda et al. 2010). Inflammatory mediators, TNBS and zymosan treatment can also activate or sensitize two different types of mechanically insensitive afferents (MIAs), also known as ‘silent afferents’.

One population is silent, responds to chemical stimuli, but doesn't subsequently display mechanosensitivity (Brierley et al. 2005a, b), whilst the other population is sensitized by mediators and develops mechanosensitivity (Feng and Gebhart 2011). The proportion of this second type of MIA is increased in a number of inflammatory and post-inflammatory states (Feng et al. 2012a, b). Another model utilizing intracolonic administration of deoxycholic acid, an unconjugated secondary bile acid, induces a mild, transient colonic inflammation within 3 days, which resolves within 3 weeks. This causes exaggerated visceromotor responses to colorectal distension, referred pain to mechanical stimulation, and increased dorsal horn neuron activity, which persists for at least 4 weeks (Traub et al. 2008).

Various stress models have been shown to increase visceral pain sensitivity (Larauche et al. 2012; Winston et al. 2010). However, stress, combined with prior acute colitis induced by *C. rodentium*, results in exaggerated peripheral nociceptive signaling of colonic afferents, their cell bodies and correspondingly visceromotor reflex thresholds via protease, β-2 adrenergic, glucocorticoid receptor and PAR2 mechanisms (Ibeakanma et al. 2011). However, such an interaction does not occur with stress and DSS treatment (Larsson et al. 2009), which again may suggest specific neuroimmune interactions in the development of neuroplasticity and chronic colonic hyperalgesia.

One of the most consistent and long-term displays of visceral neuroplasticity occurs following neonatal insult. In these cases neonatal animals receive either mechanical or chemical colonic irritation between post-natal days 8 and 21 and are then tested when they are adults (Al-Chaer et al. 2000). Colonic irritation in neonates results in chronic visceral hypersensitivity, allodynia and hyperalgesia, associated with central neuronal sensitization, in the absence of identifiable peripheral abnormalities. Evidence exists for TRPV1 (Jones et al. 2007; Hong et al. 2009; Winston et al. 2007) and TRPA1 (Christianson et al. 2010) initiating colonic hypersensitivity and TRPV1 (Winston et al. 2007), P2X (Xu et al. 2008) and TRPA1 (Christianson et al. 2010) maintaining colonic hypersensitivity induced by neonatal acetic acid or mustard oil colonic irritation. More recent studies indicate similar mechanisms in the upper gut, which may be applicable to Functional Dyspepsia. Gastric irritation in neonates results in chronic gastric hypersensitivity and gastric motor dysfunction in adults, in the absence of detectable gastric pathology (Liu et al. 2008). This gastric hypersensitivity in adults can be attenuated by the $GABA_B$ agonist baclofen, although this analgesic affect appears to occur via central rather than peripheral mechanisms (Liu et al. 2011).

Insights into the Mechanisms of Neuroplasticity Using IBS Patient Biopsies and Samples

In some subgroups of IBS patient's persistent low-grade inflammation within the gut wall (Barbara et al. 2002, 2004) and altered immunological function (Liebregts et al. 2007, 2011; Hughes et al. 2013) are evident and may lead to recurrent

re-sensitisation of nerve function within the gut (Hughes et al. 2009c, 2013). One of the first reports of this interaction demonstrated IBS patients have greater colonic mast cell infiltration and an increased release of key mediators, tryptase and histamine. Crucially these activated mast cells are in closer proximity to nerve fibres in IBS patients, which correlates with the severity and frequency of abdominal pain and discomfort (Barbara et al. 2004). Correspondingly, supernatants from IBS patient biopsies, but not healthy subjects, causes activation of afferent nerve endings and their cell bodies, via histamine H1 receptor and serine protease mechanisms (Barbara et al. 2007). Similar findings have been demonstrated using supernatants from Ulcerative Colitis patients, where application of supernatants enhances the neuronal excitability of colonic sensory DRG neurons. However, in this case the pro-inflammatory cytokine, TNFα is the key mediator, as it is elevated in Ulcerative Colitis biopsies, and acts at neuronal TNFR1 to modulate K_v and Na_v currents. These findings have increased importance as TNFα and the Ulcerative Colitis supernatants both enhance Na_V currents, and suppress K_V (I_A and I_K) currents (Ibeakanma and Vanner 2010), which are the same currents that are altered in inflammatory and post-inflammatory states (Beyak 2010).

Changes in IBS patients are also evident in peripheral blood mononuclear cells (PBMCs) (Liebregts et al. 2007, 2011; Hughes et al. 2009c, 2013). In particular several pro-inflammatory cytokines, TNF-α, IL-1β and IL-6, are all increased in PBMC supernatants from diarrhoea-predominant IBS (IBS-D) patients, which correlate with symptoms of pain frequency and intensity (Liebregts et al. 2007; Hughes et al. 2013). Notably, these supernatants from IBS-D patients evoke pronounced mechanical hypersensitivity in high- and low-threshold splanchnic and pelvic colonic afferents (Hughes et al. 2009c, 2013). As these colonic afferents express the receptors for these cytokines they can individually sensitise splanchnic and pelvic colonic afferents to mechanical stimuli (Hughes et al. 2009c, 2013). Whilst IL-1β causes direct firing of colonic afferents via a $NaV_{1.7}$ mechanism, TNF-α induces mechanical hypersensitivity, via a TRPA1 dependent mechanism (Hughes et al. 2013). This is one of numerous interactions that exist between pro-nociceptive mediators and TRP channels, which play key roles in inducing neuronal hypersensitivity and neuroplasticity.

TRP Channels: Key Roles for Neuroplasticity

In addition to its interaction with TNF-α (Hughes et al. 2013), TRPA1 also mediates the mechanical hypersensitivity induced by bradykinin (Brierley et al. 2009), as well as PAR2-induced hyperalgesia (Cattaruzza et al. 2009) (Fig. 9.2). This is important as TRPA1 plays a major role in visceral nociception, as TRPA1 deletion causes pronounced mechanosensory deficits, predominantly in high-threshold colonic afferents (Brierley et al. 2009, 2011) and correspondingly reduces viscero-motor responses to noxious colorectal distension (Brierley et al. 2009). Furthermore, activation of TRPA1 by numerous agonists, including mustard oil, cinnamaldehyde

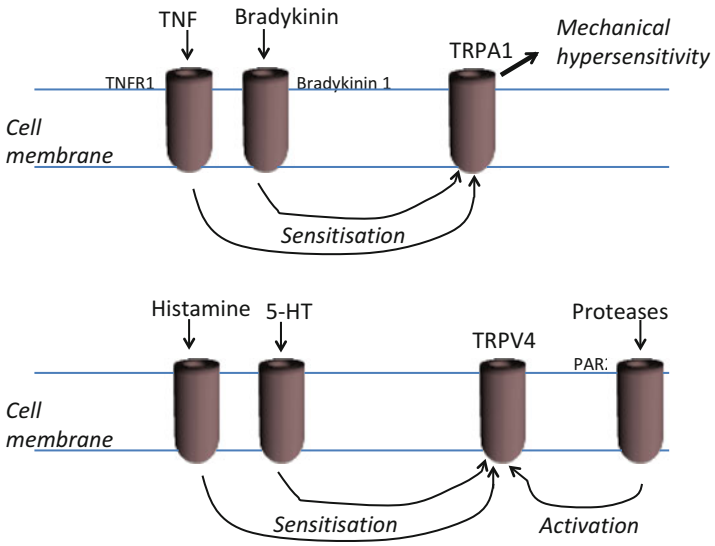

Fig. 9.2 TRP channels are key mediators of visceral afferent hypersensitivity and are downstream targets of receptor activation. (**a**) Whilst TRPA1 can be activated directly by compounds such a 4-Hydroxynonenal, mustard oil and cinnamaldehyde to induce mechanical hypersensitivity, TRPA1 can also be sensitised by interactions with TNFR1 and bradykinin 1 receptors. Binding of TNFα to TNFR1 and bradykinin to bradykinin 1 respectively can both independently evoke mechanical hypersensitivity of nociceptors by a TRPA1 dependent process. (**b**) Similarly, histamine and 5-HT can cause sensitisation of TRPV4, evoking neuronal hypersensitivity. This occurs via mitogen-activated protein kinase kinase (MAPKK) and phospholipase A2 (PLA2)-dependent mechanisms and increased TRPV4 dependent hypersensitivity in response to colorectal distension. By contrast, the interaction between TRPV4 and PAR-2 appears more fundamental, with expression of TRPV4 being required for PAR-2-induced mechanical hyperalgesia and excitation of colonic afferent neurons

and 4-hydroxynonenal, can tune nociceptor responses, inducing pronounced mechanical hypersensitivity (Brierley et al. 2009), and visceral mechanical hyperalgesia (Cattaruzza et al. 2009). Notably, TRPA1 function is increased during TNBS induced inflammation (Brierley et al. 2009) and TRPA1 deletion markedly reduces TNBS-induced colonic mechanical hyperalgesia (Cattaruzza et al. 2009), suggesting TRPA1 is also a key contributor to inflammatory pain. In addition to these effects on neurons, TRPA1 can also contribute to the inflammatory response itself, via neurogenic inflammation, as activation and sensitization of TRPA1 and release of substance P induces and maintains colitis in mice (Engel et al. 2011), which correspondingly re-sensitises nociceptors.

Another member of the TRP channel family, TRPV4, also plays a key role in nociception, neuroplasticity and pain. TRPV4 is predominantly expressed in spinal neurons innervating the colon and in the gut only contributes to the mechanosensory function of high-threshold colonic afferents (Brierley et al. 2008). These changes in colonic neuronal function translate to decreased visceromotor responses to colorectal

distension in TRPV4 -/- mice, or in mice with siRNA induced down-regulation of TRPV4 (Brierley et al. 2008; Cenac et al. 2008; Sipe et al. 2008). TRPV4 also has a crucial interaction with PAR2, whereby TRPV4 is required for PAR2-induced excitation of colonic afferent neurons and colonic mechanical hyperalgesia (Sipe et al. 2008). PAR2 is also a key receptor for inducing neuroplasticity, as PAR2 agonists can evoke sustained hyperexcitability of colonic nociceptive neurons by suppressing I_K currents, via a PKC and ERK(1/2) pathway (Kayssi et al. 2007). More recently another key PAR2-dependent mediator has been identified, cathepsin-S, which is activated in macrophages during TNBS colitis and evokes hyperexcitability of colonic nociceptive neurons and visceral hyperalgesia (Cattaruzza et al. 2011). TRPV4 can also be sensitised by a series of other mediators leading to neuronal hyperexcitability. Pre-exposure of colonic DRG neurons to 5-HT or histamine increases TRPV4 agonist induced responses and increases TRPV4 expression at the plasma membrane via PKC, PLA(2), PLCβ and MAPKK-dependent mechanisms (Cenac et al. 2010). TRPV4 can also contribute to the inflammation response itself, by inducing neurogenic inflammation, via activation of neuronal TRPV4 stimulating neuropeptide release from peripheral afferent terminals (Vergnolle et al. 2010). Secondly, TRPV4 is also expressed on intestinal epithelial cells, where its activation induces chemokine release and induces colitis (D'Aldebert et al. 2011).

TRPV1 is the most identifiable of the TRP channels and has long been implicated in gut nociception and altered neuronal function. Intra-colonic administration of the TRPV1 agonist, capsaicin, causes pronounced visceral pain (Laird et al. 2001, 2002), whilst TRPV1 -/- mice display decreases in visceromotor responses to colorectal balloon distension (Jones et al. 2005). TRPV1 appears to have a transient role in neuroplasticity, with initial increases in TRPV1 expression and function during the height of active colonic inflammation (De Schepper et al. 2008a, b; Miranda et al. 2007; Yang et al. 2008), which may return to normal levels at later post-inflammatory time points (Miranda et al. 2007). Correspondingly, TRPV1 deletion or pharmacological blockade partially reverses inflammation induced mechanical hypersensitivity and hyperalgesia (Jones et al. 2007; Miranda et al. 2007). However, a key interaction in this process appears to be via TRPV1 and the G protein-coupled receptor kinase 6 (GRK6) (Eijkelkamp et al. 2009). The pro-inflammatory cytokine IL-1β sensitizes TRPV1, which can be prevented by over-expressing GRK6. Following colitis, TRPV1-induced behavioural pain responses are more pronounced in GRK6 -/- mice than in wild-type mice, suggesting GRK6 can regulate inflammation-induced sensitization hyperalgesia (Eijkelkamp et al. 2009).

Neuroplasticity Induced by Bacterial Cell Products

Mucosal barrier function is crucial for the overall function of the gastrointestinal tract; however it is disturbed during inflammation associated with IBD (Turner 2009), whilst alterations and increased epithelial permeability are also evident in the small intestine and colon of IBS patients (Bertiaux-Vandaele et al. 2011;

Dunlop et al. 2006). These changes may allow bacteria to access the interstitial compartment of the gut and several recent studies have identified that bacterial cell products can profoundly alter gut neuronal function. In the jejunum lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, activates extrinsic sensory afferents (Wang et al. 2005; Donovan and Grundy 2012), an effect which is reduced by a non-selective cannabinoid agonist, an anandamide transport inhibitor, but not by a fatty acid amide hydrolase (FAAH) inhibitor (Donovan and Grundy 2012). Interestingly, activation of afferents appears to be specific for certain types of LPS, as luminally applied LPS from *Salmonella typhimurium*, but not LPS from *Escherichia coli,* activates these jejunal afferents (Liu et al. 2009). Increased afferent activity and an increased afferent sensitivity to a 5-HT3-receptor agonist following *Salmonella typhimurium* LPS can be blocked via a cyclo-oxygenase or EP1/EP2 mediated mechanism (Liu et al. 2009). Afferents innervating the colon can also be activated by LPS. Standard-grade LPS applied acutely for 3 min or chronically incubated for 24 h induces significant increases colonic DRG neuronal excitability (Ochoa-Cortes et al. 2010). These effects can be mimicked by acute application of bacterial lysate from *Escherichia coli* NLM28, which is exaggerated during DSS induced colitis. However, these effects cannot be blocked in TLR4 -/- mice or be replicated by the use of selected bacterial products activating individual TLRs, suggesting additive or alternate mechanisms may be involved. As ultrapure LPS cannot mimic the hyper-excitability effects of standard-LPS and lysate, but does stimulate TNF-α secretion from acutely dissociated DRG neurons, bacterial cell products may also sensitize colonic afferents via the release of pronociceptive cytokines from both immune cells and the neurons themselves. This appears to be evident by intracolonic administration of a toll-like receptor TLR7 activator, which causes inflammation, and induces short term hyperalgesia which is reduced in $Na_V1.9$ -/- mice. As wild-type and -/- mice display similar acute inflammatory responses and similar increases in pro-inflammatory cytokines, this reduction in hyperalgesia in $Na_V1.9$ -/- mice presumably occurs via the loss of neuronal $Na_V1.9$ (Martinez and Melgar 2008).

Endogenous Factors that Reduce Nociceptor Signalling

In addition to the myriad of nociceptive mediators and mechanisms described above, several key anti-nociceptive mechanisms have also been described that can reduce nociceptor signalling and prevent hyperalgesia and allodynia. Protease activated receptor 4 (PAR4) agonists suppresses the excitability of colonic DRG neurons (Karanjia et al. 2009) and significantly reduce the visceromotor response to colorectal distension in whole animal studies (Auge et al. 2009). PAR4 is actually co-localised in the same neurons as PAR2 and TRPV4 (discussed above) and correspondingly PAR4 activation attenuates both PAR2 agonist and TRPV4 agonist-induced allodynia and hyperalgesia in response to colorectal distension. Interestingly PAR4 agonist exposure inhibits free intracellular calcium mobilization induced by

the pro-nociceptive agonists of PAR2 and TRPV4 (Auge et al. 2009). As such the resultant balance between PAR2, PAR4 and TRPV4 activation is likely to determine the resultant effect on nociceptor responsiveness and therefore visceral pain.

Endogenous opioids are also key regulators of anti-nociceptive function (Karanjia et al. 2009; Verma-Gandhu et al. 2006, 2007). The lack of hyperalgesia and allodynia associated with chronic DSS colitis is actually accompanied by an increase in β-endorphin and μ-opioid receptor expression and CD4 +ve T-cells. This suggests chronic DSS induced-inflammation involves infiltration by lymphocytes, which is accompanied by μ-opioid receptor and β-endorphin up regulation, providing an anti-nociceptive input that restores normal visceral perception (Verma-Gandhu et al. 2007). In addition, colonic supernatants from chronic DSS treated mice have a 14-fold increase in β-endorphin levels, and their incubation suppresses the excitability of nociceptive colonic DRG neurons (Valdez-Morales et al. 2013). However, the timing of these effects may be disease specific as different opioid induced effects are evident in IBS. It has recently been shown that supernatants from PBMCs taken from healthy subjects actually inhibit colonic afferent mechanosensitivity, via a μ-opioid receptor mechanism (Hughes et al. 2013). Moreover, the number of β-endorphin expressing colonic mucosal lamina propria cells actually decreases in constipation predominant-IBS (C-IBS) patients compared with healthy subjects, suggesting that healthy human immune cells actively secrete β-endorphin, which dampens colonic mechanosensation (Hughes et al. 2013). As this inhibitory effect from PBMC supernatants is lost in C-IBS patients, and actually switches to sensitisation in diarrhea predominant (D-IBS) patients, where increases in pro-inflammatory cytokines are evident, these results suggests that resultant neuronal function is a constant balance between pro- and anti-nociceptive mechanisms.

More recently, it was demonstrated that inflammation can induce the function of kappa-opioid receptors, as demonstrated by the inhibitory effects of the agonist asimadoline on colonic nociceptor function (Hughes et al. 2014). Furthermore, the oxytocin receptor is not expressed in healthy colonic DRG neurons, however its expression is induced following inflammation and oxytocin receptor analogues inhibit colonic nociception in vitro and in vivo in post-inflammatory chronic visceral hypersensitivity models (de Araujo et al. 2014).

Conclusions and Future Perspectives

Recent studies have clearly demonstrated the capacity of inflammation or infection to cause long term neuroplasticity and the development of gut symptoms. In the absence of a ‘perfect’ pre-clinical model to replicate the multifactorial nature of many gut disorders, such as IBS, concurrent studies on numerous models have allowed identification of several distinct mechanisms that may potentially underlie neuroplasticity in the clinical setting. Specific immune pathways are recruited in response to different insults, which in turn leads to specific interactions between inflammatory cells, immune cells and neurons. This leads to alterations in neuronal

ion channel and receptor expression and function, leading to neuroplasticity. These studies also suggest some commonality in the mechanisms underlying neuroplasticity and that several mechanisms may have to interact to cause pronounced long term neuroplasticity. Crucially, several different therapeutic strategies may exist for the treatment and prevention of gastrointestinal dysfunction. Selective targeting of the individual neuronal populations displaying neuroplasticity is the ultimate goal for patients currently experiencing chronic pain or alterations in gut motility. However, another therapeutic window of opportunity exists, whereby reducing the initial inflammatory response, for example during the early stages of gastroenteritis, may reduce or prevent subsequent inflammation-induced neuroplasticity. Future research will need to identify how the differing extrinsic and intrinsic neural pathways communicate with one another and the complex interactions that each of them have concurrently with stress mediators, immune responses, enteric/spinal glia and gut microbiota to underlie normal gut physiology. Determining how these interactions are altered during pathophysiology will be crucial in the next phase of understanding the mechanisms of neuroplasticity, which underlie gastrointestinal dysfunction.

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Chapter 10
Purinergic Signalling in the Gut

Geoffrey Burnstock

Introduction

Parasympathetic nerve stimulation that produced atropine-resistant responses of gastrointestinal smooth muscle was recognised early (Langley 1898; McSwiney and Robson 1929; Paton and Vane 1963). However, it was not until the early 1960s that gastrointestinal neuromuscular transmission other than that mediated by the classical transmitters acetylcholine (ACh) and noradrenaline (NA) was recognised (Burnstock et al. 1964; see Burnstock 1969, 2008a). The identification of adenosine 5′-triphosphate (ATP) as the non-adrenergic, non-cholinergic (NANC) inhibitory neurotransmitter in the gut was proposed in 1970 (Burnstock et al. 1970) and the purinergic signalling hypothesis was launched in a Pharmacological Review (Burnstock 1972). This hypothesis was rejected by many people over the next 20 years and it was often ridiculed at international meetings (see Burnstock et al. 2010; Burnstock 2012a). Resistance to the concept was perhaps understandable, because ATP was established as an intracellular energy source involved in the Krebs cycle and it seemed unlikely that such a ubiquitous molecule would also act as an extracellular signaller. It is now clear that ATP, an ancient biological molecule, evolved both as an intracellular energy source and an extracellular signalling molecule. Later, after the cotransmitter hypothesis was published (see Burnstock 1976), it was recognised that nitric oxide (NO) and in some regions vasoactive intestinal polypeptide (VIP) were cotransmitters with ATP in NANC gastrointestinal inhibitory nerves.

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Strong evidence is now available in support of the purinergic hypothesis (see Olsson and Pearson 1990; Hoyle 1992; Dubyak and El Moatassim 1993; Zimmermann 1994; North 2002; Burnstock 2007a, 2012a; Burnstock et al. 2010; Burnstock and Verkhratsky 2012).

Intestinal motility, secretion and absorption can be influenced by ATP released from intrinsic enteric neurons, sympathetic nerves or sensory-motor nerves during axon reflexes, acting directly on purinoceptors on smooth muscle mediating relaxation or contraction or on epithelial cell secretion. Also, ATP released from mucosal epithelial cells can activate sensory enteric neurons involved in reflex activities. In addition, purine nucleotides and nucleosides can act on blood vessels, glia and interstitial cells of Cajal (ICCs) thereby indirectly modulating motility patterns. After breakdown to adenosine, ATP acts on prejunctional nerve terminals to modify transmitter release from motor and inhibitory neural pathways.

In the late 1980s and 1990s electrophysiological studies established that synaptic purinergic transmission was present between neurons in both myenteric and submucosal enteric plexuses (see LePard et al. 1997; Burnstock 2001a, 2007a; Galligan 2002; Bornstein 2008; Christofi 2008). The turning point for acceptance of purinergic signalling was when receptors for nucleotides and nucleosides were cloned and characterised in the early 1990s. Four subtypes of P1 (adenosine) receptors, 7 subtypes of P2X ion channel nucleotide receptors and 8 subtypes of G protein-coupled receptors were identified (see Ralevic and Burnstock 1998; Burnstock 2007b). RT-PCR and immunohistochemical studies were carried out to show the distribution of purinoceptor subtype mRNA and protein in different neurons and non-neuronal cells in different regions of the gastrointestinal tract of different species, including man (see Burnstock and Knight 2004; Burnstock 2007b, 2008a)

An exciting new field emerged when purinergic mechanosensory transduction in visceral organs was discovered (see Burnstock 1999, 2009). ATP released from mucosal epithelial cells during distension of the gut activates P2X3 receptors on submucosal sensory nerve endings (Wynn et al. 2003). Low threshold intrinsic sensory nerves mediate enteric reflex activity, while high threshold fibres mediate the initiation of pain via extrinsic sensory nerves.

There is increasing interest in the pathophysiology of purinergic signalling in the gastrointestinal tract (see Burnstock 2008b, 2014) and in this article its involvement in inflammatory bowel disease (IBD) (Yiangou et al. 2001; Wynn et al. 2004) will be considered.

The Early Discovery of Purinergic Neuromuscular Transmission

Correlated electrical and mechanical activity was recorded in the guinea pig taenia coli using the sucrose-gap technique (Burnstock and Straub 1958). After stimulation of the intramural nerves in the presence of adrenergic and cholinergic blocking

agents, hyperpolarisations and relaxations were reported (Burnstock et al. 1963, 1964; see Burnstock 2004). Tetrodotoxin, a neurotoxin that prevents the action potential in nerves without affecting the excitability of smooth muscle cells blocked the hyperpolarisations (Bülbring and Tomita 1967). This established them as inhibitory junction potentials (IJPs) in response to stimulation of NANC inhibitory nerves. Later NANC neurotransmission was shown to be mediated by intrinsic enteric neurons controlled by vagal and sacral parasympathetic nerves (Burnstock et al. 1966). NANC relaxations were identified at about the same time in the stomach upon stimulation of the vagus nerve (Martinson and Muren 1963; Martinson 1965).

The next step was to try to identify the transmitter released during NANC inhibitory transmission in the gut. Several criteria were postulated by Eccles (1964) and also by Paton (1958) that needed to be satisfied to establish a neurotransmitter: synthesis and storage in nerve terminals; release by a Ca^{2+}-dependent mechanism; mimicry of the nerve-mediated responses by the exogenously applied transmitter; inactivation by ectoenzymes and/or neuronal uptake; and parallel block of responses to stimulation by nerves and exogenously applied transmitter. Different substances were examined in the late 1960s, including amino acids, monoamines and neuropeptides, but none satisfied the criteria. However, hints in a paper by Drury and Szent-Györgyi (1929) showing extracellular actions of purines on heart and blood vessels, a paper by Feldberg and Hebb (1948) showing extracellular actions of ATP on autonomic ganglia and a paper by Holton (1959) showing release of ATP during antidromic stimulation of sensory nerves supplying the rabbit ear artery, led Burnstock and his colleagues to consider ATP and this satisfied all the criteria required to establish it as a transmitter involved in NANC inhibitory neurotransmission (Burnstock et al. 1970). The purinergic neurotransmission hypothesis was proposed in a Pharmacological Review in 1972 (Burnstock 1972).

There was early evidence for ATP as a cotransmitter in sympathetic nerves supplying the guinea pig taenia coli (Su et al. 1971). Periarterial sympathetic nerve stimulation led to release of tritium from guinea pig taenia coli preincubated in [^{3}H] adenosine (which was taken up and converted largely to [^{3}H]ATP) and the release of both tritium and NA was blocked by guanethidine. The proportion of ATP and NA in sympathetic nerves varies in different regions of the gut, between species and during development and ageing. It has been reported that ATP is the sole transmitter in sympathetic nerves supplying arterioles in the submucosal plexus of the intestine, while NA released from these nerves acts as a prejunctional modulator of transmitter release (Evans and Surprenant 1992). 'Axon reflex' activity involving sensory-motor nerves is widespread in autonomic effector systems and forms an important physiological component of autonomic control of blood vessels and visceral organs, including the gut (Burnstock 1993; Holzer 2006). ATP and glutamate are cotransmitters in primary afferent sensory nerves. Cotransmission occurs in enteric neurons and the concept of 'chemical coding' was proposed as a consequence of the patterns of colocalisation of neuropeptides defining specific neuron types (Furness et al. 1989). It is now recognised that three major cotransmitters are released from NANC inhibitory enteric nerves: (1) ATP producing fast IJPs; (2) NO also eliciting IJPs, but with

a slower time course; and (3) VIP producing slow tonic relaxations (Burnstock 2001a). In some sphincters the NANC inhibitory nerves primarily utilise VIP, in others they utilise NO, and in non-sphincteric regions of the intestine, ATP is prominent. Detailed accounts of purinergic neuromuscular transmission in different regions of the gut are available (Hoyle and Burnstock 1989; Burnstock 2001a, 2014; Burnstock and Verkhratsky 2012).

NANC inhibitory purinergic transmission to intestinal smooth muscle of laboratory animals and humans is mediated by $P2Y_1$ receptors (Wang et al. 2007; Gallego et al. 2008, 2012). α,β-Methylene ATP (α,β-meATP) has a potent relaxant action in some preparations (Johnson and Hourani 1994; Johnson et al. 1996; Pacaud et al. 1996). However, it is likely that α,β-meATP is acting on P2X3 receptors on sensory nerves (Storr et al. 2000; De Man et al. 2003) leading to reflex activation of NANC inhibitory nerves and to $P2Y_1$ receptor-mediated relaxation of smooth muscle (see King and Townsend-Nicholson 2008). Evidence was presented that ATP mediates a non-cholinergic component of the excitatory junction potential and contraction of intestinal smooth muscle (Zagorodnyuk and Maggi 1998). ATP stimulated cholinergic interneurons in the myenteric plexus to cause a fast contraction of rat ileum (Sakai et al. 1979). $P2Y_2$ and/or $P2Y_4$ receptors were shown to mediate smooth muscle contractions in the small intestine of lower vertebrates (Burnstock 1969; Sneddon et al. 1973). Contraction of rat duodenal muscularis mucosae smooth muscle was mediated by P2X receptors (Johnson et al. 1996). P2X receptors mediated contraction of guinea pig ileum (Moody and Burnstock 1982; Ivancheva et al. 2001). Ileal contractions mediated by α,β-meATP were inhibited in P2X1 receptor knockout mice (Vial and Evans 2001). mRNAs for P2X2, P2X3 and P2X4 receptors were expressed by canine colon circular myocytes, while longitudinal myocytes expressed mRNAs for P2X3 and P2X5 receptors (Lee et al. 2005).

Synaptic Purinergic Transmission in the Enteric Plexuses

Elegant electrophysiological studies, carried out during the past 20 years have demonstrated synaptic purinergic transmission between enteric neurons in both myenteric and submucous plexuses in both in situ and tissue culture preparations (see Galligan et al. 2000; Galligan 2002; Hu et al. 2003; Ren et al. 2003; Galligan and North 2004; Ren and Galligan 2005; Burnstock 2007a, 2008a; Bornstein 2008; Ren and Bertrand 2008; Valdez-Morales et al. 2011). Also, extensive immunostaining of the localisation of both P2X and P2Y receptor subtypes in the gastrointestinal tract of guinea pigs, rats and mice was carried out (Gröschel-Stewart et al. 1999; Giaroni et al. 2002, 2006; Van Nassauw et al. 2002, 2006; Xiang and Burnstock 2004a, b, 2005, 2006; Ruan and Burnstock 2005; Yu et al. 2010).

Myenteric Ganglia

The effects of ATP in single myenteric neurons from guinea pig small intestine using intracellular electrodes were first shown by Katayama and Morita (1989). They showed that ATP produced hyperpolarisation in 80 % of AH neurons and depolarisation in 90 % of S neurons. The studies of purinergic signalling in guinea pig myenteric neurons were extended by several groups. Whole-cell and outside-out patch clamp recordings were used to characterise the physiological and pharmacological features of P2X receptors on myenteric neurons of the guinea pig ileum (Barajas-López et al. 1996). Fast excitatory postsynaptic currents (fEPSCs) were recorded in primary cultures of myenteric neurons from guinea pig intestine and hexamethonium-resistant fEPSCs were abolished by the P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) (Zhou and Galligan 1996; LePard et al. 1997). Fast excitatory postsynaptic potentials (EPSPs) were mediated in part by P2X receptors in myenteric neurons in both the small and large intestine, but were rare in the gastric corpus (LePard et al. 1997). P2X2 receptors were the dominant subtype shown to be expressed by subpopulations of guinea pig enteric neurons, namely inhibitory motor neurons, vasomotor neurons, cholinergic secretomotor neurons, intrinsic sensory neurons and the endings of vagal afferent fibres in the stomach (Castelucci et al. 2002; Misawa et al. 2010). Using P2X2 receptor knockout mice it was shown that P2X2 receptors contributed to fast synaptic excitation of myenteric neurons in small intestine (Ren et al. 2003). The predominant receptors mediating fast synaptic excitation in the gut appear to be P2X2 homomeric receptors (Galligan 2002; Galligan and North 2004; Ohta et al. 2005), including intrinsic sensory neurons in the gut (Furness et al. 2004b).

P2X3 receptors were expressed by both excitatory and inhibitory motor neurons, ascending interneurons and cholinergic secretomotor neurons (Poole et al. 2002). However, it was claimed that they were not expressed by intrinsic sensory neurons in guinea pig ileum (Van Nassauw et al. 2002). In the small intestine of mice lacking P2X3 receptors peristalsis was impaired (Bian et al. 2003). The distribution of the mRNA and protein of P2X2 and P2X3 receptors were described in the enteric nervous system of the rat (Xiang and Burnstock 2004b). Most myenteric S neurons in guinea pig small intestine expressed P2X3 receptors, about half of which were inhibitory motoneurons (Ren and Galligan 2007). Nerve fibres that enveloped ganglion cell bodies in the myenteric and submucous plexuses in mouse intestine expressed P2X5 receptors, probably as heteromultimers with P2X2 receptors on enteric sensory neurons (Ruan and Burnstock 2005).

Purinergic signalling in dispersed primary cultures of guinea pig myenteric plexus was studied by the group of Mulholland. Different populations of enteric neurons responded to combinations of ATP with ACh, ATP with substance P (SP), ATP with ACh, ATP with ACh and SP, ATP with bombesin or ATP with ACh and bombesin (Kimball and Mulholland 1995). When ACh and ATP acted as cotransmitters, there was an interaction between nicotinic and P2X receptors with

cross-inhibition between α3β4 nicotinic receptors and the C-terminal tail of P2X2 receptors (Decker and Galligan 2010). Inhibitory interactions have also been shown to take place between P2X and γ-aminobutyric acid-A receptors on myenteric neurons from the guinea pig small intestine (Karanjia et al. 2006). In excitatory neuro-neuronal transmission in both ascending and descending reflex pathways to the longitudinal and circular muscles of the guinea pig ileum triggered by mucosal stimulation, a major role was played by ATP (Clark et al. 1996; Spencer et al. 2000). P2X receptor-mediated transmission from interneurons to motor neurons in guinea pig ileum underlies descending inhibitory reflexes (Bian et al. 2000; Bornstein et al. 2004).

There is expression of P2Y receptors on enteric neurons in addition to P2X receptors (Xiang and Burnstock 2005, 2006; Van Nassauw et al. 2005; Gao et al. 2006; Wood 2006). In the mouse gastrointestinal tract relaxation is mediated by $P2Y_1$ receptors on NANC myenteric neurons (Giaroni et al. 2002). Slow excitatory synaptic transmission on S-type neurons in the guinea pig enteric nervous system was mediated by $P2Y_1$ receptors (Hu et al. 2003). They also mediated slow excitatory synaptic potentials on interneurons during descending inhibition in guinea pig ileum (Thornton et al. 2013). $P2Y_2$ receptors were expressed by S-type neurons in both myenteric and submucosal plexuses of the guinea pig gut. 40–60 % of P2X3 receptor-immunoreactive neurons were immunoreactive for $P2Y_2$ receptors in the myenteric plexus and all P2X3 receptor-immunoreactive neurons expressed $P2Y_2$ receptors in the submucosal plexus (Xiang and Burnstock 2005). 30–36 % of neurons in ganglia in the myenteric, but not submucosal plexus of the guinea pig gut expressed $P2Y_6$ receptors, while 42–46 % of the neurons in both myenteric and submucosal plexuses were immunoreactive for $P2Y_{12}$ receptors (Xiang and Burnstock 2006). 28–35 % of $P2Y_6$ receptor-immunoreactive neurons coexisted with NO synthase, while all $P2Y_{12}$ receptor-immunoreactive neurons were immunopositive for calbindin, on AH intrinsic sensory neurons. $P2Y_2$ and $P2Y_{12}$ receptors were identified on enteric neurons in the rat distal colon (Van Nassauw et al. 2005). Presynaptic A_1 receptors mediated suppression of slow EPSPs and amplified slow inhibitory postsynaptic transmission to myenteric neurons (Christofi and Wood 1993; Kamiji et al. 1994).

Submucosal Ganglia

The non-reversing type of slow excitatory postsynaptic potential recorded in S neurons of the submucous plexus of the guinea pig caecum was mimicked by ATP (Mihara et al. 1985). ATP produced fast transient depolarisation of AH-type neurons (Barajas-López et al. 1994), mediated by P2X receptors (Barajas-López et al. 2000). Neurons in the submucous plexus were immunopositive for P2X3 receptors and were colocalised with calretinin, suggesting labelling of intrinsic sensory neurons (Xiang and Burnstock 2004b). Functional interactions between nicotinic and

P2X receptors were demonstrated in dissociated guinea pig submucosal neurons in primary culture (Glushakov et al. 1996; Barajas-López et al. 1998; Zhou and Galligan 1998). Inhibitory interactions between P2X and 5-HT_3 receptors in guinea pig submucosal neurons were reported (Barajas-López et al. 2002). Slow, fast and intermediate EPSPs were recorded in neurons of the submucous plexus of the guinea pig ileum (Monro et al. 2004). The slow and intermediate EPSPs were blocked by the $P2Y_1$ receptor selective antagonist MRS2179. $P2Y_1$ receptor signalling involved in synaptic transmission in the human submucous nerve plexus was reported to be predominant (Wunderlich et al. 2008).

Intrinsic Sensory Neurons

Intrinsic sensory neurons are located in the submucosal and myenteric ganglia and their terminals are largely in a subepithelial plexus (Furness et al. 2004a). Intrinsic sensory neurons have been identified electrophysiologically as AH-type and morphologically as Dogiel type II cells. Most AH cells express calbindin and/or calretinin. Synaptic transmission to intrinsic sensory neurons is mediated by P2X receptors (Bertrand and Bornstein 2002), of the P2X2 receptor subtype in guinea pig intestine (Castelucci et al. 2002). Postsynaptic inhibition via P2Y receptors has also been identified on intrinsic sensory nerves (Bertrand 2003, 2004). P2X3 receptors were shown to be expressed by intrinsic sensory nerves in rat ileum and distal colon (Xiang and Burnstock 2004b). $P2Y_{12}$ receptors were expressed by sensory neurons in guinea pig myenteric plexus (Xiang and Burnstock 2006).

Enteric Glial Cells and Interstitial Cells of Cajal

Enteric glial cells respond to ATP and uridine 5′-triphosphate, increasing intracellular calcium via $P2Y_2$ and/or $P2Y_4$ receptors (Kimball and Mulholland 1996; Sarosi et al. 1998). Immunohistochemical studies also showed expression of P2X7 receptors on enteric glial cells (Vanderwinden et al. 2003) and $P2Y_4$ receptors (Van Nassauw et al. 2006). It was proposed that ATP released from sympathetic nerves activates enteric glia (Gulbransen et al. 2010). Purinergic neuron-glia interactions in the enteric nervous system have been reported, reflecting similar mechanisms in the CNS (Gulbransen and Sharkey 2009). From an electrophysiological study of a mouse enteric neuron-glial culture preparation, it was concluded that neuronal cells primarily express P2X receptors, while glial cells primarily express P2Y receptors (Gade and Akbarali 2013).

ICCs are a specialised cell type that act as pacemakers to regulate the activities of smooth muscle cells in the gut. P2X2 and P2X5 receptors were shown to be expressed on ICC's in guinea pig intestine (Burnstock and Lavin 2002). Later, $P2Y_4$ receptors were also identified on ICCs in guinea pig gastrointestinal tract

(Van Nassauw et al. 2006). It is likely that ATP is released as a cotransmitter from enteric nerves and glial cells to regulate the activities of ICCs (Burnstock and Lavin 2002). Modulation of pacemaker $[Ca^{2+}]_i$ activity in ICC's was mediated by P2X receptors (Furuzono et al. 2005). It was reported that ICCs in human and murine small intestine expressed $P2Y_1$ and $P2Y_4$ receptors (Chen et al. 2007). 'Fibroblast-like cells', that form a network of cells distinct from ICCs, located between intestinal circular and longitudinal smooth muscle near terminals of enteric motor neurons and with gap junction connectivity with muscle cells, express $P2Y_1$ receptors (Kurahashi et al. 2011). $P2Y_1$ receptor antagonists blocked the activation of currents and increase in $[Ca^{2+}]_i$ by adenosine 5′-diphosphate in these cells. The majority of subserosal ICCs or perhaps fibroblast-like cells in the guinea pig proximal colon responded to ATP via $P2Y_1$ receptors and it was suggested that this may contribute to smooth muscle relaxation (Tamada and Hashitani 2014).

Purinergic Mechanosensory Transduction: Enteric Reflexes and Pain

Both submucosal intrinsic sensory neurons and extrinsic sensory nerves show positive immunoreactivity for P2X3 receptors (Xiang and Burnstock 2004b). It has been proposed that during intestinal distension ATP is released from mucosal epithelial cells to activate P2X3 receptors on both low threshold enteric sensory nerve fibres to mediate enteric reflexes (including peristalsis) and high-threshold extrinsic enteric sensory fibres leading to initiation of nociceptive impulses that pass messages through sensory ganglia to pain centres in the CNS (Burnstock 2001b, 2009). This hypothesis has been supported by experiments on a rat pelvic sensory nerve-colorectal preparation (Wynn et al. 2003). Distension of the colorectum led to increase in release of ATP from mucosal epithelial cells and evoked pelvic sensory nerve excitation. This excitation was mimicked by application of ATP and was attenuated by the selective P2X3 and P2X2/3 antagonist, 2′(3′)-O-(2,4,6-trinitrophenyl) ATP, and by PPADS. The sensory activity in the nerves was potentiated by ARL-67156, an ATPase inhibitor. It has been claimed recently that subepithelial fibroblasts in rat ductal villi also release ATP by mechanical stimuli to activate P2X3 receptors on subepithelial sensory nerves (Furuya and Furuya 2013).

Purinergic Signalling in Inflammatory Gut Disorders

P2X3 receptors are upregulated on enteric sensory neurons in inflammation and hypersensitivity (Wynn et al. 2004). Intestinal inflammation also increased the expression of $P2Y_6$ receptors on epithelial cells and uridine diphosphate, a potent

$P2Y_6$ receptor agonist, released CXCL8, a chemokine known for chemoattraction to recruit neutrophils during the acute phase of colitis (Grbic et al. 2008, 2012). ATP may be beneficial in the treatment of intestinal disorders where intestinal permeability changes are involved (Bours et al. 2007). It was reported that $P2Y_2$ receptor expression was upregulated in intestinal epithelial cells by the transcription factor C/EBPβ during inflammation (Degagné et al. 2012).

Expression of P2X3 receptors was increased in enteric plexuses of human IBD, suggesting a role in dysmotility and pain (Yiangou et al. 2001) and the possibility that P2X receptor antagonists could be used for the treatment of irritable bowel syndrome (IBS) was raised (Galligan 2004). It was also suggested that P2X receptors on intrinsic enteric neurons may mediate enhanced gastrointestinal propulsion and secretion and might be used for treating constipation-predominant IBS, while P2X receptor antagonists might be useful for treating diarrhoea-predominant IBS. It has been suggested that sensitisation of P2X3 receptors on vagal and spinal afferents in the stomach may contribute to the development of visceral hyperalgesia (Dang et al. 2005). In inflamed gastrointestinal tract, glial cells proliferate and produce cytokines, indicating that P2X7 receptors may play a role in the response of enteric glia to inflammation (Vanderwinden et al. 2003).

ATP release and P2X3 and P2X2/3 receptor-mediated nociceptive sensory nerve responses were enhanced in the rat trinitrobenzene sulfonic acid (TNBS) model of colitis (Wynn et al. 2004). Different mechanosensory information from the colon to the spinal cord is mediated by lumbar splanchnic (LSN) and sacral pelvic (PN) nerves. It was shown that 40 % of LSN afferents responded to α,β-meATP compared to 7 % of PN afferents (Brierley et al. 2005). There is enhancement of P2X3 receptor-mediated signalling in the TNBS colitis model, which was due, at least in part, to the appearance of P2X3 receptor expression in a greater number of calcitonin gene-related peptide-labelled small nociceptive neurons in the dorsal root ganglia (DRG) (Wynn et al. 2004). There is also increased release of ATP from mucosal epithelial cells with distension in TNBS-treated rats. Purinergic mechanosensory transduction has been shown to contribute to post-infectious mechano-hypersensitivity (Rong et al. 2009). In TNBS-induced colitis in mice, P2X1 receptor expression on colonic submucosal arterioles was increased (Lomax et al. 2007). Propulsive motility was attenuated in the ulcerated region of the TNBS-inflamed colon and this was associated with a decrease in the purinergic component of the descending inhibitory limb of the peristaltic reflex circuit (Strong et al. 2010).

Substances are released from mucosal epithelial cells during distension that often act synergistically to cause sensitisation of afferent nerves to mechanical or chemical stimuli (Wynn and Burnstock 2006). Thus, receptors to a variety of substances including ATP are potential targets for drug treatment for inflamed bowel function and visceral pain (see Kirkup et al. 2001; Holzer 2004). The sensitising effects of P2X3 receptor agonists on mechanosensory function were also demonstrated in oesophagitis (Page et al. 2000). Visceral hyperalgesia was shown to be

associated with an increase in ATP activity and enhanced expression of P2X3 receptors in colonic sensory neurons (Xu et al. 2008). Selective P2X3 and P2X2/3 receptor antagonists that are orally bioavailable and do not degrade in vivo are in clinical trials for the treatment of visceral pain (see Gever et al. 2006; Donnelly-Roberts et al. 2008). P2X3 receptor mRNA expression in DRG was significantly decreased in an ovariectomized rat model of colitis, which was reversed by oestrogen (Fan et al. 2009). It was suggested that ATP is a critical autocrine regulator of mechanosensitive 5-hydroxytryptamine (5-HT) release, also involved in the pathogenesis of IBD and it was shown that P2X3 receptors on enterochromaffin cells were downregulated in ulcerative colitis (Liñán-Rico et al. 2013). CD39 (NPTDase 1) was upregulated in the submucosa during colitis, resulting in compromise of epithelial barrier function (Neshat et al. 2009). It was reported that dysregulation occurs in 59 % of purinoceptor genes in IBD, including $P2Y_6$, $P2Y_{13}$, $P2Y_{14}$, P2X5, A_{2A} and A_{2B} receptors (Rybaczyk et al. 2009).

P2X7 receptors play a pivotal role in intestinal inflammation and are involved in the development of visceral hypersensitivity (Keating et al. 2011). Epithelial and immune cells express P2X7 receptors, which are implicated in the pathogenesis of IBD based on the dysregulation of immune responses in (de Campos et al. 2012). Activation of neuronal P2X7 receptor/pannexin 1 mediates death of enteric neurons during colitis (Gulbransen et al. 2012). This supported an earlier study of TNBS-induced colitis, using high-density oligonucleotide microassay analysis and oral N^6-(3-iodobenzyl)-adenosine-5-N-methyluronamide, an A_3 receptor agonist, blocked the colitis-induced upregulation of P2X1, P2X4, P2X7, $P2Y_2$ and $P2Y_6$ receptors (Guzman et al. 2006). Extracellular ATP largely via P2X7 receptors evoked cell death in human intestinal epithelial cells and the implication of this in inflammatory conditions and immune responses was explored (Souza et al. 2012). It has also been shown that ATP mediated mast cell-dependent intestinal inflammation via P2X7 receptors (Kurashima et al. 2012).

An adenosine A_3 agonist has been recommended to be protective in two murine models of colitis (Mabley et al. 2003). A_{2B} receptor expression and signalling in intestinal epithelium in colitis was upregulated by tumour necrosis factor-α (Kolachala et al. 2005). A_{2B} receptor blockade ameliorated mouse colitis (Kolachala et al. 2008a) as did A_{2B} receptor gene deletion (Kolachala et al. 2008b). The inhibitory effects of adenosine on enteric neuromuscular activities were diminished in inflamed colon (Antonioli et al. 2005). Oxidative stress disrupted purinergic neuromuscular transmission in the inflamed colon (Roberts et al. 2013). A_{2B} receptors appear to play a role in the control of T cell-mediated colitis by suppressing the expression of pro-inflammatory cytokines, while sparing anti-inflammatory activity mediated by interleukin (IL)-10 and transforming growth factor-β (Naganuma et al. 2006). A_{2A} receptors were also reported to mediate the inhibitory effects of adenosine on colonic motility in the TNBS model of experimental colitis (Antonioli et al. 2006; Rahimian et al. 2010). Adenosine deaminase inhibition attenuates

inflammation in experimental colitis (Antonioli et al. 2007) through the recruitment of A_{2A} and A_3 receptors (Antonioli et al. 2010). Adenosine, acting via A_3 receptors, has been shown to be involved in intestinal anti-inflammation activities (Guzman et al. 2006; Gessi et al. 2008). A_{2A} receptors were also involved in the anti-inflammatory actions of adenosine (Odashima et al. 2005) and A_{2A} receptor agonists are being developed for the treatment of IBD (El-Tayeb et al. 2011). A_{2B} receptors mediate regulation of 5-HT synthesis and release from hypoxic enterochromaffin cells in IBD (Damen et al. 2013). A_{2B} receptor antagonists were reported to be effective against murine colitis (Kolachala et al. 2008c). The involvement of adenosine A_1 and A_{2A} receptors (Antonioli et al. 2011) and A_3 receptors (Ren et al. 2011) in colitis has been reported. Recent reviews of the roles of adenosine signalling in gastrointestinal inflammation are available (Estrela and Abraham 2011; Colgan et al. 2013). The involvement of adenosine deaminase in patients with Crohn's disease has been explored (Maor et al. 2011). Adenosine kinase inhibition by GP515 has also been investigated as a potential target for the treatment of colitis (Siegmund et al. 2001). It was concluded in a review about purinergic receptors in gastrointestinal inflammation (Kolachala et al. 2008a) that P1 (A_{2A} and A_{2B}) and P2Y receptor-based therapy is highly promising for treatment of inflammatory conditions of the gut (see Michael et al. 2010). Serum adenosine deaminase activity has been proposed as a predictor of disease severity in ulcerative colitis (Beyazit et al. 2012). Ecto-nucleoside triphosphate diphosphohydrolase 7 was preferentially expressed in epithelial cells of mouse small intestine (Kusu et al. 2013). ATP released from colonic mucosal epithelial cells of IBS patients excited via P2X receptors enteric cholinergic motor neurons (Balestra et al. 2012). The role of adenosine as an immune modulator of IBD has been considered (Ye and Rajendran 2009). Polymorphisms of CD39 have been linked to Crohn's disease (Künzli et al. 2011). Release of ATP by activated neutrophils and necrotic intestinal epithelial cells stimulates epithelial cell P2X7 receptors leading to activation of caspase 1 and secretion of IL-1β proinflammatory cytokine (Cesaro et al. 2010).

Concluding Comments

In this brief review, the focus has been on purinergic neuromuscular transmission, synaptic purinergic transmission in the enteric nerve plexuses, purinergic mechanosensory transduction in initiation of enteric reflexes and intestinal nociception and the involvement of purinergic signalling in inflammatory gut disorders. Figure 10.1 summarises the complex distribution of purinoceptor subtypes in the gut. For fuller coverage of the involvement of purinergic signalling in the physiology and pathophysiology of the gastrointestinal system, readers are recommended to refer to the following recent reviews (Burnstock 2008a, b, 2011, 2012b, 2014).

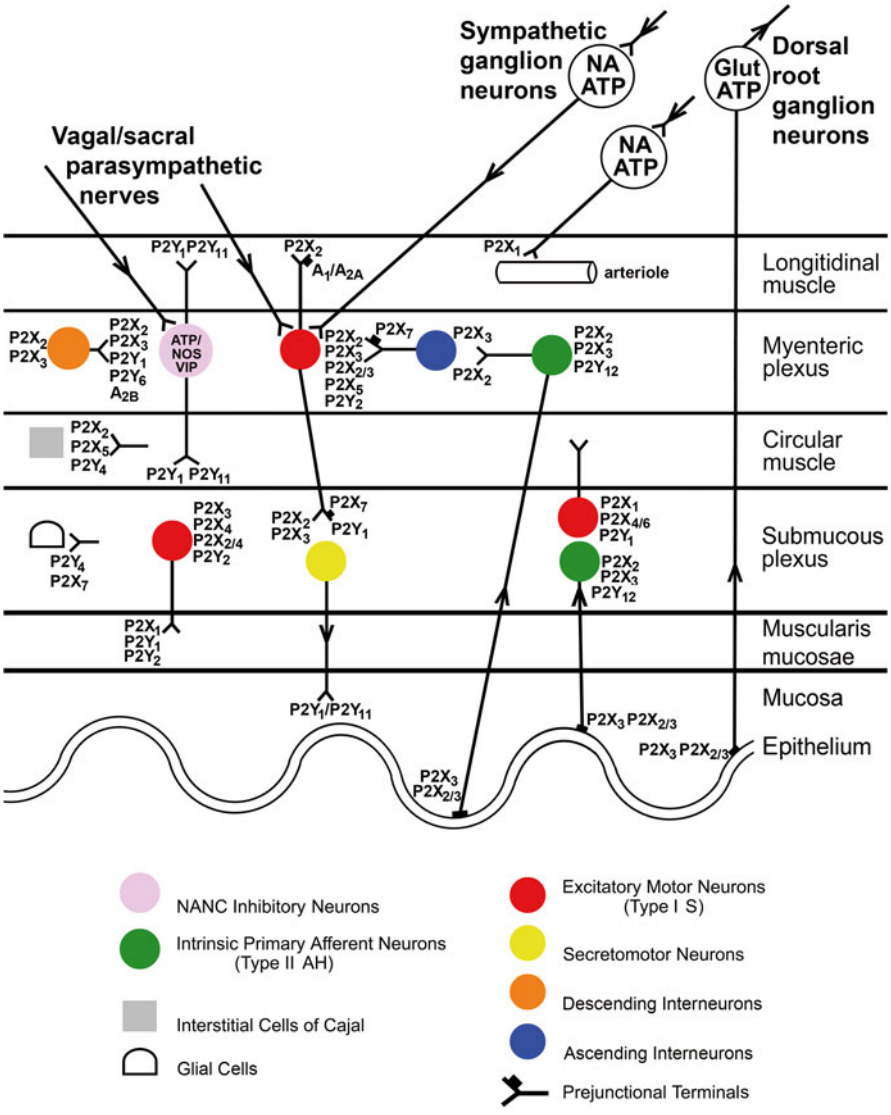

Fig. 10.1 Schematic showing the localisation of receptors to purines and pyrimidines on neurons and non-neuronal effector cells in the gut, although some of the interacting pathways are not yet known. Extrinsic vagal and sacral parasympathetic nerves connect with NANC inhibitory neurons in the myenteric plexus expressing P2X2, P2X3, $P2Y_1$, $P2Y_6$ and A_{2B} receptors, as well as with cholinergic motor neurons; these neurons are also activated by descending interneurons. Extrinsic sympathetic nerves modulate motility via excitatory motor neurons and constrict blood vessels in the gut via P2X1 receptors. Extrinsic sensory nerves arising from cell bodies in dorsal root ganglia and with subepithelial terminals mediate nociception. Intrinsic sensory neurons in both myenteric and submucosal plexuses express P2X2 and P2X3 receptors, while a subpopulation also express $P2Y_{12}$ receptors; they connect with motor pathways involved in peristalsis. Excitatory motor neurons express P2X2, P2X3, P2X2/3, P2X5 and $P2Y_2$ receptors and connect with both interneurons and secretomotor neurons. Interneurons express P2X2 and P2X3 receptors. Enteric glial cells express $P2Y_4$ and P2X7 receptors, while interstitial cells of Cajal express P2X2, P2X5 and $P2Y_4$ receptors. P2X7 and P1 receptors appear to act as prejunctional modulators of both motor and interneurons. (Reproduced from Burnstock 2008b, with permission.)

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Chapter 11
Is There a Role for Endogenous 5-HT in Gastrointestinal Motility? How Recent Studies Have Changed Our Understanding

Nick J. Spencer and Damien J. Keating

Introduction

Over the past few years, there have been dramatic changes in our understanding of the role of endogenous 5-hydroxytryptamine (5-HT) in the generation of gastrointestinal (GI) motility patterns in the small and large intestine. The idea that endogenous 5-HT played a major role in the generation of peristalsis in the small intestine was first proposed in the mid 1950s, after it was discovered that endogenous 5-HT could be released from the mucosa at a similar time that peristalsis occurred; and that exogenous 5-HT could potently stimulate peristalsis. The fact that exogenous 5-HT stimulated peristalsis and that there was a similarity in timing between the release of 5-HT from the mucosa and the onset of peristalsis led investigators to propose that release of endogenous 5-HT from the mucosa was *causally* related to the generation of peristalsis. In further support of this, other studies showed that selective 5-HT antagonists could inhibit or block peristalsis, and other motor patterns, such as the migrating motor complex. Taken together, based on these findings, some laboratories believed that endogenous 5-HT (synthesized in the gut wall) was an important mediator, or initiator, of different propulsive motor patterns in the lower GI tract. This notion changed dramatically in the past few years, however, after it was discovered that removal of the mucosa abolished all cyclical release of endogenous 5-HT, but did not block peristalsis, nor the cyclical migrating complex. Furthermore, other laboratories revealed that genetic deletion of the gene tryptophan hydroxylase 1 (TPH-1) (that synthesizes endogenous 5-HT in the mucosa)

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actually had no inhibitory effect on transit of intestinal contents in live animals. Then, perhaps one of the most startling of all observations was the discovery that selective 5-HT receptor antagonists actually have the same inhibitory effects on peristalsis and the migrating complex in segments of intestine that had been depleted of all endogenous 5-HT. Taken together, these recent findings have led to a major revision in our understanding of the functional role of endogenous 5-HT in the generation of propulsive motor patterns in the lower GI tract. This review will focus on how our understanding of endogenous 5-HT in the GI tract has changed substantially in recent times.

Early Hypotheses Regarding the Role of Mucosally-Derived 5-HT and Gut Motility

In the early to mid 1950s, the laboratory of Professor Edith Büllbring performed a number of in vitro experiments on isolated segments of guinea-pig small intestine that addressed the functional role of endogenous and exogenous serotonin in the generation of peristalsis, evoked by intraluminal fluid infusion (Büllbring and Lin 1957, 1958; Büllbring et al. 1958). Following their work which showed that endogenous 5-HT could be released into the intestinal lumen at a similar time as the onset of distension-evoked peristalsis (Büllbring and Lin 1957, 1958; Büllbring et al. 1958), other investigators some years later also proposed that endogenous 5-HT release from the mucosa played a major role in the initiation of peristalsis in the large intestine (Grider et al. 1996; Jin et al. 1999) and even a "critical" role in the generation of colonic migrating complexes in the mouse colon (Heredia et al. 2009). Remarkably, these major conclusions in the mouse colon were made without ever recording 5-HT release from the mucosa (Heredia et al. 2009). Rather, these conclusions were proposed after the mucosa was dissected away from the colon and it was stated that removal of the mucosa "…*appeared to block*" CMMC generation in four mice tested (Heredia et al. 2009). It was rationalized by other laboratories that if release of 5-HT from the mucosa was critical for CMMC generation (Heredia et al. 2009), then deletion of the gene that synthesizes 5-HT from the mucosa would be expected to also block CMMCs. Not long after it was proposed that release of 5-HT from the mucosa was critical for CMMC generation (Heredia et al. 2009), this conclusion was disproved by the same authors, when they published that mice lacking the TPH-1 gene (that synthesizes mucosal 5-HT) still generated robust CMMCs (Heredia et al. 2013). Also, when other investigators repeated these experiments they found that by carefully removing the mucosa and submucosa from the colon (without damaging the underlying myenteric plexus), it was possible to still record cyclical CMMCs, even though all release of 5-HT had been prevented (Keating and Spencer 2010) (Fig. 11.1).

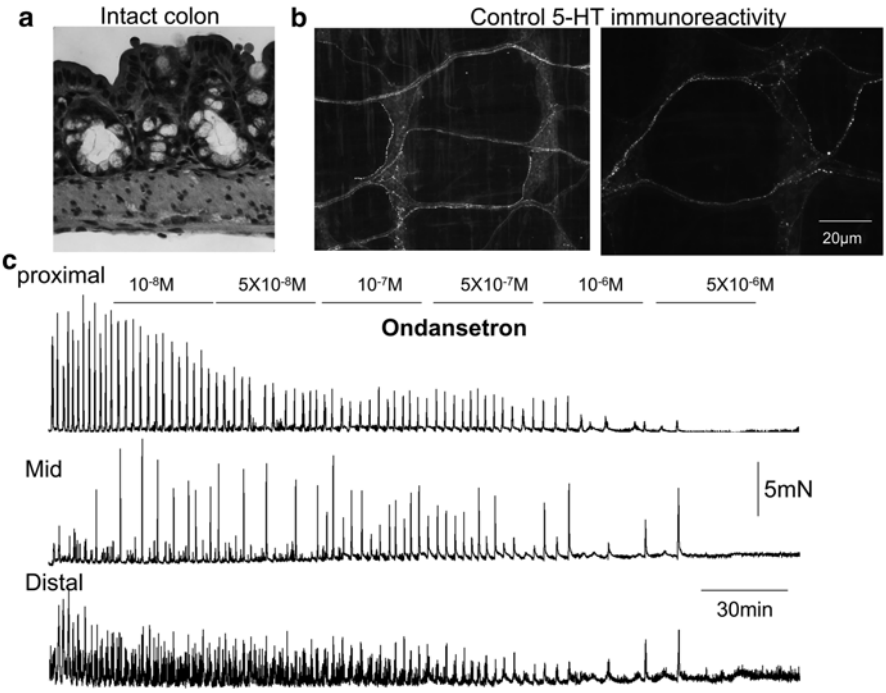

Fig. 11.1 Effects of ondansetron on colonic migrating motor complexes recorded from whole isolated control mouse colon, where 5-HT immunoreactivity is detected in the myenteric plexus. (**a**) Cross section of intact mouse colon, showing mucosa, smooth muscle layers, and enteric ganglia. (**b**) Immunohistochemical staining for 5-HT in the myenteric plexus. 5-HT immunoreactive fibres were found to exist in a small proportion of axons in myenteric ganglia and some internodal strands. (**c**) Shows effects of ondansetron at incremental increases in concentration from 10^{-9} M to 10^{-5} M on spontaneous CMMCs recorded from intact whole colon (containing mucosa). Each concentration of ondansetron was applied for 30 min increments. CMMCs were abolished at 10 μM in this preparation

Mechanisms Underlying Endogenous 5-HT Release from the Mucosa

Definitive proof that intrinsic sensory neurons exist in the intestine was finally demonstrated about 20 years ago (Kunze et al. 1995; Bertrand et al. 1997, 2000) and convincing evidence was presented that these neurons respond directly to stimulants applied to the mucosa. Although the functional role of intrinsic sensory neurons during peristalsis still remains unclear, studies have clearly demonstrated that the nerve endings of intrinsic sensory neurons do project into the mucosa (Song et al. 1991) and their nerve endings lie in close apposition to the mucosal border, where they can be activated directly by exogenous 5-HT (Kunze et al. 1995; Bertrand et al. 1997, 2000), or electrical stimulation of the mucosa as in the colon

(Neunlist et al. 1999). However, the significance of any possible release of 5-HT from enterochromaffin (EC) cells in the mucosa was seriously questioned after studies revealed that removal of the mucosa, or disruption to the mucosal membrane did not prevent peristalsis in the same preparation used by Professor Büllbring and colleagues (Ginzel 1959; Diament et al. 1961; Tsuji et al. 1992). In more recent studies, Bertrand and colleagues developed an amperometric approach to record the dynamic release of 5-HT, in real time, from the intestinal mucosa during fluid-induced peristalsis of the small intestine and during spontaneous and pharmacologically-evoked contractions (Bertrand 2006). He provided compelling evidence that the cyclical release of 5-HT from the mucosa was actually a *consequence* of peristalsis and was clearly *not* the underlying *cause* of peristalsis. His data completely supported studies that showed removal of the mucosa from the small bowel did not block peristalsis (Ginzel 1959; Diament et al. 1961; Tsuji et al. 1992). Indeed, work from Dr. Bertrand's laboratory clearly demonstrated that mechanical deformation of EC cells (caused by the contraction of the gut wall. e.g. during peristalsis) is the stimulus for the release of 5-HT from EC cells, rather than release of 5-HT being a catalytic step required for the initiation of peristalsis (Bertrand 2006). This now explains why Professor Büllbring and colleagues observed release of 5-HT every time a peristaltic contraction occurred. Then, studies by Keating and Spencer arrived at the same conclusion as (Bertrand 2006) for the generation of colonic migrating motor complexes in the isolated mouse colon (Keating and Spencer 2010). In that study, the first direct real time recordings of 5-HT release from the colonic mucosa were made and it was also shown that, similar to the guinea-pig small intestine, endogenous 5-HT could be released in a cyclical fashion, at the same time as CMMC generation (Keating and Spencer 2010). However, when care was employed to sharp dissect away the mucosa from the colon, it was revealed that all release of 5-HT was blocked, but CMMCs were still reliably recorded (Keating and Spencer 2010). Similarly, in the guinea-pig colon, real time amperometric recordings showed that removal of the mucosa abolished all release of 5-HT release, but did not block distension-evoked peristalsis (Spencer et al. 2011).

Soon after these amperometric studies were performed, the laboratory of Gershon and colleagues (Yadav et al. 2010; Li et al. 2011) took a genetic approach to determine the functional role of endogenous 5-HT release in intestinal motility. They demonstrated that selective deletion of the gene responsible for 5-HT synthesis in enterochromaffin (EC) (Tryptophan Hydroxylase 1—TPH-1) led to *no* inhibitory effects on GI-transit in live mice (Yadav et al. 2010; Li et al. 2011). These recent findings are important because the vast majority of 5-HT in the body (>95 %) is synthesized within the intestinal mucosa, with only very minor quantities synthesized in the enteric nervous system. Taken together, these studies, at last, provided definitive proof that endogenous release of 5-HT from the mucosa was not required for peristalsis in vitro, nor the colonic migrating motor complex in the large bowel in vitro, nor was there any detectable delay in GI transit in live animals if mucosal 5-HT synthesis had been prevented.

How Recent Findings Have Changed Our Understanding of the Role of Endogenous 5-HT in Neurogenic Gastrointestinal Motility Patterns

After recent studies confirmed that the mucosa, or release of 5-HT from the mucosa was not required for distension-evoked peristalsis or the cyclical migrating complex, studies then focused on the possible functional role of endogenous 5-HT in enteric neurons, bearing in mind that only ~1 % of enteric neurons synthesize 5-HT (Costa et al. 1996). Dr. Gershon's laboratory deleted the gene (TPH-2) that synthesizes 5-HT in enteric neurons from mice. They demonstrated that intestinal transit was reduced, but this genetic deletion also led to major neuroanatomical and neurochemical changes in the ENS, which could also have been responsible for the altered transit (Li et al. 2011). To test this further, and avoid any genetic side effects related to the ENS neurochemistry, a recent showed that in preparations of colon that had had the mucosa and submucosal plexus removed, acute depletion of endogenous 5-HT from enteric neurons (using reserpine) had no effects on distension-evoked peristalsis (Sia et al. 2013a) or the colonic migrating motor complexes (CMMCs) in the mouse colon (Spencer et al. 2013). It seemed difficult to believe that if deletion of gene of that synthesized mucosal 5-HT has no effect on motility in vivo (Yadav et al. 2010), that deleting 5-HT from 1 % of enteric neurons would lead to major changes in motility, especially since endogenous 5-HT has not been shown to cause any synaptic potentials in mouse ENS (Furukawa et al. 1986; Nurgali et al. 2004) (Fig. 11.2).

By What Mechanism Do 5-HT Antagonists Inhibit Intestinal Motility If Depletion of Endogenous 5-HT Has Such Minor Effects on Motor Patterns?

Specific antagonists of 5-HT receptors such as ondansetron (Zofran) and alosetron (Lotronex), had been commonly prescribed in clinics to provide relief of symptoms in patients with diarrhea-predominant irritable bowel syndrome (D-IBS). Despite this, there is still no clear understanding where these antagonists act to cause their inhibitory effects on GI-motility. We once believed (and published) that endogenous 5-HT played an important role in the control of the propulsion in the small and large intestine since 5-HT3 antagonists could abolish or potently inhibit the migrating motor complex in the small bowel and colonic migrating motor complex in the large bowel (Bush et al. 2001). However, our recent findings have led to completely revised view of this hypothesis, because we discovered that 5-HT3 and 5-HT4 antagonists can still inhibit or block peristalsis and the migrating complex even in preparations that have been depleted of all detectable endogenous 5-HT

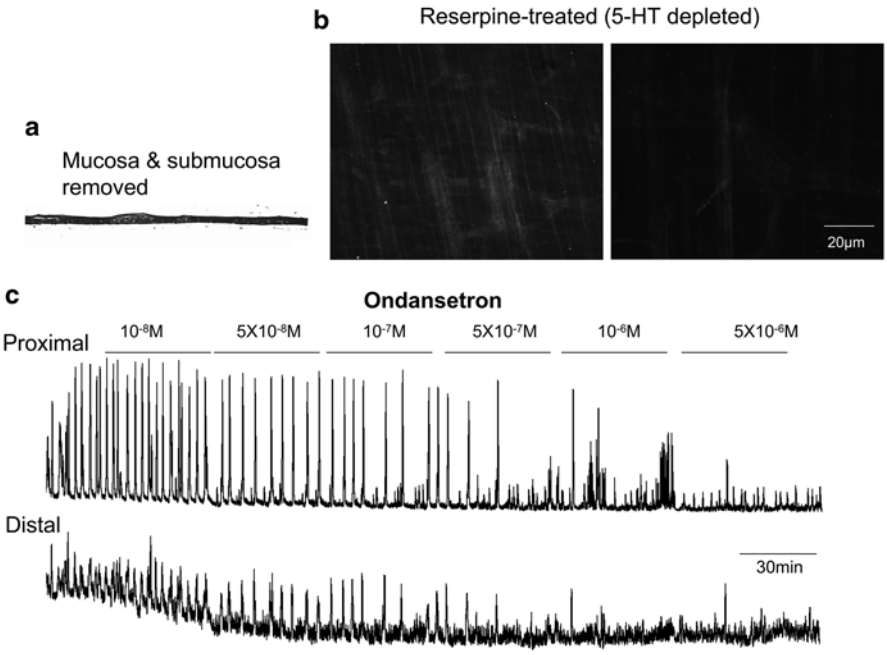

Fig. 11.2 Effects of ondansetron on colonic migrating motor complexes recorded from whole isolated control mouse colon that has been depleted of endogenous neuronal 5-HT and after the mucosa and submucosal plexus had been removed. (**a**) Shows a cross section of the preparation of colon from which mechanical recordings were made in panel (**c**). The preparation consists only of muscle layers and myenteric ganglia. (**b**) After 24 h pretreatment with reserpine, immunohistochemical staining for 5-HT revealed that all endogenous 5-HT had been depleted from axons in myenteric ganglia and internodal strands. (**c**) Shows effects of ondansetron at incremental increases in concentration from 10^{-9} M to 10^{-5} M on CMMCs recorded from intact whole colon (with entire mucosa and submucosal plexus removed). Note, not only do CMMCs still occur in reserpine-treated preparations that are also devoid of mucosa and submucosal plexus, but that ondansetron induced a progressive reduction in the frequency of occurrence of CMMCs, similar to control CMMCs in Fig. 11.1. Ondansetron was applied for 30 min increments

(confirmed depletion of 5-HT with mass spectrometry (Sia et al. 2013a, b) and immunohistochemistry) (Spencer et al. 2013). One possible explanation for these findings with 5-HT3 and 5-HT4 antagonists is that 5-HT3 and 5-HT4 receptors are constitutively active in the absence of any endogenous ligand. That is, endogenous 5-HT itself if not required for the 5-HT3 or 5-HT4 receptor to remain active and contribute to the resting conductance of the enteric neuronal cell membrane. Indeed, the ligand-gated 5-HT3 receptor (Hu and Peoples 2008) and the G-protein coupled 5-HT4 (Berthouze et al. 2005) receptor have both been reported to display constitutive activity. If 5-HT3 and 5-HT4 antagonists reduce this constitutive activity (acting as inverse agonists), this could reduce background excitability in enteric neurons that express these receptors and inhibit the ENS circuitry. These findings

are highly significant because it means that supreme caution should be exercised in future studies when interpreting data that involve selective 5-HT antagonists applied to the isolated preparations of intestine.

Why 5-HT Fails the Criteria To Be a Neurotransmitter in the ENS of Most Species

Whilst serotonin is generally considered to be a neurotransmitter in some parts of the central nervous system, it has been very difficult to obtain direct and consistent evidence that serotonin is a neurotransmitter in the enteric nervous system. According to the classic definition of a neurotransmitter it is "*..a substance that is released at a synapse of by one neuron and that affects another cell, either neuron or effector organ, in a specific manner…*" (Kandel et al. 2000). In other words, for a substance, such as 5-HT, to be considered a neurotransmitter in the ENS it must be demonstrated to cause a postsynaptic response following stimulation of the ENS. Despite exhaustive attempts from different laboratories, intracellular electrophysiological recordings from enteric neurons in mice (Furukawa et al. 1986; Nurgali et al. 2004), rats (Brookes et al. 1988) and humans (Brookes et al. 1987) have not be able to record any synaptic potentials that could be attributed to endogenous 5-HT. In these latter three species, all fast synaptic potentials in enteric neurons are abolished by hexamethonium and it is conspicuous that no serotonergic synaptic potentials have ever been identified. Because of this, 5-HT does not satisfy the criteria as an enteric neurotransmitter in these species. In guinea-pig intestine, small fast excitatory post synaptic potentials have been reported to occur in about 10 % of enteric neurons (Furness 2006). The lack of evidence for 5-HT as a neurotransmitter in the human ENS has been concerning for drug companies and laboratories that have promoted 5-HT as a major neurotransmitter in ENS. Despite the fact that 5-HT does not satisfy the criteria as a neurotransmitter in human, rat or mouse ENS, 5-HT antagonists can clearly reduce intestinal transit in these species (Balfour et al. 2000; Bush et al. 2001)? It is important to note that there are many other "putative" neurotransmitters which are also clearly synthesized in enteric neurons and can be demonstrated immunohistochemically, but also fail to be classified as an ENS neurotransmitter because they do not cause any known postsynaptic responses.

Why Would Serotonin Be Synthesized in the Gut Wall If It Has No Function?

One major misconception that has hindered research in GI motility is the notion that all neurotransmitters synthesized in the ENS must have a function. This is invalid. There is now overwhelming evidence in mammals that a variety of proteins,

receptors, neurotransmitters, ion channels, even whole organs have no essential function for survival of mammals. For example, there is no essential function of nipples on men, the appendix, the coccyx, the helix in the ear, the tonsils, the arrector pili (goose bumps), wisdom teeth, plica semilunaris (nictitating membrane) in the eye lid; and even the sinus cavities in the skull. The question that is then commonly raised is why would these cell types (tissues) and other receptors, neurotransmitters, ion channels, even whole organ (such as the gall bladder) be synthesized in the body if they do not have an essential function for normal day-to-day life? The answer is they are known to be evolutionary vestiges. Evolutionary pressure and environmental change determine their relative importance in each species. This is important to recognize because in the enteric nervous system there about 30 different neurotransmitters are known to be synthesized (Furness 2006), yet there is actually only functional evidence that a very small number actually satisfy the criteria as a neurotransmitter (Galligan et al. 2000; Furness 2006) and generate any postsynaptic response following electrical stimulation (Galligan et al. 2000; Furness 2006). Our view is that endogenous 5-HT is an either an evolutionary vestige in the ENS and that current lifestyle and dietary intake does not necessitate 5-HT as an ENS neurotransmitter in day-to-day function. Or, that perhaps 5-HT only plays a major functional role in the bowel during disease.

Concluding Remarks

Recent studies have revealed major new insights into the functional role of endogenous 5-HT in neurogenic GI motor patterns in the lower GI tract. Whilst it is well accepted that the majority of serotonin (>95 %) in the body is synthesized within the mucosa of the gut wall, there is now unequivocal evidence that endogenous serotonin in the mucosa is not required for the generation of distinct neurogenic motor patterns, such as peristalsis and the migrating motor complex in healthy mammals. Of course, it is entirely possible that during intestinal inflammation, such as inflammatory bowel disease (IBD), an upregulation of serotonergic signaling pathways may underlie, or contribute, to changes in gastrointestinal motility. However, it should be noted that following intestinal inflammation literally thousands of genes have been shown to change their expression; and identifying which genes are directly responsible for intestinal dysmotility has not be resolved. To date, no studies have demonstrated that during inflammation changes in serotonergic signaling are a ***cause*** of intestinal dysmotility, as opposed to an ***effect*** of the inflammation. Understanding the functional role of endogenous 5-HT per se in intestinal inflammation and GI dysmotility will have important ramifications for the future development of therapeutic agents.

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Chapter 12
Enteric neuropathies: Yesterday, Today and Tomorrow

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Introduction

A significant proportion of people (approximately 20–30 %) in the Western population experience unexplained gastrointestinal (GI) symptoms referred to as 'functional', i.e. disturbs typically unrelated to underlying major organic diseases. Although generally mild or moderate, a small subset of cases shows GI functional symptoms, i.e. nausea, vomiting, bloating, abdominal distension, intractable constipation and chronic pain, of such severity to hamper normal feeding and compromise considerably patients' quality of life (Thompson et al. 1999). In addition, this subset of patients may also have recurrent intestinal sub-occlusive episodes, which occur in the absence of demonstrable mechanical causes, leading to numerous hospitalizations as well as useless and potentially harmful surgical interventions (Stanghellini et al. 2005). A number of diagnostic approaches, e.g. radiological and manometric tests, revealed severe abnormalities of gut transit and motor coordination

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(Stanghellini et al. 2005; De Giorgio et al. 2011) likely due to changes of the morpho-functional integrity of the enteric smooth muscle (the main effector system of gut propulsion) and/or neuromuscular systems (mainly the enteric nervous system, ENS—a collection of several million neurons that controls the vast repertoire of gut functions, including motility) (Knowles et al. 2013; Furness 2012). The possibility to investigate these severely ill patients with GI full-thickness surgical specimens opened to histopathology as a mean to actually demonstrate the type and extent of derangements occurring in the enteric neuromuscular systems. In this line, the histopathological analyses revealed degenerative or inflammatory abnormalities/loss of ganglia, neuronal cell bodies and nerve endings (and associated glial cells) of the ENS, which taken together can be labeled as 'enteric neuropathies' (ENs) (for review see Knowles et al. 2013; Furness 2012).

Despite consistent progress, the mechanisms of ENs are only partially understood and the therapeutic approaches to these patients (mainly with predominant small bowel involvement, as exemplified in the clinical phenotype referred to as chronic intestinal pseudo-obstruction—CIPO) are mainly supportive, rather than curative (Stanghellini et al. 2005; De Giorgio et al. 2011; Knowles et al. 2013). Histopathological analysis of gut full-thickness biopsies from patients with severe gut dysmotility is expected to improve the knowledge on ENs and pave the way to new management strategies and therapeutic options.

The purpose of this chapter is to review the advancements that have been done in the knowledge of ENs identified in adult patients, starting from a brief historical background, focusing on where are we and where we are headed in terms of future research and related clinical implications. The present paper will cover only adult forms of ENs and the reader is referred to excellent reviews on enteric aganglionosis, i.e. Hirschsprung disease, and other rare congenital forms of ENs (Panza et al. 2012; Laranjeira and Pachnis 2009).

'Yesterday': A Brief Historical Background on Gut Neuropathology

A synoptic view of the major milestones in ENS neuropathology has been reported in Fig. 12.1. The neuropathology of the ENS dates back to the nineteenth century (specifically 1886) when Harald Hirschsprung, a Danish pediatrician, described the disease which still carries its name. He recognized ENS abnormalities, i.e. the absence of myenteric and submucosal ganglia, in newborn babies presenting with congenital megacolon (Hirschsprung 1886). After that milestone finding most of the ENS neuropathology acquisitions revolved around the assessment of colonic tissues obtained mainly in pediatric patients undergoing surgery for megacolon. In those studies, an important advancement was obtained by the application of the Golgi's (or variations of) silver staining technique to better investigate the ENS abnormalities. In that respect, papers published by Whitehouse and Kernohan (Whitehouse and

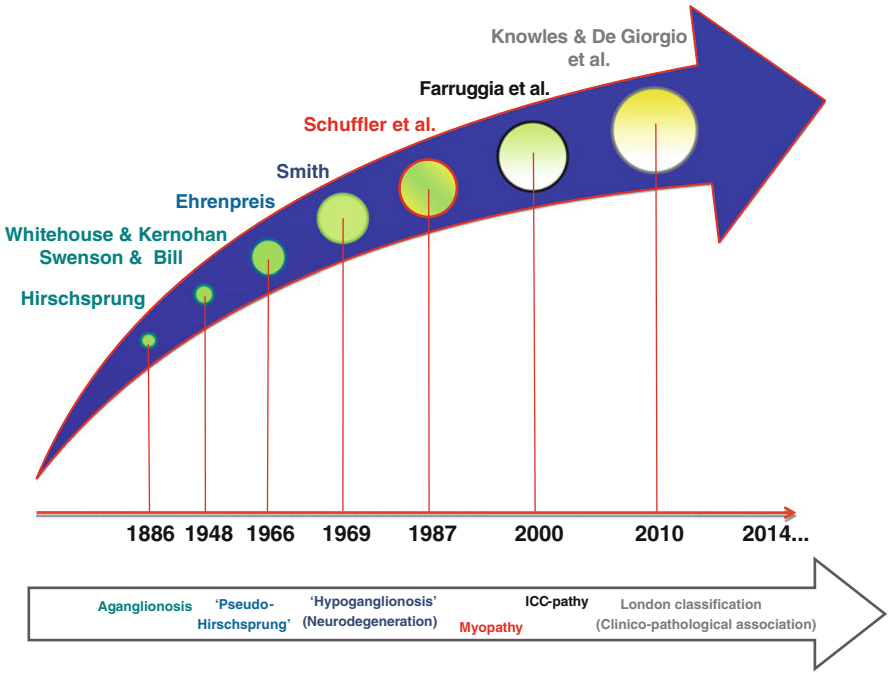

Fig. 12.1 Synopsis summarizing some of the major milestones in ENS neuropathology over the years, i.e. from Hirschsprung's seminal description of congenital aganglionosis of the colon up to the London classification. Colors shown here couple the author(s) (above the *curvilinear arrow*) with the corresponding neuropathological acquisition (reported in the *horizontal arrow*)

Kernohan 1948), Swenson and Bill (Swenson and Bill 1948), and Bodian et al. (Bodian et al. 1949) provided a better definition of the aganglionosis and associated nerve fiber abnormalities in patients with severe gut dysmotility mainly due to massive colonic (and sometimes small bowel) dilatation. Ehrenpreis was the first to introduce the term of 'pseudo-Hirschsprung' to denote the occurrence of cases with megacolon not necessarily associated with aganglionosis (Ehrenpreis 1965); Smith used tangential (not only transverse) cutting of gut specimens for a better evaluation of ENS changes in adult patients with idiopathic megacolon (Smith 1967). During the 1980s, Schuffler and collaborators used silver staining to investigate any case operated on for severe dysmotility and proposed a classification of enteric neuromuscular disorders (Krishnamurthy and Schuffler 1987). Although accurately detailed, this classification progressively lost its value as a result of the overwhelming knowledge emerging from basic studies on enteric neuromuscular structure and function (e.g., milestone work from Farrugia's group (He et al. 2000) as well as (Vanderwinden et al. 1993) and other authors on interstitial cells of Cajal and ENS abnormalities—for review also see (Knowles et al. 2013; Furness 2012; Panza et al. 2012; Laranjeira and Pachnis 2009). Finally, because of the growing knowledge on ENs and the need to provide guidelines on enteric neuropathology, a panel of experts (the

Gastro 2009 International Working Group, IWG) elaborated consensus papers regarding tissue collection, processing, staining and histopathological reporting as well as classification of gut motility disorders and related histopathological features (Knowles et al. 2009, 2010).

'Today': ENs—Where Are We?

Over the years the interest for gut histopathology in patients with suspected ENs has been challenged by the evidence that traditional surgery was commonly associated with a deterioration of the clinical picture characterized by severe impairment of gut motility and the formation of adhesions, thus introducing a mechanical component in a functional GI context. In addition, pathologists often discouraged clinicians as an 'apparently normal' neuromuscular layer was reported even in cases characterized by marked intestinal dilatation. In recent years, however, impressive technological advancements have fully regenerated the physicians' interest for gut full-thickness biopsy in ENs. Several minimally invasive procedures, including laparoscopic surgery or more recently endoscopic approaches (e.g. natural-orifice transluminal endoscopic surgery) (Song et al. 2012) showed a high diagnostic yield and safety (Knowles et al. 2008). Gut full-thickness histopathology should be considered a diagnostic 'gold-standard' as it indicates the possible abnormalities affecting the main control mechanisms regulating gut physiology, including the ENS. The demonstration of histopathological features indicative of ENs may provide not only pathophysiological information but also clinically useful insights regarding diagnosis, prognosis and possible treatment options for patients with severe gut dysmotility.

ENs can be classified as primary when the disease targets primarily the ENS or secondary if the ENS abnormalities are part of a systemic condition with multiorgan involvement (e.g. diabetes mellitus, systemic sclerosis, amyloidosis and other conditions) (Knowles et al. 2013; De Giorgio and Camilleri 2004). Most of the primary ENs are also idiopathic in origin as no evident causes can be identified. In some cases of primary ENs, however, recent data indicate that genetic abnormalities can play an aetiologic role. Herein, primary/idiopathic and genetic ENs will be reviewed, while secondary ENs lack systematic analysis based on gut full-thickness biopsy.

According to histopathological features, primary ENs can be either degenerative or immune-mediated/inflammatory. Degenerative neuropathies are still not completely understood. In fact, some cases may disclose an apparently normal ENS and therefore a quantitative analysis of enteric ganglia and neuronal cell bodies would be required (Accarino et al. 2012). However, quantitative analysis of the ENS is a complex and time-consuming approach not readily feasible in routine pathology. In addition, the lack of normative data in the human ENS contributes to increase the uncertainty experienced by pathologists facing with cases of ENs requiring a quantitative analysis. Quantitative assessment (based on control values of individual laboratories) in ENs may reveal an 'oligoneuronal hypoganglionosis', i.e. a reduced

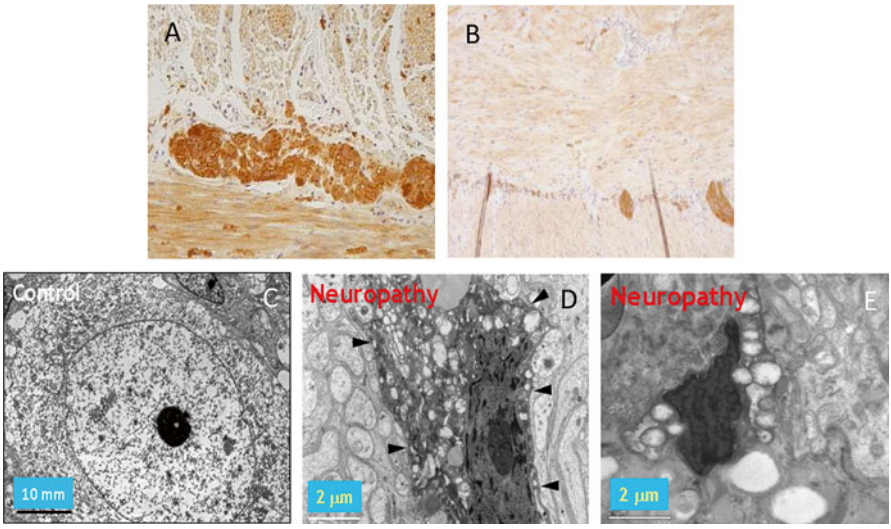

Fig. 12.2 Representative photomicrographs illustrating a small bowel section of a 21-year old female patient with a clinical diagnosis of neuropathic CIPO (**b**) compared to a control (**a**—48 year old female undergoing surgery for uncomplicated right colon cancer). Note the smaller appearance of NSE labeled myenteric ganglia as well as less intensely stained neuronal cell bodies in (**b**) (patient's section) as compared to control (**a**). Streptavidin-biotin complex peroxidase immunohistochemical technique using a specific anti-NSE mouse monoclonal antibody, to identify enteric neuronal cell bodies and processes. Original magnifications X200 in (**a**) and X100 in (**b**). (**d**) and (**e**) (Labeled as 'Neuropathy') are representative electron microscopic photomicrographs taken from the ileum of a 20-year-old man with recurrent sub-occlusive episodes related to a CIPO. Compared to a control picture (**c**), a number of ultramicroscopic abnormalities, including cytoplasmic vacuolization (*arrowheads*), mitochondrial abnormalities (*arrowheads*) and a shrunken nucleus are detectable in (**d**), while an apoptotic body is visible in (**e**). Calibration bars: 10 mm in (**c**); 2 µm in (**d**) and (**e**)

number of myenteric (usually more affected than the submucosal) ganglia and ganglion cell bodies (Wedel et al. 2002). Conversely, a neuropathic pattern may also be identified in conditions characterized by 'giant' enteric ganglia likely due to an increase in the number of neurons (and most likely glial cells) as it occurs in some peculiar disorders with a genetic origin (i.e. ganglioneuromatosis—see below) (Raue and Frank-Raue 2010). Qualitative findings in degenerative ENs include altered expression of a variety of neuronal markers (e.g. neuron specific enolase, NSE; or protein gene product 9.5, PGP9.5; or HuC/D) (Fig. 12.2b) swollen ganglion cell bodies, aberrant mitochondria, cytoplasmic vacuolization (Fig. 12.2d, e), nerve fragmentation and loss of axons (De Giorgio and Camilleri 2004; Wedel et al. 2002; Sarnelli et al. 2005). Neurodegenerative mechanisms in the ENS may include an altered calcium signaling, mitochondrial dysfunction, production of free radicals and neuronal apoptosis (Hall and Wiley 1998). Abnormalities of enteric glial cells also may play a role in enteric ENs, since consistent evidence points to a key

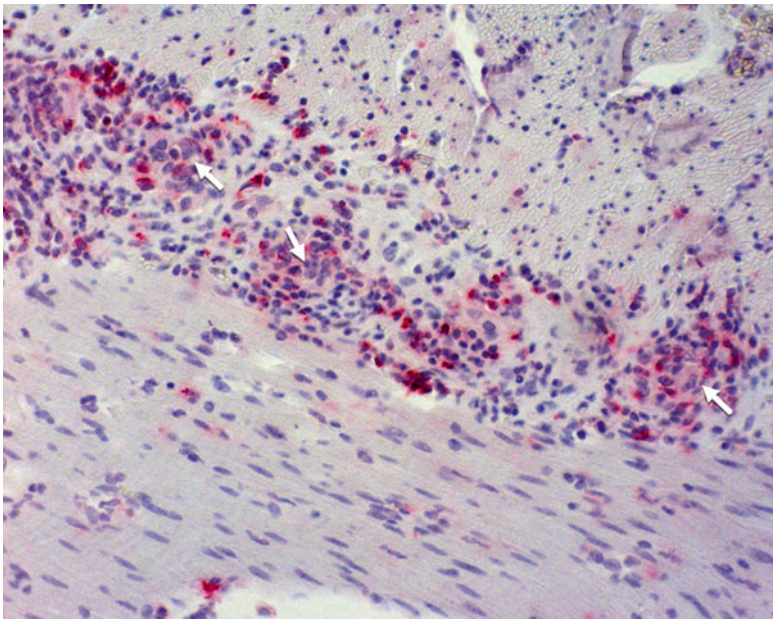

Fig. 12.3 Representative photomicrograph illustrating a small bowel section of a 32-year old male patient with a clinical diagnosis of neuropathic CIPO. The surgical specimen was obtained in the operative room and processed for histopathological analysis. Note the intense immune-mediated (lymphocytic)/inflammatory infiltrate throughout a myenteric plexus of the ileum (hence, 'myenteric ganglionitis'). The *arrows* indicate residual neuronal cell bodies surrounded or in close vicinity to CD8 positive T cells (*pink staining*). The longitudinal smooth muscle layer appears apparently not invaded by immune infiltrate. Alkaline phosphatase antialkaline phosphatase immunohistochemical technique using specific anti-CD8 monoclonal antibodies, to identify a subset of T lymphocytes. Original magnification X200

role exerted by these cells in enteric neuron maintenance and survival (De Giorgio et al. 2012).

Immune-mediated/inflammatory ENs can be due to lymphocytes (mainly CD3+ T cells), eosinophils and mast cells infiltrating the ganglionated plexuses of the ENS (hence the term 'enteric ganglionitis') (De Giorgio et al. 2004). Commonly, immune/inflammatory cells target primarily myenteric as well as axons running throughout the muscular layer of the gut. The definition of lymphocytic ganglionitis is easily applicable when a massive infiltration of lymphocytes can be detected within myenteric ganglia (Fig. 12.3); it is less easily definable in cases with a low-grade infiltrate where a quantitative assessment of the number of lymphocytes may be necessary. The Gastro 2009 IWG proposed that ≥5 lymphocytes/ganglion identify an enteric ganglionitis (Knowles et al. 2009). Overt and low-grade lymphocytic ganglionitis can be identified in severe generalized gut motility disorders (usually CIPO). In two distinct studies on CIPO the analysis of intestinal full-thickness biopsies showed lymphocytic ganglionitis in 29 % (Knowles et al. 2004) and 34 % (Lindberg et al. 2009) of patients. A lymphocytic ganglionitis may be associated

with neuronal degeneration and loss up to complete ganglion cell depletion in the most severe cases. In addition to a cell-mediated response, patients with histopathologic evidence of lymphocytic ganglionitis may develop anti-neuronal antibodies referred to as anti-HuC/D (based on the molecular target) or anti-nuclear neuronal antibodies (ANNA-1) (based on nomenclature) (De Giorgio et al. 2004). In addition to their usefulness in the diagnostic work-up, the anti-HuC/D antibodies are known to affect the ascending reflex pathway of peristalsis in vitro, evoke neuronal apoptosis and elicit autophagic mechanisms in primary culture of myenteric neurons or neuronal cell lines (De Giorgio et al. 2004). Taken together, the lymphocytic infiltrate in enteric ganglia and anti-neuronal autoantibodies provide a basis to understand the origin of severe dysmotility in patients with an inflammatory neuropathy related to generalized dysmotility.

ENs with Genetic Abnormalities

A wide array of genes are now known to regulate enteric neuron migration, development, maturation and maintenance and their mutations in animal models determine ENs often with a syndromic (multisystemic) phenotype (Laranjeira and Pachnis 2009). The genes involved in the pathogenesis of Hirschsprung disease (for review see Panza et al. 2012; De Giorgio and Camilleri 2004) do not appear to play a role in adult ENs except *SOX10*, a transcription factor exerting a key role in neuronal survival and maintenance. Sporadic patients carrying de novo *SOX10* heterozygous mutations showed a clinical phenotype of ENsS with CIPO and features of Waardenburg-Shah syndrome (i.e., pigmentary anomalies and sensorineural deafness) (Pingault et al. 2002). To our knowledge only few histopathologic pictures can suggest genetic disorders, including neuronal intranuclear inclusion disease (NIID), multiple endocrine neoplasia type 2B (MEN-2B) and mitochondrial neurogastrointestinal encephalomyopathy (MNGIE).

NIID may be considered a polyglutamine disease characterized by eosinophilic intranuclear inclusions (the hallmark of the disease) in neurons of the central, peripheral and enteric nervous systems along with progressive degeneration and neuronal loss. Molecular analysis indicates that inclusions contain expanded polyglutamine tracts, immunopositive for ubiquitin and, especially, for small ubiquitin-like modifier (SUMO)-1 (Panza et al. 2012). In cases with predominant autonomic dysfunction the GI tract is involved with a severe dysmotility affecting the esophagus (dysphagia), stomach (gastroparesis) and the whole gut (CIPO). Although most NIID cases are sporadic, occasional familial recurrence indicates a genetic basis, but the responsible gene(s) remains unidentified. NIID overlaps with familial visceral neuropathy, a heterogeneous, poorly defined group of disorders due to abnormalities of the ENS (Kimber et al. 1998).

MEN-2B is a rare autosomal-dominant syndrome characterized by the early development of medullary thyroid cancer in all affected individuals. Pheochromocytomas occur in 50 % of patients along with other features, mainly marfanoid habitus, “blub-

bery lips" (due to mucosal neuromas), and neuromas of the eyelids (Raue and Frank-Raue 2010). A diffuse ganglioneuromatosis, i.e. transmural 'giant ganglia' with increased number of neurons and (likely) glial cells, of the GI tract occurs in about 40 % of patients and is associated with severe constipation/megacolon. A specific germ-line point mutation (methionine → threonine) in *RET* in exon 16 at codon 918 (M918T) occurs in 95 % of patients. Other rarer (5 %) mutations involve exon 15 at codon 883 (A833F) in the *RET* tyrosine kinase domain (Nguyen et al. 2006).

A recessive form of CIPO is the mitochondrial neurogastrointestinal encephalopathy (MNGIE). In addition to severe gut dysmotility, patients with MNGIE manifest with cachexia, ptosis, ophthalmoparesis, peripheral neuropathy and exhibit white matter changes (leukoencephalopathy) on magnetic resonance imaging of the brain. This syndrome is caused by mutations in the thymidine phosphorylase gene (*TYMP*, also known as endothelial cell growth factor-1, *ECGF1*) or in the polymerase gamma gene (*POLG*, a form of MNGIE without leukoencephalopathy). Gut tissue analysis showed that CIPO in patients with MNGIE show a peculiar histopathologic pattern characterized by underlying enteric neuromuscular abnormalities mainly in the small bowel (Giordano et al. 2008).

Finally, in a consanguineous Turkish family, we have demonstrated an autosomal recessive idiopathic form of CIPO in addition to megaduodenum, long-segment Barrett esophagus, and different cardiac abnormalities of variable severity identifiable in the affected members (OMIM 611376; Mungan syndrome) (Mungan et al. 2003). Notably, full-thickness intestinal biopsies from two affected individuals revealed a severe reduction of the myenteric and submucosal neurons, suggesting an underlying intestinal neuro-myopathy. (Deglincerti et al. 2007). Genome-wide linkage analysis and homozygosity mapping approach identified a maximum multipoint lod score of 5.01 in a critical interval of about 13 Mb between D8S1830 and D8S1799 on the chromosome 8q23-q24 (Deglincerti et al. 2007). Using whole exome sequencing analysis we have shown a novel mutation in the *RAD21* gene, cosegregating with the disease phenotype in this family (Bonora et al. 2015). Therefore, it is clear that even severe forms of ENs show a high degree of genetic heterogeneity, hindering the identification of the molecular causes and of the deranged molecular pathways shared by the different affected individuals. Nevertheless, recent advances in omics technologies, including the massive next-generation sequencing approaches leading to the availability of entire exomes/transcriptomes will improve the identification of molecular defects leading to ENs, likewise for other highly heterogeneous diseases such as cancer (Dienstmann et al. 2014) and intellectual disability (Gilissen et al. 2014).

'Future': Where Are We Headed?

Although rare, ENs are highly disabling conditions characterized by very severe clinical phenotypes (e.g., CIPO) usually associated to a poor prognosis mainly because of the lack of available effective therapeutic strategies. Nonetheless, research

data obtained in recent years open to an optimistic view for the future. Indeed, we are now beginning to decipher molecular (genetic abnormalities; neurodegeneration) and cellular (immune-mediated) mechanisms underlying ENs (Knowles et al. 2013). Furthermore, an emerging investigational field is represented by the gut microbiota and its impact on the ENS maturation, differentiation and maintenance. Initial evidence suggests that the gut microbiota (Anitha et al. 2012) may influence the ENS maturation, chemical coding and function. Also, nutrients, alone or in combination with microbiota, have been shown to have a role in ENS neuroplasticity and gut physiology (De Giorgio and Blandizzi 2010). However, whether an altered interplay between the gut microbiota/nutrients and ENS is actually pathogenetically relevant for ENs will be matter of future study. Other challenges in the research agenda will concern: (1) a better standardization of methods in order to quantify and characterize enteric neurons; we do believe that modern medicine cannot avoid thorough (e.g., quantitative) analysis and this should be made routinely applicable in patients with ENs; (2) the acquisition of normative (control) data in each referral laboratory; (3) the combination of clinical and histopathological quali-quantitative findings in order to establish a solid relationship between clinical phenotype (for example CIPO) and the underlying morphological correlates, i.e. ENs; (4) the identification of molecular or histopathological biomarkers of ENs for improving clinical management and therapeutic option discovery.

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Chapter 13
Postnatal Development of the Mouse Enteric Nervous System

Jaime Pei Pei Foong

Owing to over three decades of research, we now have a good understanding of the genetic and molecular control of enteric nervous system (ENS) development during embryonic and prenatal stages. On the other hand, it has only just become clear that a substantial process of ENS maturation occurs after birth (Hao et al. 2013a). During postnatal stages, in addition to genetic influences, ENS development is also potentially affected by the external environment. Thus it is possible that manipulating certain environmental factors could help prevent or reduce motility disorders. However the genetic and environmental factors that regulate postnatal ENS development remain unknown. Researchers have used a variety of animal models that are easy to manipulate genetically or experimentally, and have short gestational periods, to understand the development of the ENS. Notably, due to the availability of mouse models for several human enteric neuropathies, many studies have used the mature and developing murine ENS as a model. Here, I will discuss recent advances in knowledge about postnatal development of the murine ENS, and highlight future directions for this emerging research field.

Development of Enteric Ganglionated Plexuses

The ENS is a network of neurons and glia residing within two major ganglionated plexuses embedded along the wall of the gastrointestinal tract (Furness 2012). The submucous plexus lies between the mucosa and circular muscle, the myenteric plexus is sandwiched between the longitudinal and circular muscles.

The ENS derives mainly from the vagal neural crest. Similar to other parts of the nervous system, development of the ENS comprises of a series of overlapping

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stages: migration, proliferation, differentiation and formation of synapses (Hao et al. 2013a; Sasselli et al. 2012). While the formation of the myenteric plexus precedes the submucous plexus in mice (Jiang et al. 2003; Uesaka et al. 2013), much is still unknown about how development of neurons in one plexus influences the other. The mature and developing myenteric plexus has been well studied compared to the submucous plexus.

Maturation of Cholinergic and Nitrergic Neurons

In the mature ENS, cholinergic (express the enzyme choline acetyltransferase, ChAT) and nitrergic (express the enzyme neuronal nitric oxide synthase, nNOS) neurons are typically characterized as separate neuronal populations (Foong et al. 2014; Sang and Young 1998). Cholinergic neurons are excitatory neurons and function as motor neurons, interneurons and sensory neurons, while nitrergic neurons can be inhibitory motor neurons or interneurons.

Significant numbers of myenteric neurons are still being born (exiting the cell cycle) between P0 and P10 in mice (Bergner et al. 2014; Laranjeira et al. 2011; Pham et al. 1991). Submucosal neurons tend to exit the cell cycle and differentiate later than myenteric neurons (McKeown et al. 2001; Pham et al. 1991). Some neuronal precursors transiently express certain neurotransmitter synthetic enzymes during fetal or early postnatal development (Baetge and Gershon 1989; Bergner et al. 2014; Gershon et al. 1993; Hao et al. 2013b; Obermayr et al. 2013). For example, in the small intestine, some submucosal and myenteric neurons that do not express nNOS in the mature gut transiently express nNOS during embryonic and early postnatal development (Bergner et al. 2014; Young and Ciampoli 1998). Nonetheless, segregation between nitrergic and cholinergic neurons seems to be established early in development (Hao et al. 2013b).

The proportion of nNOS myenteric neurons in the small intestine does not change significantly postnatally, whereas nNOS submucosal neurons decline from 50 % at P0 to only 3 % in adult mice (Young and Ciampoli 1998). Research into the development of ChAT neurons has been limited by technical difficulties until very recently. Uptake of 3[H]-choline at E10–E12 suggested that ChAT+ cells appear very early in development (Rothman and Gershon 1982). However, several ChAT antisera failed to detect any neurons in the gut until late embryonic stages. To overcome this problem, two recent studies used mouse fluorescent reporter lines to detect ChAT (Hao et al. 2013b; Erickson et al. 2014). The first used Cre-lox technology (*ChAT-Cre* mice in combination with a fluorescent reporter line) and detected the first ChAT+ neurons in the gut at E11.5. In this *ChAT-Cre:R26R* line, cholinergic neurons gradually increase in number during late embryonic and postnatal stages, and at birth, the proportion of cholinergic neurons at birth is still less than half of that in the adult small intestine (Hao et al. 2013b). The second study extended these findings and found that in *ChAT-GFP* mice GFP+ neurons are already present at E10.5; this study was also able to detect ChAT immunoreactive neurons in the gut

E10.5 (Erickson et al. 2014). Furthermore, the proportion of cholinergic neurons in the small intestine was found to achieve adult levels as early as E13.5 (Erickson et al. 2014).

Little is known about the mechanisms regulating neuronal differentiation postnatally, although the role of two transcription factors in postnatal neuronal differentiation has been examined. *Hand2* haploinsufficiency results in a reduction of nNOS neurons in embryos, and this reduction persists through to postnatal weeks 6–8 (D'Autreaux et al. 2011). Homeodomain interacting protein kinase 2 (*HIPK2*) is a transcriptional cofactor of bone morphogenetic proteins. Loss of *HIPK2* did not affect enteric neurons during embryogenesis, but caused a progressive decrease in the number of myenteric and submucosal neurons in the caudal gut with increasing postnatal age (Chalazonitis et al. 2011). Thus *HIPK2* may be important for survival and differentiation of enteric neurons postnatally (Chalazonitis et al. 2011). Although not specifically studied, many other transcription factors, including *Ascl1* (formally known as *Mash1*) (Okamura and Saga 2008), implicated in prenatal neuronal differentiation could also be involved in the postnatal development of enteric neurons.

Some external factors have been reported to affect enteric neuron differentiation postnatally. For example, the colonization of the intestine by microbiota has been shown to affect ENS postnatal development. The jejunum and ileum of germ-free mice have decreased myenteric nerve density and ganglion size, but an increase in proportion of myenteric nitrergic neurons (Collins et al. 2014). However, whether this is a direct effect of a lack of microbiota remains to be elucidated, as there are other defects in germ-free mice and the ENS defects might be indirect (Yi and Li 2012). Diet has also been shown to affect the postnatal development of the ENS; mice fed on a high fat diet for 8 weeks have decreased numbers of myenteric neurons in the small and large intestine, but submucosal neurons were unaffected (Stenkamp-Strahm et al. 2013; Voss et al. 2013). Notably in the duodenum, nitrergic neurons were particularly affected, while cholinergic neurons were spared (Stenkamp-Strahm et al. 2013). In contrast, a high fat diet was reported to be neuroprotective in the stomach, preventing age-associated loss of nitrergic myenteric neurons (Baudry et al. 2012).

Maturation of Neuronal Morphology

As in other species, adult enteric neurons in the mouse have either a single axon or multiple axons (Dogiel type II, DII) (Foong et al. 2012, 2014; Nurgali et al. 2004; Wong et al. 2008). Most myenteric uniaxonal neurons only have lamellar dendrites, while DII neurons have smooth cell bodies and typically project their axons circumferentially (Foong et al. 2012; Nurgali et al. 2004).

The first enteric neurons to differentiate have single long, anally-projecting axon-like processes (Hao et al. 2013a; Young et al. 2002, 2014). Orally and circumferentially-projecting neurons appear later. Unlike adults, most uniaxonal

embryonic and early postnatal myenteric neurons have multiple filamentous dendrite-like structures instead of lamellar dendrites (Foong et al. 2012). Maturation of dendritic morphology and axonal projection lengths of uniaxonal neurons persists in postnatal stages, even after P10. DII neurons are present by birth and their long processes project nearly around the entire gut circumference at P10. Despite significant growth in gut circumference between P10 and adult, the lengths of DII neuron axons do not increase after P10 (Foong et al. 2012). The morphological changes in dendrites and axons after birth suggests that synapse formation remains dynamic postnatally; raising the possibility that ENS connectivity is vulnerable to environmental factors.

Maturation of Electrophysiological Properties

Enteric neurons are usually characterized into two classes. One group fires action potentials with short repolarizations (S-type), while the other group of neurons fires action potentials with long after-hyperpolarization potentials (AH-type). S-type myenteric neurons are typically uniaxonal while AH-type neurons exhibit DII morphology (Foong et al. 2012; Nurgali et al. 2004).

The electrophysiological properties of early postnatal myenteric neurons have been examined only very recently. At birth, the two adult-like classes of myenteric neurons are present, with distinctive electrophysiological and corresponding morphological properties, but these properties are not yet mature (Foong et al. 2012). Furthermore, the two classes of neurons develop asynchronously. S-type neurons seem to mature electrophysiologically first. A prominent Ca^{2+}-mediated afterdepolarizing potential is observed in DII neurons at P0 and P10 that is significantly larger than that recorded in their adult counterparts (Foong et al. 2012). In other parts of the nervous system, neuronal activity and increased intracellular calcium concentration affect development (Young et al. 2014), however, the mechanisms regulating the postnatal maturation of enteric neurons remain to be identified.

Maturation of Synaptic Profile

Adult enteric neurons receive a variety of synaptic inputs including fast and slow excitatory postsynaptic potentials (EPSPs), and inhibitory postsynaptic potentials (IPSPs) (Foong et al. 2012, 2014; Nurgali et al. 2004; Wong et al. 2008). The vast majority of enteric neurons exhibit fast EPSPs and only the neurotransmitters mediating fast transmission have been investigated in mice to date. Acetylcholine activating nicotinic receptors is the main mode of fast transmission, but ATP acting via P2X receptors is also involved (Foong et al. 2012, 2014; Nurgali et al. 2004; Wong et al. 2008).

At P0, S-type neurons exhibit fast EPSPs, and by P10, like adults, these fast EPSPs are mediated by nicotinic receptors (Foong et al. 2012). At P10-11, there is still few, if any, slow EPSPS or IPSPs (Foong et al. 2012). Thus, in combination with the significant growth of gut size, maturation of dendritic structure and axonal projection that occurs from P10 to adulthood, it is evident that the formation and maturation of synapses occurs over a protracted period of time.

Little is known about the regulation of synaptogenesis in the ENS. However, loss of *Hipk2* and the absence of gut microbiota reduced intraganglionic synapses and nerve density respectively in postnatal myenteric plexus (Chalazonitis et al. 2011; Collins et al. 2014).

Maturation of Neurally-Mediated Motility Patterns

Motility in the mature intestine involves interactions between enteric neurons, interstitial cells of Cajal and intrinsic smooth muscle (Huizinga and Lammers 2009). Motility patterns differ in different regions of the gut, but mainly involves a combination of mixing behaviours to facilitate digestion and absorption of nutrients, and propulsion, to push the gut contents along the GIT (Bornstein et al. 2004; Burns et al. 2009; Costa et al. 2013; Huizinga and Lammers 2009).

It is essential for the duodenum to be functional by birth in order for the newborn to digest and absorb milk nutrients. The first propagating motility patterns detected in the gut are non-neuronal. In the duodenum, neurally-mediated motility commences just prior to birth, at E18.5 (Roberts et al. 2010). Conversely, neurally-mediated motility in the colon commences several days after birth. By P6, the neural circuit underlying colonic migrating motor complexes (CMMCs) is present; however, CMMCs are only induced by blocking NOS (Roberts et al. 2007). In E18.5 and P0 duodenum, inhibition of nNOS induced or increases the frequency of contraction complexes (Roberts et al. 2010). In addition, during development the circular muscle appears to receive innervation from nNOS+ fibres prior to ChAT+ fibres. The number and density of ChAT+ fibres increase significantly after birth (Hao et al. 2013b; Roberts et al. 2007). Thus overall these pharmacological and immunohistochemical studies suggest that smooth muscle cells are tonically inhibited by NO until sufficient ChAT+ fibres are present to induce contractions. However, a re-examination of the number and density of ChAT+ fibres during development is now necessary since ChAT+ neurons are recently reported to achieve adult proportions by E13 in the small intestine and by P0 in the proximal colon (Erickson et al. 2014). In both duodenum and colon, motility patterns seem to be mature by P10 (Hao et al. 2013b; Roberts et al. 2007, 2010).

Some genetic and environmental factors that affect postnatal development of neurons also elicit profound effects on gut motility. Indeed, abnormal numbers of neuronal subtypes and nerve density due to impaired trophic factor signalling, absence of gut microbiota and prolonged ingestion of high fat diets all led to altered gut motility (Baudry et al. 2012; Chalazonitis et al. 2011; Collins et al. 2014; Mushref and Srinivasan 2013).

Postnatal Gut and Therapy

Understanding how the ENS develops postnatally could assist in developing therapies for gut motility disorders. Defects in ENS development result in pediatric motility disorders including Hirschprung disease, a congenital disorder affecting 1:4000–5000 newborns, in which enteric neurons are absent from the distal gut. These patients suffer from severe constipation and require surgery. Current therapies for Hirschprung patients and other gut motility disorders are life-saving, but remain inadequate in addressing other complications including dysmotility and incontinence that can last for years (Hotta et al. 2009).

A potential treatment plan would be to restore the ENS at the aganglionic region of Hirschprung patients. In recent years, the idea of transplanting progenitor cells as therapy for gut motility disorders has generated a lot of interest (Burns and Thapar 2014). Diagnosis of enteric neuropathies typically occurs after birth, thus cell therapy has to be administered postnatally. In fact, it is ideal for progenitor cells to be obtained from a "healthy" gut region of the patients themselves. Neural progenitors can be obtained the postnatal mouse and human gut and that their developmental potential appear to be similar to those obtained from fetal gut (Bondurand et al. 2003; Hotta et al. 2013; Metzger et al. 2009). Furthermore, it has recently been shown that after transplantation into the colon of postnatal mice, ENS progenitors isolated from postnatal gut successfully developed into neurons with the appropriate enteric neuronal subtype properties, including electrophysiological properties (Hotta et al. 2013). While this shows that cell therapy is a promising strategy, there are still significant obstacles to be overcome. In particular, these progenitors need to be able to generate a functional ENS over the patient's aganglionic region (average length 7–10 cm) (Burns and Thapar 2014).

External factors such as diet and gut microbiota found to affect postnatal development could potentially be manipulated to prevent or help rescue the defected ENS. This strategy can be applied on its own or coupled with cell therapies to improve treatment of enteric neuropathies.

Conclusions

Substantial development of enteric neuronal properties continues postnatally. During this time the ENS is vulnerable to factors present in the extra uterine environment. Interaction between the environment and the yet to be identified intrinsic factors regulating ENS maturation will be a fertile source of research in the future.

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Chapter 14
G Protein-Coupled Receptor Trafficking and Signalling in the Enteric Nervous System: The Past, Present and Future

Daniel P. Poole and Nigel W. Bunnett

Introduction

G protein-coupled receptors (GPCRs) enable cells to detect and respond to changes in their extracellular environment. With over 800 members, the GPCR family includes receptors for a diverse range of agonists including olfactants, neurotransmitters and hormones. Importantly, GPCRs represent a major therapeutic target, with approximately 50 % of all current drugs acting at some aspect of GPCR signalling (Audet and Bouvier 2008). GPCRs are widely expressed by all cell types in the gastrointestinal (GI) tract and are major regulators of every aspect of gut function. Many GPCRs are internalised upon activation, and this represents one of the mechanisms through which G protein-signalling is terminated. The latency between the endocytosis of GPCRs and their recycling and resensitization is a major determinant of the cell's ability to respond to subsequent exposure to agonists.

This article focuses on GPCR signalling and trafficking in the enteric nervous system (ENS), with an emphasis on key studies from the past, the current state of the field and potential areas for future investigation.

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Past

The mid to late 1990s was a period in which extensive characterisation of GPCR endocytosis in myenteric neurons was conducted, with most research focussing on the neurokinin 1 receptor (NK_1R). It was during this time that the first evidence for internalisation of GPCRs in enteric neurons was reported, demonstrated using uptake of fluorescently-labelled agonist (Bunnett et al. 1995). Subsequent antibody-based studies identified significant endocytosis of NK_1R in both myenteric and submucosal neurons following exposure to *C. difficile*-derived Toxin A (Mantyh et al. 1996), which stimulated SP release from extrinsic primary afferent nerves. This study demonstrated that receptor endocytosis could be used to measure and localise release of proinflammatory neuropeptides in digestive disease, and that interaction between the extrinsic and intrinsic innervation of the GI tract mediated the effects of Toxin A. Sternini et al. (1996) examined endocytosis of the therapeutically-relevant mu opioid receptor (MOR), which mediates the inhibitory effects of morphine on gut motility. In this study, MOR was internalised in myenteric neurons in a ligand-dependent manner. Importantly morphine was found not to simulate endocytosis of MOR. In addition, the physiologically-relevant reflex release of neurotransmitter in response to mechanical stimulation of the mucosa was demonstrated using NK_1R endocytosis as a biomarker (Southwell et al. 1998). An example of agonist-induced receptor endocytosis is presented in Fig. 14.1.

The basic mechanisms through which GPCRs are internalised and the role of endocytosis in regulating GPCR desensitization were also delineated during this period. A series of elegant studies characterized agonist-induced recruitment of key

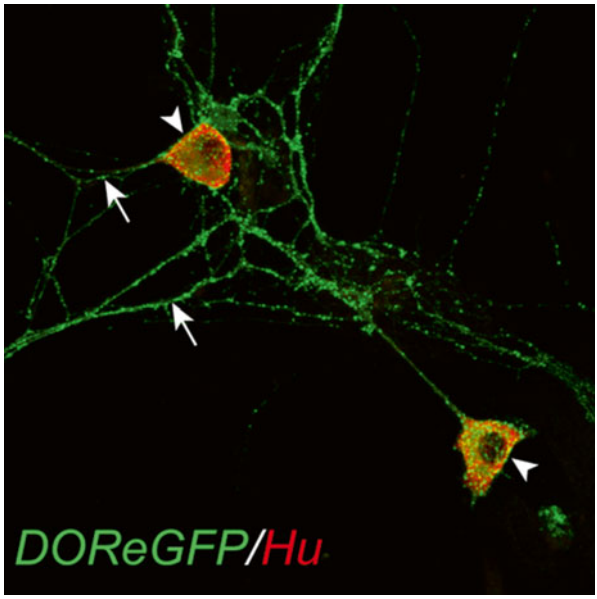

Fig. 14.1 Endocytosis of the eGFP-tagged delta opioid receptor (DOReGFP) in cultured myenteric neurons following treatment with the DOR agonist SNC80 (100 nM, 30 min). DOR endocytosis was observed in both the soma (*arrow heads*) and neurites (*arrows*) of these neurons

molecules, including beta arrestin, clathrin, and GPCR-related kinases (GRKs) in myenteric neurons and demonstrated that the acidic environment of the endosome was essential for effective recycling of internalised NK_1R (Grady et al. 1996; McConalogue et al. 1998).

Subsequent studies examined the GPCR-dependent activation of downstream signalling kinases at the level of the individual neuron using immunofluorescence-based techniques. The coupling of GPCRs, including the neurokinin 3 receptor and protease activated receptor 2, to protein kinases C (PKC) and D was characterised (Poole et al. 2007, 2008). A key role for PKC as a mediator of inflammation-associated hyperexcitability of enteric neurons was defined (Nguyen et al. 2005; Poole et al. 2007). These studies identified PKC isoform specific effects and also demonstrated distinct spatiotemporal dynamics of PKC and PKD translocation and activation.

Present

Studies of GPCR localisation and function in the ENS are still continuing. For example, the distribution and role of TGR5, a novel membrane associated bile acid receptor, has recently been characterised in the mouse ENS (Poole et al. 2010; Alemi et al. 2013). The basic examination of GPCR trafficking has shifted focus in line with current trends in this field.

There have been a number of major conceptual advances in the area of GPCR signalling. There is a greater appreciation that although endocytosis effectively prevents further G protein-dependent signalling, this internalisation does not necessarily represent a termination of either cellular signalling or the functional consequences of GPCR activation. It is now established that the internalised, activated GPCR can continue to signal from within endosomal signalling complexes, or ‘signalosomes’, through a G protein-independent, beta arrestin-dependent mechanism (Murphy et al. 2009) (Fig. 14.2).

The physiological effects of cell surface (G protein-dependent) and endosomal (beta arrestin-dependent) signalling can be distinctly different, and much interest has been given to defining these differences. Furthermore, the development of ligands that act at the same receptor, but preferentially favour different signalling pathways within the same cell (biased ligands) and the use of beta arrestin-deficient mice have provided the tools with which to examine the functional significance of endosomal signalling in the ENS. With respect to GI physiology, a major focus has been in the area of opiate analgesics. A limiting side-effect of current opiate-based drugs is the development of opioid-induced bowel dysfunction, of which chronic constipation is the most-debilitating symptom. Unlike other side-effects, constipation is sustained throughout the treatment period and does not diminish with the development of tolerance. The inhibitory effects of opiates on gut motility and secretion are primarily mediated through actions on MOR expressed by enteric neurons (Wood and Galligan 2004). Studies utilising beta arrestin 2 deficient mice have

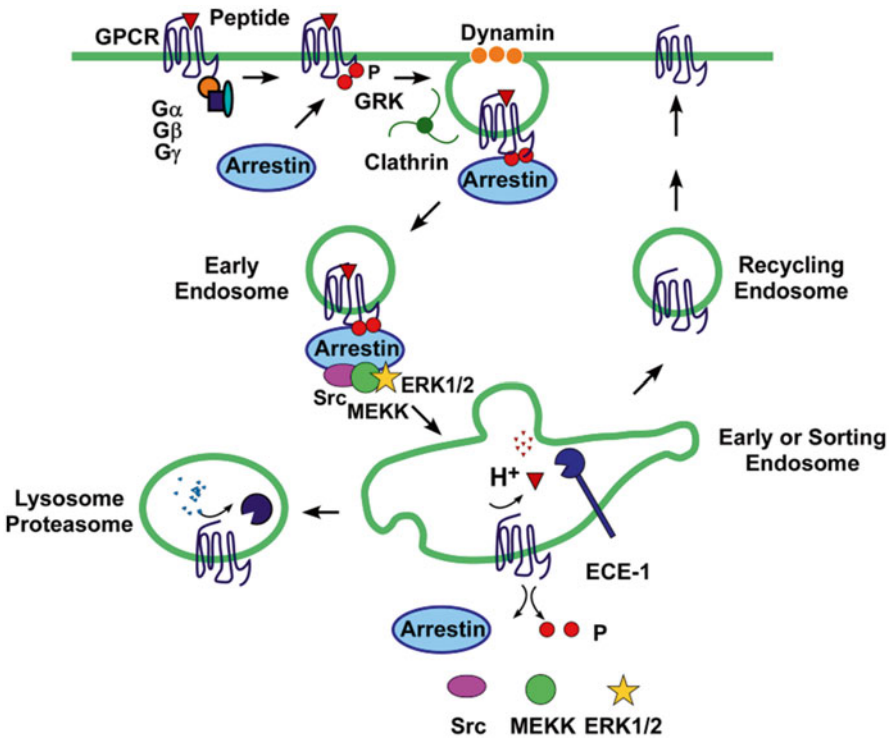

Fig. 14.2 Diagram summarising agonist-dependent regulation of GPCR signalling and trafficking. Modified from Murphy et al. (2009)

demonstrated that they exhibit greater analgesia in response to morphine, while constipatory effects are diminished relative to wild type mice (Bohn et al. 2000; Raehal et al. 2005). This has since been confirmed in the isolated colon (Kang et al. 2012; Maguma et al. 2012). The translation of these findings to the development of therapeutic biased ligands has led to some success, including the identification and characterisation of TRV130, which may preferentially signal through a G protein-dependent mechanism (DeWire et al. 2013). It should be noted that despite the obvious therapeutic potential, almost nothing is known of the effects of beta arrestin 2 deletion or of biased ligands on mu opioid receptor signalling and trafficking in the ENS.

The functional significance of endosomal signalling in the GI tract remains elusive, with the limited published research focusing on the ENS itself. All studies of endocytic signalling in the ENS have examined the role that the metalloendopeptidase endothelin converting enzyme 1 (ECE-1) plays in determining the duration of endosomal signalling. Experiments utilising heterologous systems have determined that ECE-1 cleaves neuropeptides within the acidified environment of early endosomes to remove bound agonist, leading to destabilisation of the signalosome. This serves two functions. Firstly, the agonist-free receptor is now capable of recycling back to

the cell surface for resensitization, thereby enabling subsequent responses by the neuron to agonist stimulation. Secondly, the degradation and effective removal of the bound ligand serves as a terminator of endosomal signalling. Thus, ECE-1 is a major determinant of the type and duration of GPCR signalling. Immunoreactivity and mRNA for ECE-1 is expressed by the majority of enteric neurons as well as other cell types in the intestine (Cottrell et al. 2009; Pelayo et al. 2011). The selective inhibition of ECE-1 activity has defined a physiological role for ECE-1 in controlling recycling and resensitization of the neurokinin 1 receptor in myenteric neurons (Pelayo et al. 2011). Moreover, ECE-1 controls the duration of MAPK signalling and may play a neuroprotective role in the ENS (Cottrell et al. 2009; Pelayo et al. 2011). A similar role for ECE-1 in regulating recycling of the somatostatin 2A receptor in myenteric neurons has recently been reported (Zhao et al. 2013).

Future

At present we have a good understanding of the fundamental processes underlying agonist-evoked GPCR signalling and trafficking in the ENS. However, almost nothing is known of the significance of receptor endocytosis to basic gut function beyond the initial termination of G protein-dependent signalling. Furthermore, the dysregulation of signalling under pathophysiological conditions, where there may be chronic exposure of GPCRs to agonists, is largely unexplored. This is of direct significance to conditions including chronic inflammation as well as to pharmaceutically-mediated opioid-induced bowel dysfunction.

There is now a greater appreciation that the spatiotemporal dynamics of GPCR signalling are highly-dependent on the types of ligands involved. This has been suggested by the ECE-1 studies outlined above, where non-peptidic or ECE-1-resistant agonists exhibited markedly different receptor trafficking and beta arrestin recruitment profiles relative to ECE-1 sensitive peptide agonists (Zhao et al. 2013). The development of novel *F*luorescence/*F*örster *R*esonance *E*nergy *T*ransfer (FRET) based probes (Harvey et al. 2008) has enabled the examination of cellular signalling at high resolution. Moreover, targeting of these biosensors to distinct subcellular compartments has allowed the spatiotemporal characterisation of GPCR signalling, as we have demonstrated for DOR signalling in dorsal root ganglion neurons (Poole et al. 2015). However, there is still great difficulty in introducing recombinant DNA or siRNA into enteric neurons. This technical limitation precludes the expression of FRET biosensors in enteric neurons at this stage.

The investigation of the functional selectivity of endogenous ligands is another research area of interest. The key questions remain as to why so many endogenous ligands capable of activating the same GPCR exist, and what the physiological basis underlying such diversity may be. For example SST-14, SST-28, thrittene and cortistatin all activate SSTR2A, and a diverse array of opioid ligands is expressed in the gut (Thompson et al. 2015). The tools and models are now available to examine whether there is functional selectivity across the different endogenous ligands,

although their direct application to studies of enteric neurons and of GI physiology will be challenging.

The use of novel inhibitors of dynamin and clathrin-dependent endocytosis and the identification of non-internalising agonists should enable a systematic investigation into the physiological significance of endocytosis beyond the fundamental role in controlling cell surface GPCR expression.

GPCR trafficking and signalling may be altered in disease states. It is known that enhanced neuropeptide release during inflammation stimulates receptor endocytosis in target cells (Mantyh et al. 1996; Cattaruzza et al. 2013). Research in this area has almost exclusively focused on the NK_1R due to its key role in pain transmission and neurogenic inflammation. Questions remain regarding the effect of chronic and acute inflammation on receptor trafficking and signalling. Recent work by our group suggests that there are significant delays in NK_1R recycling in myenteric neurons during colitis (Poole, 2015). It is possible that GPCR trafficking may also be altered in other conditions where there is chronic exposure of receptors to agonists, such as occurs in opioid tolerance. For example, it has recently been demonstrated that there are clear alterations in MOR internalization in myenteric neurons from opiate-tolerant guinea pigs that are associated with changes in dynamin expression (Patierno et al. 2011).

Closing Remarks

The translation of experimental findings from laboratory animals to human tissues is essential. There has been increasing utilisation of human tissues for studies of motility and in Ca^{2+} imaging of neuronal activity. In contrast, there have been only limited studies of GPCR expression, and no studies that we are aware of that have systematically examined receptor trafficking in the human ENS. The application of state-of-the-art molecular and cellular approaches to enteric neuroscience is likely to lead to great progress in this field.

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Chapter 15
The Intrinsic Reflex Circuitry of the Inflamed Colon

Gary M. Mawe and Keith A. Sharkey

In 1899, Bayliss and Starling determined that the innervation of the intestines differs from that of other organs. They found that local neuronal networks are capable of generating reflex responses without the involvement of the central nervous system (Bayliss and Starling 1899). Once this unique feature of the enteric nervous system (ENS) was identified, it took roughly a century for enteric neurobiologists to accomplish the task of being able to identify the components of this "intrinsic neural mechanism", including intrinsic primary afferent neuron, ascending and descending interneuron, and excitatory and inhibitory motor neurons (Bayliss and Starling 1899). Once this was possible, we and others began to investigate the intrinsic circuitry of the colon and ileum to systematically determine the cellular mechanisms that explain the changes in motility and secretion that occur in intestinal inflammation. We wanted to establish what changes occur in the enteric neural circuitry, where they occur, the mechanisms responsible for these changes, and how these changes in the neural circuitry impact intestinal function.

Serotonin Availability Is Increased

In the epithelial layer of the intestines, serotonin (5-hydroxytryptamine; 5-HT) is synthesized by enterochromaffin (EC) cells and released in a regulated manner in response to mechanical and chemical stimuli (Mawe and Hoffman 2013). Locally released

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5-HT acts on 5-HT receptors located on nearby intrinsic and extrinsic afferent nerve fibers in the lamina propria. The serotonergic signaling is terminated by the action of the serotonin-selective reuptake transporter (SERT), which moves 5-HT into epithelial cells, all of which express SERT. Because of the importance of mucosal 5-HT signaling in the activation of intrinsic and extrinsic reflexes, many studies have been carried out to evaluate whether the elements of 5-HT signaling are altered in response to inflammation (Costedio et al. 2007). In IBD and related animal models, inflammation is centered in the mucosal layer of the gut; therefore, it was not too surprising to discover that mucosal 5-HT signaling is altered in these conditions. In animal models of inflammation, there tends to be an increase in 5-HT content and numbers of EC cells, whereas in the more chronic condition of human IBD, 5-HT levels appear to decrease (Costedio et al. 2007), but release is unchanged. In both animal and human samples, there is a decease in expression of the SERT, which is responsible for removal of 5-HT from the interstitial space following release from EC cells. Based on studies of epithelial cell lines, it appears that this involves interferon-gamma and tumor necrosis factor-alpha.

Intrinsic Sensory Neurons Are Hyperexcitable

The AH neurons of the submucosal and myenteric plexuses project to the lamina propria of the mucosal layer, where inflammatory responses are centered in IBD. A consistent feature of inflammation-induced neuroplasticity in the inflamed gut is that AH neurons are hyperexcitable. This has been demonstrated in allergen models (Frieling et al. 1994; Liu et al. 2003) and *T. spiralis* enteritis (Palmer et al. 1998; Chen et al. 2007), where a decrease in IK_{Ca} appears to be responsible, based on the depolarization and decrease in input resistance that is observed. AH neurons are also hyperexcitable in the myenteric (Linden et al. 2003) and submucosal (Lomax et al. 2005) plexuses of the TNBS inflamed colon. In the myenteric plexus, an increase in the hyperpolarization-activated cation current (I_h) is a major factor (Linden et al. 2003), It is important to note that spontaneous activity is increased in these neurons. Under basal conditions, few AH neurons exhibit spontaneous activity, whereas about 50 % generate spontaneous action potentials in the TNBS inflamed colon. COX-2 activation is a critical component of the inflammation-induced increase in excitability (Linden et al. 2004). In these neurons, chronic exposure to prostaglandin E2 causes a similar increase in excitability (Manning et al. 2002).

Interneuronal Synaptic Transmission Is Enhanced

Evoked synaptic potentials are enhanced in S neurons of the submucosal and myenteric plexus in colitis (Linden et al. 2003; Lomax et al. 2005). In the submucosal plexus, this involves a shift from purely nicotinic events, to synaptic events that also

include purinergic and/or serotonergic contributions (Lomax et al. 2005). In the myenteric plexus, synaptic pharmacology is not altered, but there seems to be an increase in the readily releasable pool of neurotransmitters (Krauter et al. 2007a). Detection of EPSP activity in AH neurons is also increased in TNBS colitis (Linden et al. 2003) and T. spiralis enteritis (Palmer et al. 1998).

Purinergic Neuromuscular Transmission Is Attenuated

The ENS regulates smooth muscle tone in the muscularis in the form of excitatory and inhibitory junction potentials that are mediated by excitatory and inhibitory neurons, respectively. Excitatory neurons release acetylcholine and substance P to transmit their signals whereas inhibitory neurons use nitric oxide and purines. Evoked and spontaneous excitatory junction potentials are not altered in the inflamed colon, but inhibitory junction potentials (IJPs) are significantly reduced in both guinea pig TNBS colitis and murine DSS colitis (Strong et al. 2010; Roberts et al. 2013). Pharmacological analysis of IJPs revealed that the nitrergic component of the IJP is unchanged, whereas the purinergic IJP is attenuated in colitis (Strong et al. 2010). Given that the same nerve fibers are likely to mediate both nitrergic and purinergic IJPs it was hard to imagine how one would be affected without the other. Recently, we demonstrated that the reduction in purinergic neurotransmission is due to an oxidative stress-mediated reduction in mitochondrial purine synthesis in nerve terminals of the inflamed colon (Roberts et al. 2013). The purinergic neuromuscular transmission deficit is prevented by treating animals with a free radical scavenger during the development of colitis.

Neurons Die

In TNBS colitis, inflammation is associated with a permanent loss of about 20 % of the neurons (Linden et al. 2005). In this model, it appears that the loss of neurons in indiscriminate because the loss of neurons is comparable amongst different neuronal subpopulations identified by immunohistochemistry. This neuronal loss is an early event in the inflammatory process (Linden et al. 2005), and it involves activation of a neuronal signaling complex composed of P2X7 receptors, pannexin-1 channels, the Asc adaptor protein and caspases (Gulbransen et al. 2012).

Inflammation-Induced Dsymotility Involves Neuroplasticity

Propulsive motility can be most readily be evaluated in the ex vivo guinea pig distal colon preparation (Hoffman et al. 2010). While in this assay, propulsive motility is typically very linear, movement of fecal pellets is obstructed or halted in inflamed

regions of the guinea pig distal colon (Strong et al. 2010). With the knowledge that AH neurons are hyperexcitable, synaptic activity is facilitated, and purinergic neuromuscular transmission is attenuated in the inflamed colon, studies have been carried out to evaluate propulsive motility when these changes are pharmacologically replicated in normal tissue or reversed in inflamed preparations. In normal preparations, the velocity of propulsive motility is significantly reduced IK_{Ca} is inhibited to increase excitability of AH neurons, and further reduced when synaptic transmission is augmented by a 5-HT4 receptor agonist (Hoffman et al. 2011). In addition, propulsive motility is slowed by an antagonist of the P2Y1 receptor, which mediates the IJP (Strong et al. 2010). Conversely, propulsive motility is significantly improved in inflamed preparations when the hyperexcitability of AH neurons is dampened by inhibition of I_h (Hoffman et al. 2011), and in colons from animals treated with a free radical scavenger (Roberts et al. 2013). When colons from TNBS-inflamed animals that have been given a free radical scavenger in their drinking water are treated with an I_h blocker, the rate of propulsive motility is restored to the control level (Roberts et al. 2013).

Inflammation-Induced Neuroplasticity Persists

An important feature of inflammation-induced neuroplasticity in the gut is that changes persist long after complete recovery from inflammation. Changes that persist include AH neuron hyperexcitability, synaptic facilitation, and disrupted propulsive motility (Krauter et al. 2007b; Lomax et al. 2007). This is important because it underscores the likelihood that persistent inflammation-induced changes in the neuronal circuitry of the gut could be a contributing factor in functional GI disorders.

In summary, understanding the cellular mechanisms of intestinal inflammation-induced neural plasticity has provided new insights into the control of gut function in health and disease.

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Chapter 16
Integrated Neural and Endocrine Control of Gastrointestinal Function

John B. Furness

Organisation of Nerve Pathways

The gastrointestinal tract differs from all other peripheral organs in that it has an extensive intrinsic nervous system, the ENS, that can control functions of the intestine even when it is completely separated from the CNS. The ENS, however, is not autonomous. Indeed, neuronal control of gastrointestinal function involves interactions between local enteric reflexes, reflexes that pass through sympathetic ganglia and reflexes that pass to and from the gut through the CNS (Fig. 16.1). Conventional textbook descriptions of the autonomic nervous system depict efferent pathways from the CNS as two neurons in series, a preganglionic and a postganglionic neuron, and depict sensory information flowing from the periphery to the CNS through spinal and cranial primary afferent neurons. The organisation of the ENS and of neuronal pathways to and from the intestine does not follow these conventional concepts of the organisation of the nervous system (Fig. 16.1). For example, axons of neurons with cell bodies in the ENS (intestinofugal neurons) project to sympathetic ganglia, the pancreas, gallbladder and trachea, and to the spinal cord and brain stem (Furness 2012). Vagal pathways to the gastrointestinal tract impinge on enteric neurons that are themselves involved in intrinsic reflexes. In the stomach, the target neurons include gastric interneurons and motor neurons. To call the vagal neurons 'preganglionic' is to suggest that they are involved in conventional preganglionic/postganglionic pathways to control the digestive tract. It is preferable to call the

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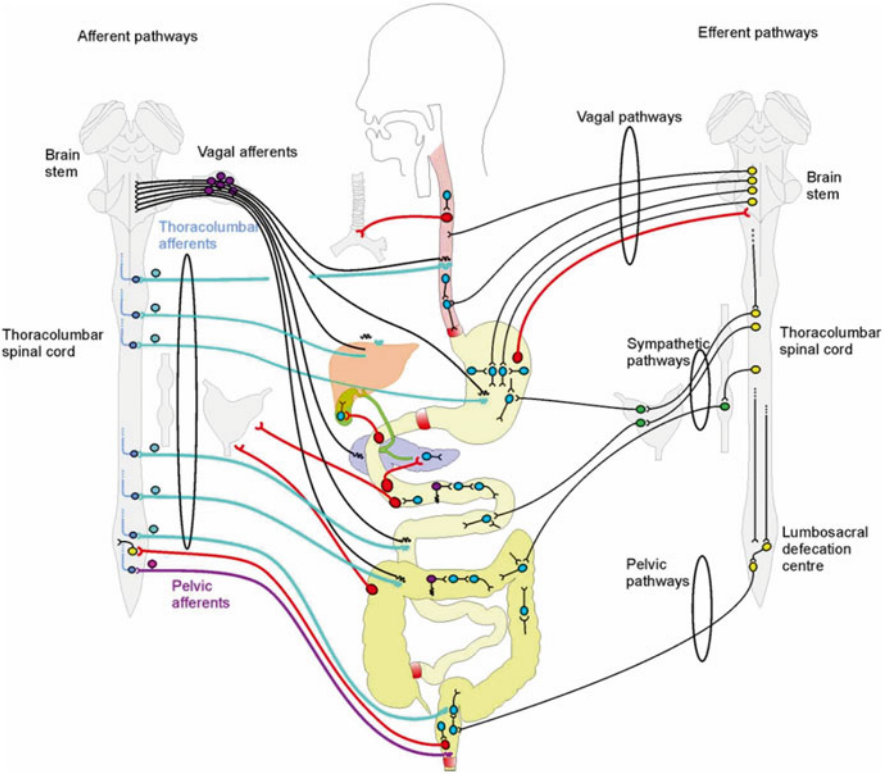

Fig. 16.1 The innervation of the gastrointestinal tract. Connections that carry signals from the ENS to other organs and the CNS are at the left, and pathways from the CNS are at the right. The small and large intestines (*middle of figure*) contain full ENS reflex circuits (motor neurons and interneurons in *blue*, sensory neurons in *purple*). Intestinofugal neurons (*red*) project from the gut, to the CNS, sympathetic ganglia, gallbladder, pancreas and trachea. Some neurons in sympathetic prevertebral ganglia (*green* neurons) receive both CNS and ENS inputs. Sensory information goes both to the ENS, via intrinsic primary afferent (sensory) neurons (*purple*) and to the CNS via extrinsic primary afferent neurons. Cervical afferents connect the esophagus to the cervical spinal cord. Pathways from the CNS reach the ENS and gastrointestinal effector tissues through vagal, sympathetic and pelvic pathways (*right of figure*). Vagal medullary and pelvic spinal outflows include pre-enteric neurons (ending in enteric ganglia) and most gut-projecting sympathetic neurons with cell bodies in PVG are also pre-enteric neurons. Modified from Furness (2012)

neurons emanating from the lower brain stem pre-enteric. The same is true of neurons in the pelvic pathways to the distal gut. In sympathetic pathways to the intestine, what would conventionally be called postganglionic neurons impinge on enteric neurons within enteric circuits. The postganglionic neurons are thus preganglionic (or perhaps interneurons)! They can also be referred to as pre-enteric neurons.

ENS and CNS Integration, Regional Differences in Motility Control

The muscle of the gastrointestinal tract directs propulsion, mixing of contents, reservoir capacity (e.g., in the stomach) and expulsion of pathogens and noxious chemicals. The relative roles of the CNS and ENS for co-ordinated muscle function differ with the region of the gastrointestinal tract.

Movements of the esophagus are largely determined by neural pattern generators in the lower brain stem. Although there is a rather extensive ganglionated myenteric plexus in both the striated muscle and smooth muscle parts of the esophagus (Neuhuber et al. 2006), the CNS provides the primary control of propulsion. Indeed, if both vagi are severed the upper part of the esophagus is paralysed and food is no longer propelled (Cannon 1907; Ingelfinger 1958). The motor pathways from the CNS (nucleus ambiguus) to the striated muscle part of the esophagus are direct, they do not connect through or even form collaterals in enteric ganglia (Neuhuber et al. 1998; Wu et al. 2003).

The CNS, through vagal pathways from the lower brain stem and vagal esophagogastric and gastro-gastric reflexes, has a major role in monitoring the state of the stomach and, in turn, controlling its volume and strengths of contractions (Hasler 2003). An effect of truncal vagotomy is dysregulation of gastric emptying; this is relieved surgically by pyloromyotomy (Seymour and Andersen 1999).

The CNS has significant control of defecation, through the defecation centres in the lumbosacral spinal cord (de Groat et al. 1981). Cutting the pelvic nerves, or otherwise interrupting connections from the CNS to the colorectum, removes the pathways that control defecation, and individuals suffer from constipation and overflow incontinence due to their not being able to empty the bowel voluntarily (Lynch et al. 2001). About 40–50 % of people with spinal cord injury, whose voluntary bowel emptying is lost because the spinal defecation centres are isolated from cortical control, lose control of the colorectum (Widerström-Noga et al. 1999).

The ENS dominates the control of the motility of the small and large intestines (Furness 2006), and the involvement of the CNS in the control of movement of the small bowel and the proximal and mid-colon appears to be minor; there is little apparent change in motility following vagal, sympathetic or pelvic nerve section. In the case of the sympathetic influence on small intestine motility, this appears to have only a minor role in normal healthy conditions (Furness and Costa 1974). Conversely, as detailed below, loss of ENS function can be devastating.

Importance of the ENS

The ENS has multiple roles: determining the patterns of movement of the gastrointestinal tract; controlling gastric acid secretion; regulating movement of fluid across the lining epithelium; changing local blood flow; modifying nutrient handling; and interacting with the immune and endocrine systems of the gut. The ENS

contributes, along with enteric glial cells, to the maintenance of the integrity of the epithelial barrier between the gut lumen and cells and tissues within the gut wall (Toumi et al. 2003; Savidge et al. 2007). The importance of the ENS is highlighted by the range of enteric disorders that occur if it fails.

In Hirschsprung disease, the ganglia of the ENS fail to develop in the distal bowel, although all other tissue components are intact and functional (Swenson 2002). Propulsion fails in the aganglionic bowel, and the newborn child will almost always die if this region is not removed. Degeneration of colonic enteric neurons in Chagas disease, which is precipitated by infection with the protozoan *Trypanosoma cruzi*, also causes colorectal propulsion to fail and megacolon develops in adults, similar to that associated with Hirschsprung disease in children (Matsuda et al. 2009). An underlying occult tumour (that is, paraneoplastic syndrome) can also cause gut propulsion to fail, often with evidence of bowel dilatation (De Giorgio et al. 2004; Knowles et al. 2010). Other enteric neuropathies that have severe effects on the motor functions of the digestive tract include esophageal achalasia, gastroparesis, hypertrophic pyloric stenosis and intestinal pseudo-obstruction (Di Nardo et al. 2008). By contrast, removal of neural connection between the CNS and the stomach, small or large intestine causes minor pathology.

The control of fluid movement between the intestinal lumen and body fluid compartments, as discussed below, is also subject to pathological, life-threatening, influences. Fluid movement is controlled in part by enteric secretomotor neurons that are abnormally activated by certain infective agents or their products. These pathogens or products, including *Vibrio cholerae* (which secretes cholera toxin) and rotavirus (which secretes the enterotoxin NSP4), act directly on secretomotor neurons and mucosal epithelial cells (Lundgren 2002), triggering hypersecretion and subsequent diarrhea. Infectious diarrhea causes approximately 1.5 million deaths a year, primarily in underdeveloped tropical countries (Podewils et al. 2004).

The Gut Enteroendocrine System

The endocrine cells of the gut (EEC) are scattered as single cells in the mucosa of the stomach, small and large intestines. EEC of the pancreas (and the small populations in the biliary tracts) are not reviewed here. The EEC are usually divided into 12 major cell types (Table 16.1), coded by a single letter and based on their content of a single hormone, except for L cells that are defined by containing PYY and products of the proglucagon gene (GLP-1, GLP-2 and oxyntomodulin).

The single letter, single hormone classification is being rapidly superseded; it is now clear that the classification by letter code and associated hormone is deeply flawed (Brubaker 2012; Egerod et al. 2012; Habib et al. 2012; Engelstoft et al. 2013; Sykaras et al. 2014). Recent investigations have analysed EEC isolated from transgenic mice in which a fluorescence marker is under the control of a specific promoter. The fluorescent reporters were used to separate cells by FACS, following which expression of other genes was investigated. Using a fluorescent marker under

Table 16.1 The traditional, but outdated, classification of enteroendocrine cells of the mammalian gastrointestinal tract

Letter code for EEC	Traditional defining hormones	Recently documented other hormones/ comments	Classical locations
A (X-like) cells and subtypes	Ghrelin	Nesfatin-1. Duodenal ghrelin cells contain CCK	Stomach
ECL cell	Histamine		Stomach
G cells	Gastrin		Stomach
D cells	Somatostatin	Somatostatin cells appear to belong to a different lineage than CCK expressing cells that also express GIP, GLP, secretin and neurotensin, but there is colocalisation of somatostatin and GIP expression	Stomach, small intestine (and pancreas)
EC (enterochromaffin) cells	5HT	5HT is contained in sub-groups of CCK, GIP, GLP and secretin cells. Defined by the chromaffin reaction, these would all be EC cells	Stomach, small and large intestine
I cells	CCK	5HT is in a high proportion of CCK cells. Proportions of CCK cells also contain GIP, PYY and ghrelin	Proximal small intestine
K	GIP	GIP is commonly co-localised with GLP, PYY and CCK	Proximal small intestine
L cells, and subtypes	GLP-1, GLP-2, PYY, Oxyntomodulin	All duodenal proglucagon cells expressed CCK. Some ileal and colonic cells contain GIP	Distal small intestine, colon
M cells	Motilin	Motilin cells occur in some species (e.g., pig, human), but not in others (e.g., mouse, rat)	Small intestine
N cells	Neurotensin	CCK cells are enriched for neurotensin	Small and large intestine
P cells	Leptin	Gastric chief cells may also contain leptin	Stomach
S cells	Secretin	Secretin and CCK are colocalised in some EEC. Ablation of secretin cells causes substantial loss of 5HT, CCK, GLP, somatostatin and PYY cells	Proximal small intestine

the control of the promoter for proglucagon (a classical marker of L cells), subpopulations of proglucagon reporter cells from the duodenum were found to express transcripts for CCK, GLP, PYY, secretin and neurotensin (Habib et al. 2012). Quantitatively, 100 % expressed CCK, which in the absence of other information, would classify them as I cells, and 15 % contained the K cell marker, GIP.

A study of EEC isolated by fluorescence under the control of the promotor for GIP (classical marker of K cells), revealed that there are gradients of colocalisation of hormonal markers (Habib et al. 2012). Subpopulations of GIP reporter cells in

the proximal small intestine expressed genes for CCK, secretin, somatostatin, proglucagon, PYY and islet amyloid polypeptide. In other studies, in which a fluorescent protein was under CCK promotor control, CCK was found to colocalise, in different combinations, with message for GIP, secretin, GLP-1, PYY and neurotensin (Egerod et al. 2012; Sykaras et al. 2014).

Ablation studies lead to similar conclusions. Induced ablation of secretin-positive cells, in vivo, resulted in more than 80 % loss of small intestinal cells containing CCK, proglucagon derived peptides and PYY, and 30–50 % loss of cells staining for somatostatin and 5HT (Rindi et al. 1999).

Immunohistochemistry confirms overlaps that are indicated by gene expression studies. The hormones, CCK, ghrelin, GIP and PYY, all occurred in CCK gene-expressing duodenal EEC (Sykaras et al. 2014). About half of duodenal CCK cells expressed ghrelin. CCK colocalisation with 5HT is common in EEC of mice (Cho et al. 2014a). A high proportion of the gastric ghrelin containing A cells express nesfatin (Stengel et al. 2010a). In a recent immunohistochemical study in pigs and mice, we have found all combinations of GIP, GLP-1 and PYY, that is, cells containing only GIP (GIP/- cells), or only GLP-1 or PYY (GLP-1/- and PYY/- cells), cells containing each combination of two hormones (GIP/GLP-1, GIP/PYY and GLP-1/PYY cells, and cells with immunoreactivity for all three hormones (GIP/GLP-1/PYY cells). An earlier study provided evidence of cells in the ileum containing CCK, GIP and neurotensin (Roth et al. 1992).

The differences in combinations of hormones within EEC cell types, and the merging of cell types (for example the clearly demonstrated existence of cells containing both the K cell marker GIP and the L cell marker, GLP-1) means that the 12 cell type categorisation is untenable.

The hormones released from the EEC cells can act locally, on other cells including immune cells, on nerve endings, or at a distance on other organs including the pancreatic islets and the CNS (Dockray 2013; Furness et al. 2013). The effects that are exerted include changes in food intake (appetite and satiety), changes in gastric emptying and intestinal transit, release of digestive enzymes, induction of nutrient transporters, pancreatic insulin secretion, modulation of immune responses and tissue growth. The majority of the hormones act on ENS or CNS pathways. In several cases, release of hormones from EEC cells is under neuronal control. This applies to release of ghrelin, gastrin, 5HT, motilin and GLP and very likely to other EEC products.

Integration Around GLP/PYY Enteroendocrine Cells

One of the prominent features of the digestive tract is the close integration of its responses to lumenal contents, which includes interactions of the enteroendocrine, neural and tissue defence systems. This can be illustrated at the level of cells containing GLP-1, GLP-2 and PYY in the distal small intestine. The GLP/PYY cell (Fig. 16.2) has luminal receptors for fats, carbohydrates, protein

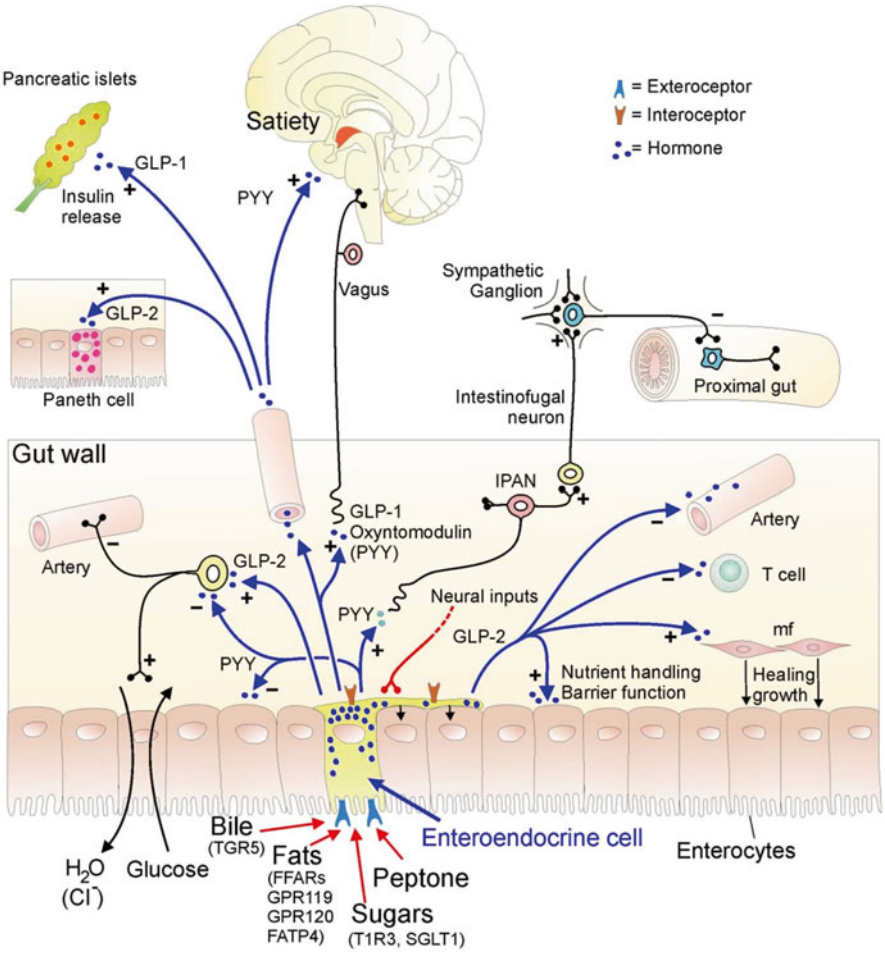

Fig. 16.2 Integration of CNS, ENS and hormonal signalling around the GLP/PYY cell. The GLP/PYY cell has exteroceptors for free fatty acids, sugars, protein fragments (peptone) and bile acids. These receptors face the contents of the intestine. The EEC releases the hormones GLP-1, GLP-2, PYY and oxyntomodulin. These hormones have actions on a range of effectors, including enterocytes, enteric neurons, vagal sensory neurons (vagus; the effect of PYY probably being indirect) and IPANs, blood vessels, lymphocytes, myofibroblasts and the hypothalamus. Downstream effects of GLP-1 on vagal afferents include slowed gastric emptying, inhibition of gastric acid secretion and satiety. In addition, vagal efferent pathways increase hormone release. GLP/PYY cells have processes that run adjacent to the basal surfaces of enterocytes. GLP/PYY cells also express interoceptors that receive signals from the internal milieu, including from neurons and hormones. *Abbreviations*: + stimulation, enhancement of function, – inhibition, including dilatation of blood vessels, *FFARs* free fatty acid receptors, *GLP* glucagon-like peptide, *IPAN* intrinsic primary afferent neuron, *mf* myofibroblast, *PYY* peptide YY. Modified from Furness et al. (2013)

metabolites and bile salts that regulate its responses (Engelstoft et al. 2008; Thomas et al. 2009; Geraedts et al. 2012). It also receives input from enteric neurons—activation of a vago-vagal reflex, and local nerve stimulation, can both enhance hormone secretion from the GLP/PYY cell (Brubaker and Anini 2003; Sandoval et al. 2013).

The products of these EEC have different, but overlapping, physiological effects. A major role of GLP-1 is as an incretin—it potently increases glucose stimulated secretion of insulin (Holst 2007). Agonists of the GLP-1 receptor on pancreatic islets or the inhibitor of the GLP-1 degrading enzyme, DPPIV, are used to treat diabetes (Kumar 2012; Russell-Jones et al. 2012). By contrast, PYY has no significant effect on basal insulin secretion and rather reduces glucose induced insulin secretion (Szecowka et al. 1983; Böttcher et al. 1989). PYY is a satiety factor, an inhibitor of gastric emptying and an inhibitor of intestinal fluid secretion (Cox 2007). GLP-1 mimics PYY in being a satiety factor and in gastric inhibition, but there is no evidence that it inhibits fluid secretion (Holst 2007). GLP-2 stimulates fluid secretion (see below). GIP, which is in some GLP/PYY cells of the proximal and mid small intestine, is also an incretin.

Many of the actions of hormones released by GLP/PYY cells are indirect, through activation of neurons. This is certainly the case for GLP-1 and PYY mediated satiety. GLP-1 is so potently inactivated by DPPIV that it does not reach sites other than vagal nerve endings in sufficient concentration to influence feeding behaviour (Holst 2007).

GLP-2 also enhances mucosal growth and repair, increases amino acid and fat absorption, increases activities of digestive enzymes, enhances intestinal barrier function (Brubaker et al. 1997; Cani et al. 2009; Hsieh et al. 2009) and has anti-inflammatory effects (Drucker et al. 1996; Rowland and Brubaker 2011). Because of these actions, GLP-2 receptor agonists have potential in the treatment of short bowel syndrome and inflammatory bowel disease (Buchman et al. 2010; Jeppesen et al. 2011).

In contrast to GLP-1 and GLP-2, PYY reduces water and electrolyte secretion, primarily by acting to inhibit enteric secretomotor neurons, but also by acting on the enterocytes (Hyland et al. 2003; Cox 2007).

It has been difficult to understand why hormones with different, and in some instances opposite, actions, are in the same EEC. However, this may now be explicable, at least in theory. High resolution confocal microscopy and super-resolution optical techniques have provided clues. These methods show that ghrelin, a hunger hormone, and nesfatin, that causes satiety, are in separate subcellular stores in gastric EEC (Stengel et al. 2010b). Likewise, GLP and PYY, which have opposite effects on fluid secretion and on glucose stimulation of insulin secretion, are separately stored in the same small intestinal EEC (Cho et al. 2014b). It remains to be shown that these hormones can be differentially released from the same cells.

Fluid Secretion, an Example of Integrated CNS, ENS and EEC Control

Movement of fluid between the lumen of the intestine and body fluid compartments is tightly regulated. Over two blood volumes cross the mucosal epithelial surfaces each day, and disruption of fluid transport regulation is life-threatening. One reason for the large flux is that the absorption of sugars (monosaccharides) and amino acids is through cation-coupled transporters. Thus, when glucose is absorbed through the sodium/glucose linked transporter it is internalised with a sodium ion and counter ions, the majority of which are chloride ions. Absorption of 100 g glucose is estimated to equate to absorption of 1.8 L of water (Wright and Loo 2000; Furness 2006). Enteric reflexes, through activation of secretomotor neurons, move water and electrolytes from the interstitium of the lamina propria to the lumen (Fig. 16.3). This water and electrolyte is drawn from the circulation and from the absorbed fluid. Enteric secretomotor reflexes cannot act in isolation and are modulated to control whole body fluid balance. This control is exerted through blood volume and blood pressure detectors that change the activity of two sympathetic pathways, vasoconstrictor pathways and secretomotor inhibitory pathways (Fig. 16.3) (Sjövall et al. 1986; Furness 2006), so that fluid balance is maintained. Glucose receptors are located on enteroendocrine cells (Young 2011). Stimulation of these cells releases GLP-2, along with other hormones. Receptors for GLP-2 are on non-cholinergic secretomotor neurons, which are activated by this hormone (Sigalet et al. 2007; Shirazi-Beechey et al. 2011). Thus, activation of the enteric receptor for glucose by glucose or artificial sweeteners stimulates secretomotor neurons to return water and electrolytes to the lumen (Fig. 16.3). In addition, the neurons activated by GLP-2 increase glucose uptake through SGLT1 (Shirazi-Beechey et al. 2011).

As previously mentioned, the fine control of fluid balance through local (ENS) and systemic (sympathetic) reflexes is thrown into chaos when the lumen contains an excess of certain pathogens or their toxins, including cholera toxin, rotavirus and pathogenic *Escherichia coli*, which activate enteric secretomotor neurons (Lundgren 2002; Gwynne et al. 2009). In mild cases, diarrhea is induced that helps to expel the pathogens and their toxic products. However, high levels of pathogens or toxins overwhelm the intestine and pathological, life-threatening diarrhea can develop.

Gastric Acid Secretion, a Further Example of Integrated Control

Acid secretion is controlled from the CNS and through vago-vagal reflexes (Schubert and Peura 2008). If the vagus nerves are cut, there is a loss of regulation of acid secretion, which is suppressed. Before the advent of histamine (H2) receptor

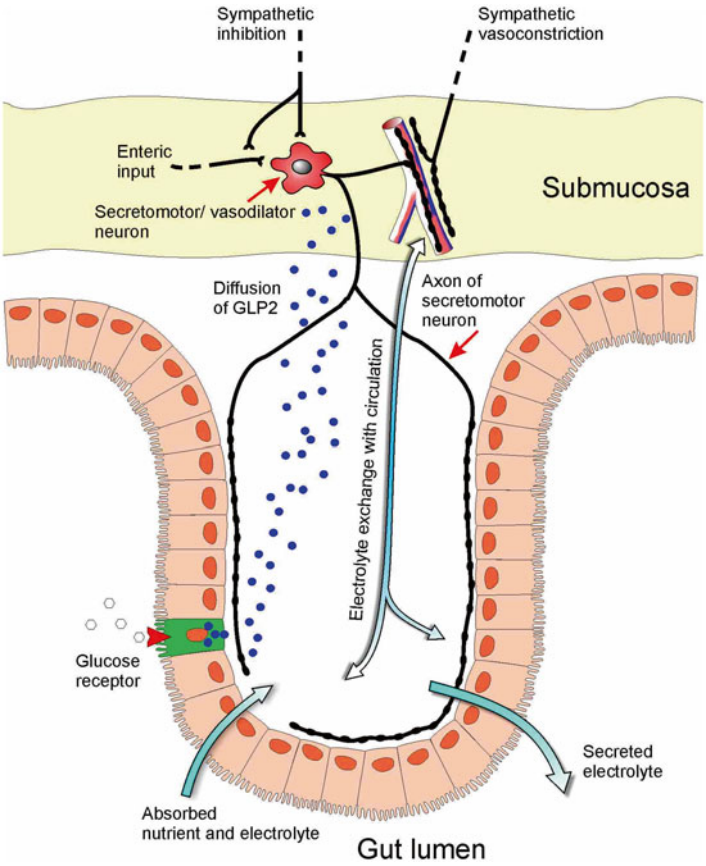

Fig. 16.3 Integrated neuronal and hormonal control of transmucosal water and electrolyte movement in the small intestine. The final secretomotor/vasodilator neuron that plays an essential role in balancing local fluid fluxes and in whole body water and electrolyte balance is illustrated. Large volumes of fluid are absorbed from the lumen with nutrients, such as glucose. The absorption of nutrients with fluid activates enteric secretomotor reflex pathways that impinge on the secretomotor neurons and returns fluids to the lumen. It is important that the balance of this fluid exchange is modulated by sympathetic vasoconstrictor and secretomotor inhibitory pathways. Activity in these sympathetic pathways, which inhibit secretion and reduce local blood flow, is determined by whole body fluid status, which includes sensory detection through blood volume detectors, baroreceptors and osmoreceptors. Modified from Furness (2006)

blockers and proton pump inhibitors, this loss of regulation was exploited through the use of vagotomy to reduce secretion and consequently protect against acid-dependent gastric ulcers (Dragstedt 1945).

Control of gastric acid secretion involves the integration of neural and endocrine signalling (Fig. 16.4). Neural influences are through extrinsic, vagal and sympathetic, pathways and intrinsic enteric neurons. Endocrine influences are through

release of the gastric hormones, gastrin, somatostatin and histamine. There is also evidence of neural and hormonal influences from the small intestine.

Three natural stimulants have direct roles to cause acid secretion from the parietal cell: acetylcholine, released from enteric neuron terminals, gastrin, released from antral EEC, and histamine, released from enterochromaffin-like cells (ECL cells) of the lamina propria, close to parietal cells (Fig. 16.4). The parietal and gastrin cells are under vagal control (Norlén et al. 2005). The vagus acts to stimulate the release of gastrin, gastrin reaches the ECL cells through the circulation, gastrin increases histamine secretion from the ECL cells, and histamine acts synergistically with nerve-released ACh to stimulate the parietal cells (Furness 2006).

The nerve-mediated, stimulation of gastrin-release is via a final neuron that utilises gastrin-releasing peptide (GRP) as a neurotransmitter. GRP is a potent

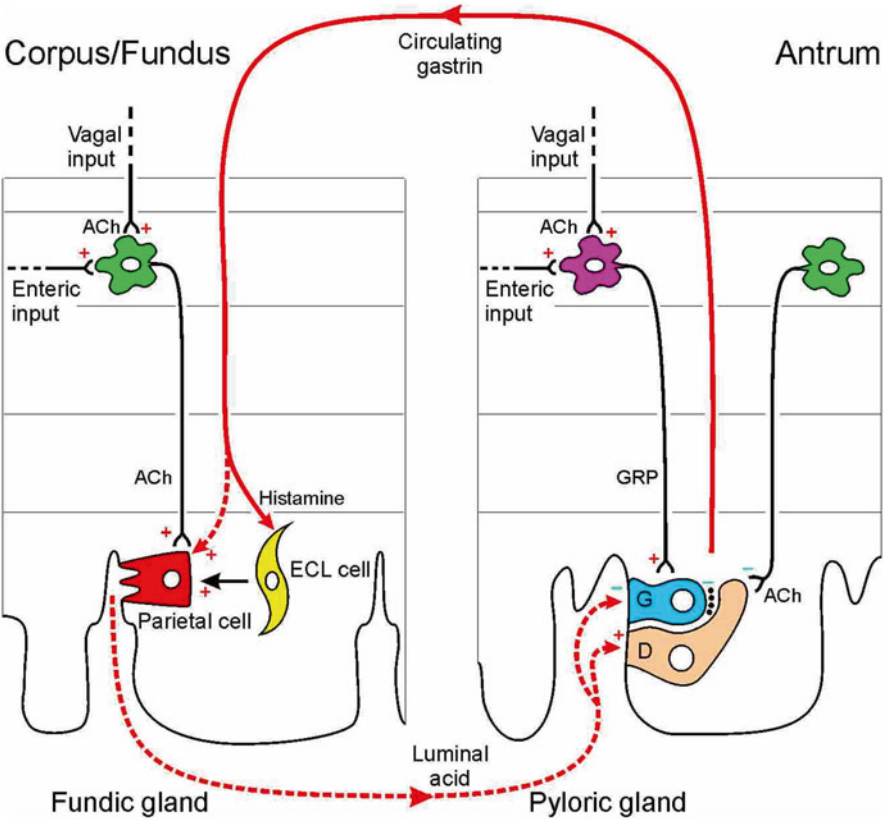

Fig. 16.4 Integrated neural and hormonal control of gastric acid secretion. The acid-secreting parietal cell is stimulated by acetylcholine (Ach), released from enteric neurons, histamine from entero-chromaffin-like (ECL) cells and gastrin from antral EEC (G cells). Enteric neurons also innervate the G cells, and the adjacent somatostatin-secreting D cells. The primary transmitter of neurons innervating the gastrin containing EEC cells is gastrin-releasing peptide. Modified from Furness (2006)

stimulant of gastrin release, by its direct action on gastrin cells (Basso et al. 1974). GRP occurs in nerve fibres but not in endocrine cells of the gastric mucosa and the GRP immunoreactive nerve fibres are found close to the gastrin cells (Holst et al. 1987; Miller et al. 1989; Sjövall et al. 1990). Desensitisation of GRP receptors abolishes the release of gastrin from the porcine stomach in response to vagus nerve stimulation (Holst et al. 1987) and a GRP receptor antagonist blocked the gastrin release caused when a vago-vagal reflex was induced by gastric distension (Weigert et al. 1997).

Conclusions

In this short review I have provided an overview, with examples, of the integrated nature of gastrointestinal control, especially in relation to the adjustments the gastrointestinal tract necessarily makes to its varied contents.

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Chapter 17
Enteric Neurobiology: Discoveries and Directions

Jackie D. Wood

NANC Hypothesis

The noncholinergic, nonadrenergic hypothesis (NANC hypothesis), espoused by Geoffrey Burnstock in Australia was a giant step forward in understanding of gastrointestinal motility and for neurogenic secretion (Fig. 17.1). It emerged in 1964 when he, Graham Campbell and Michael Bennett, at the University of Melbourne, together with Mollie Holman at Monash University, reported that inhibitory responses to sympathetic nerve stimulation were blocked by bretylium and guanethidine; whereas, a strong inhibitory component of transmural electrical stimulation was unaffected by these agents (Burnstock et al. 1964). This suggested that the inhibitory neurotransmitter was not a catecholamine and led to Burnstock's canonical hypothesis that the transmitter was a purine nucleotide (Burnstock 1972). Existence of the NANC neurons is revealed during transmural electrical field stimulation as inhibition of ongoing muscular electrical and contractile activity, followed by postinhibitory rebound excitation at the offset of stimulation (Fig. 17.1a). Removal of ganglion cell bodies from the myenteric plexus of a strip of guinea pig taenia coli eliminated inhibitory junction potentials that could normally be evoked by nicotinic agonists (Burnstock et al. 1966). Thereby, reinforcing the hypothesis that the NANC inhibitory musculomotor neurons were in the myenteric plexus.

Subsequent workers found that subpopulations of NANC inhibitory musculomotor neurons were firing spontaneously and that a major function in the intestine was continuous suppression of the autogenous activity of the unitary type smooth musculature of the circular muscle coat. Enteric neurons in multiple species were found to fire continuously (Wood 1970, 1973; Ohkawa and Prosser 1972;

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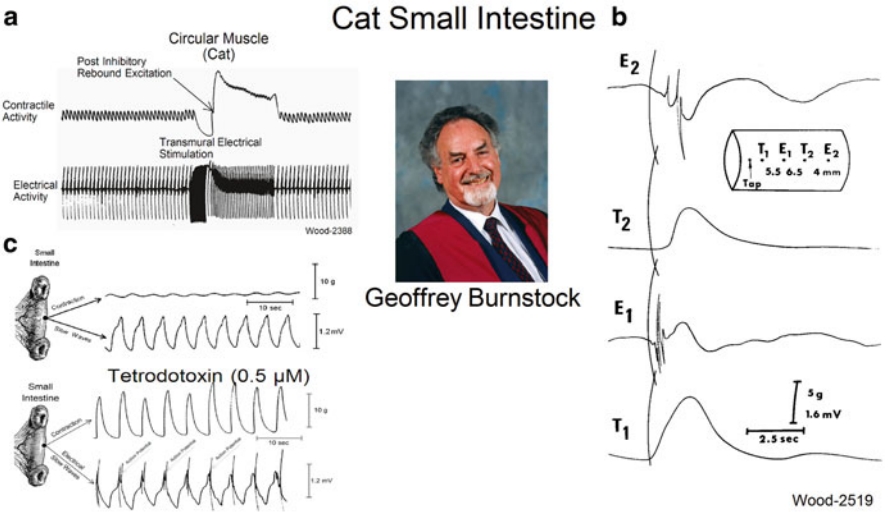

Fig. 17.1 Significance of Burnstock nonadrenergic-noncholinegic (NANC) inhibitory musculomotor neurons. (**a**) Inhibition of ongoing electrical and contractile activity and post inhibitory rebound excitation in the circular muscle coat in response to transmural electrical stimulation. (**b**) Records from 2 force transducers (T_1 and T_2) and 2 electrodes (E_1 and E_2) of propagated muscle action potentials and associated contractions evoked by a mechanical stimulus (tap) in the presence of ENS neural blockade by tetrodotoxin. Blockade of NANC neurons permitted propagation of the action potential in the aboral direction in the electrical syncytium throughout the length preparation. (**c**) Electrical slow waves have no action potentials at their crests and evoke only small amplitude contractions when NANC neurons are firing spontaneously in the upper two traces. Blockade of ongoing NANC firing by tetrodotoxin results in the appearance of muscle action potentials and large amplitude contractions associated with each slow wave (Wood 1972; Wood and Marsh 1973)

Nozdrachev et al. 1975). In intestinal segments in vitro, when firing was prevalent, muscle action potentials and associated contractile activity were absent. However, myogenic electrical slow waves were always present. Blockade of firing by tetrodotoxin resulted in every cycle of the electrical slow waves triggering intense discharge of muscle action potentials and large amplitude contractions (Fig. 17.1c) (Wood 1970, 1972; Ohkawa and Prosser 1972; Wood and Harris 1972; Wood and Marsh 1973; Biber and Fara 1973; Tonini et al. 1974; Bortoff and Muller 1975). When firing of the NANC inhibitory motor neurons was ongoing, neither mechanical stimulation nor transmural electrical stimulation effectively elicited contractile responses of the intestinal circular musculature (Wood 1972; Wood and Perkins 1970). Following neuronal blockade, both electrical and mechanical stimulation readily triggered muscle action potentials and waves of contractile activity that were propagated for extended distances in either direction along the longitudinal axis of an intestinal segment (Fig. 17.1b) (Wood 1972; Wood and Perkins 1970).

Various specialized patterns of intestinal motility are dependent upon integrated disinhibition of the intestinal circular muscle coat, which owing to interfiber gap junctions, behaves as a functional electrical syncytium activated by electrical slow

wave pacemaker potentials. In the normally functioning bowel, integrative control of the activity of NANC inhibitory neurons determines; (1) whether a particular cycle of an electrical slow wave triggers a response in the circular muscle; (2) the force of the contractile response triggered by a slow wave; (3) distance over which a muscle action potential and its associated contraction spreads from muscle fiber to fiber within the electrical syncytium; (4) the direction of spread of action potentials and associated contractions within the syncytium along a segment, which can be in the oral or aboral direction according to the motility pattern (see Fig. 17.5).

Enteric Neuronal Electrophysiology

Following in the wake of the NANC hypothesis, major progress in the neurobiology of the enteric nervous system (ENS) came from microelectrode recording of electrical behavior of single neurons. I published, in 1970, records of single unit activity of neurons in the myenteric plexus of the cat that were immediately confirmed by Ohkawa and Prosser in the United States and AD Nozdrachev and associates in Russia (Wood 1970; Ohkawa and Prosser 1972). Research on the cellular neurophysiology of ENS neurons gained significant momentum with reports of results of intracellular recording, with "sharp" microelectrodes describing electrical and synaptic behavior in guinea pig ENS neurons (Hirst et al. 1974; Nishi and North 1973; Wood and Mayer 1978a). Two types of ENS neurons were identified based on their electrophysiological and synaptic behavior and named either AH-type 2 or S-type 1 neurons (Fig. 17.2). The names were an arbitrary combination of terms that recognized G. David Hirst, who was working in the Department of Physiology, Monash University, Clayton, Victoria, Australia and R. Allan North working first as a student in the Department of Pharmacology, University of Aberdeen, Aberdeen, Scotland, UK and later as a postdoctoral fellow in the Neurophysiology Laboratory, Department of Pharmacology, Loyola University Medical Center in Maywood, Illinois, USA as the first to describe the two kinds of neurons (Hirst et al. 1974; Nishi and North 1973). The names combined the alphabetical terms used by Hirst and numerical designations used by North. The terms are facilitating because AH-type 2 neurons usually have Dogiel Type 2 multipolar morphology and S type 1 neurons generally are unipolar like Dogiel Type I neurons (Fig. 17.2).

Also taking place in this heyday for ENS neuronal electrophysiology was the discovery of slow excitatory postsynaptic potentials (Slow EPSPs) which hitherto was unknown elsewhere in the nervous system (Wood and Mayer 1978b; Katayama and North 1978). Inhibitory sympathetic noradrenergic synaptic transmission was also discovered at this time (Hirst and McKirdy 1975; North and Surprenant 1985). The competitive nature of ENS research at the time was exposed when the group of R. Alan North published evidence that slow EPSPs were mediated by substance P and Jackie Wood's group was resolute that serotonin was the neurotransmitter (Wood and Mayer 1978b; Katayama and North 1978; Grafe et al. 1979). As it played out, both were right.

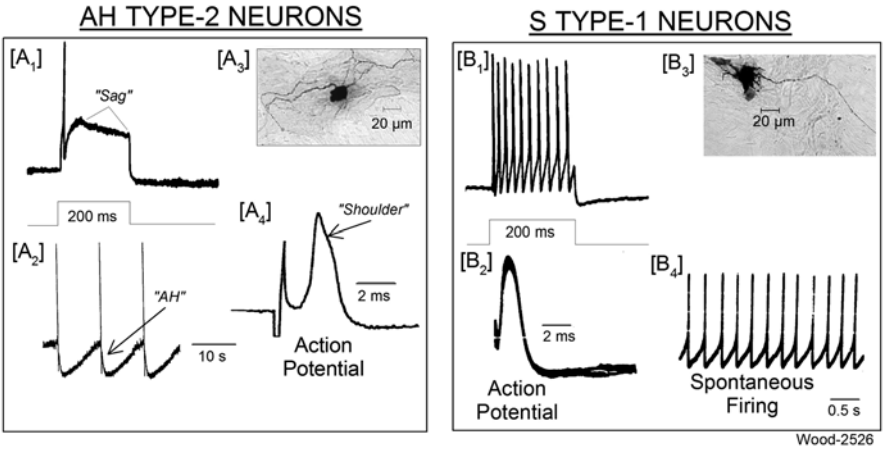

Fig. 17.2 AH type-2 and S type-1 ENS neurons, first described by Hirst et al. (1974) and Nishi and North (1973) are distinguished by specific electrophysiological behavior. (A_1) Intraneuronal injection of a 200 ms depolarizing current pulse could evoke only a single action potential, regardless of the strength of the depolarizing pulse. Neuronal excitability in AH neurons is low and membrane depolarization evokes none or only one or two action potentials at the onset of a depolarizing current pulse. The action potential is followed by a "sag" in the electrotonic potential, which reflects an increase in ionic conductance. (A_2) A long-lasting after hyperpolarization (AH) is associated with the action potentials in AH type-2 neurons. (A_3) Multipolar morphology of the AH Dogiel Type II neuron from which the records were obtained as revealed by intraneuronal injection of the marker, biocytin, from the microelectrode. (A_4) A "shoulder" on the falling phase of the action potential reflects opening of voltage-gated Ca^{2+} channels. (B_1) S type-1 ENS neurons are more excitable that AH neurons and fire repetitively during intraneuronal injection of depolarizing current pulses with the firing frequency increasing in direct relation to the strength of membrane depolarization. (B_2) Unlike AH neurons, S type-1 neurons have no "shoulder" on the action potentials. (B_3) Morphology of the uniaxonal neuron from which the records were obtained. (B_4) Unlike AH neurons, S type-1 neurons fire spontaneously, with each action potential preceded by a ramp-like prepotential

Slowly-activating membrane depolarization, which continues for several seconds to minutes and sometimes hours after termination of stimulation and release of the neurotransmitter from the presynaptic terminal, was found to be a major characteristic of slow EPSPs (Fig. 17.3). Excitatory mediators released in paracrine fashion from enteric mast cells, and other inflammatory/immune cells were found to also evoke slow EPSP-like responses (Frieling et al. 1994a, b; Liu et al. 2003a). Slow inhibitory postsynaptic potentials (IPSPs), reported at the time by Hirst and McKirdy (1975) and the partnership of North and Surprenant (1985), were described as hyperpolarizing synaptic potentials found in both myenteric and submucosal ganglion cell somas. IPSPs are now known to be more common in the submucosal than in the myenteric plexus. In the submucosal plexus, they were found to be mediated by norepinephrine release from sympathetic postganglionic neurons and predominate in the non-cholinergic secretomotor/vasodilator neurons that release vasoactive intestinal peptide at junctions with secretory glands in the mucosa (North and Surprenant 1985; Bornstein et al. 1988).

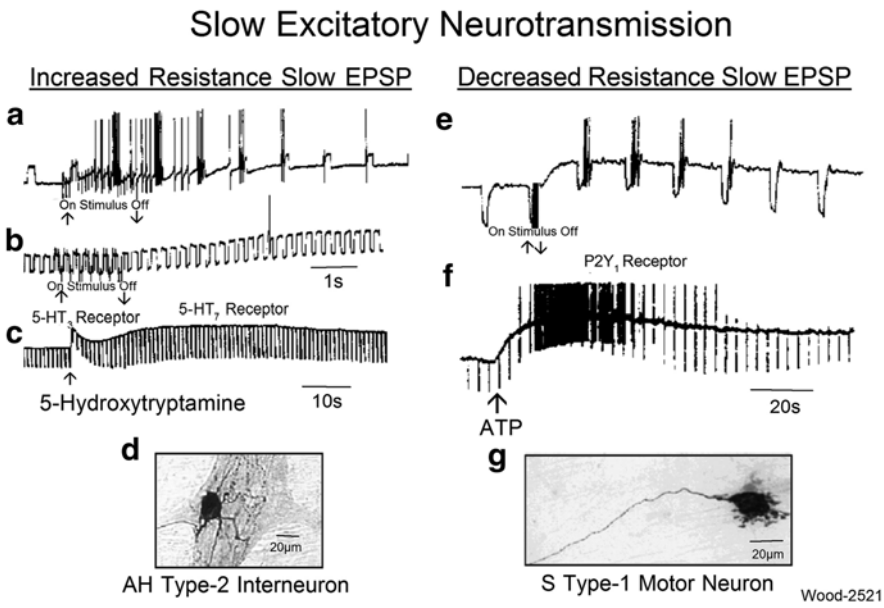

Fig. 17.3 Two kinds of slow excitatory postsynaptic potentials (EPSPs) are identified by changes in membrane conductance (i.e., input resistance) during the depolarization phase. (**a**) Slow EPSPs in AH type-2 ENS neurons are characterized by long-lasting membrane depolarization, suppression of the AH and associated elevation of excitability that continues for extended periods after termination stimulus-evoked transmitter release. (**b**) Input resistance increases during the depolarization phase of slow EPSPs in AH type-2 ENS neurons. Downward deflections are electrotonic potentials evoked by repetitive intraneuronal injection of constant-current hyperpolarizing pulses. Increased amplitude of electrotonic potentials reflects closing of ionic channels (i.e., increased input resistance). (**c**) Application of 5-hydroxytryptamine by pressure microejection from fine tipped pipettes evokes two kinds of slow EPSPs in the same AH type-2 ENS neurons. One kind evoked by stimulation of the 5-HT_3 receptor subtype is associated with a decrease in input resistance; a second kind evoked by the 5-HT_7 receptor subtype is associated with increased input resistance. (**d**) Multipolar morphology of the AH Dogiel Type II neuron from which the records were obtained. (**e**) Slow EPSPs in S type-1 neurons are characterized by long-lasting membrane depolarization associated with elevated excitability and increased membrane conductance reflected by increased input resistance. (**f**) Application of ATP by pressure microejection from fine tipped pipettes evokes slow EPSP-like excitation mediated $P2Y_1$ receptors in S type-1 ENS neurons. (**g**) Morphology of the uniaxonal neuron from which the records were obtained

Neurochemical Coding of Enteric Neurons

Application of emerging immunohistochemical and projection methodologies in parallel with the electrophysiological work, which was moving forward at the same time in Australia, was responsible for major expansion of insight in the rapidly developing importance and recognition of ENS neurobiology in the late 1980s and 1990s. Marcello Costa and John Furness working at Flinders University, and Furness, later working at the University of Melbourne, were the pioneers in major

progressive steps that established the concept of chemical coding for functional identification of neurons in the ENS synaptic networks. Their two books continue to be mandatory reading for anyone working in the ENS (Furness and Costa 1987; Furness 2006). Immunohistochemical approaches became underpinning, in concert with intraneuronal marking during electrophysiological recording, for numerous studies by others of the cellular neurobiology of the kinds of neurons comprising the synaptic microcircuits in the ENS and continues at this writing.

Inflammation

Discoveries in the 1980s–1990s that inflammatory-immune cells communicate in paracrine fashion with the ENS set the stage for understanding motility and secretory pathophysiology in food allergies, infectious enteritis, radiation induced enteritis, idiopathic enteritis (e.g., ulcerative colitis), as well as, stress-evoked events in functional gastrointestinal disorders (e.g., irritable bowel syndrome) (Hu et al. 2003; Wood 2004, 2007a, b, 2012; Tamura and Wood 1992; Liu et al. 2003b). Examples include demonstrations that AH-type neuronal electrophysiology in states of mucosal inflammation, induced by instillation of toxic chemicals, exhibit persistent enhanced excitability (Hoffman et al. 2011; Krauter et al. 2007; Linden et al. 2003; Lomax et al. 2006, 2007; Mawe et al. 2009). Enhanced excitability in these cases mimics the excitatory paracrine actions of several different mast cell mediators to augment excitability by suppressing the postspike after-hyperpolarization of AH-type neurons and increasing their input resistance (Frieling et al. 1994a; Tamura and Wood 1992; Liu et al. 2003b; Gao et al. 2002; Nemeth et al. 1984; Starodub and Wood 2000a, b).

Hardwired Polysynaptic Propulsive Motor Circuit

Bayliss and Starling's (1899) discovery that local intestinal stimulation evokes inhibition below the point of stimulation and excitation above it in association with a wave of circular muscle contraction traveling in the aboral direction ushered in ENS neurobiology. Although referred to as "the peristaltic reflex" over subsequent years; whether reflex is appropriate in the Sherringtonian sense has been opened to question (Wood 2008). On the other hand, there is little question that a hardwired polysynaptic reflex circuit at a low to intermediate level of neural organization in the ENS underlies the propulsive motility patterns in the small and large intestine and esophagus. The circuit for propulsion in the direction of the anus is "wired" in such a way that it evokes relaxation of the circumferentially oriented muscle layer and contraction of the longitudinal muscle below the initiation area and contraction of the circumferentially oriented muscle layer above the initiation site (Fig. 17.4A, B). The "wiring" is reversed for propulsive motility moving luminal contents in the oral

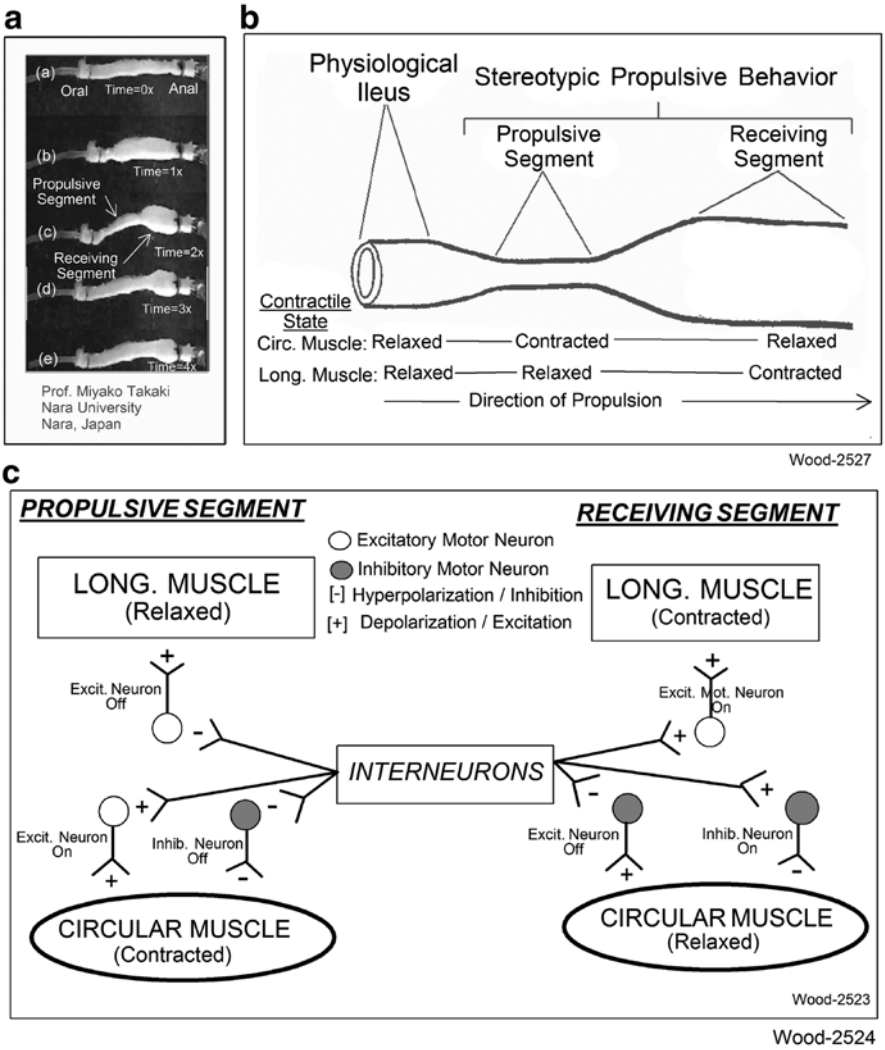

Fig. 17.4 A "hardwired" polysynaptic peristaltic reflex circuit in the ENS underlies integrated control of intestinal and esophageal propulsive motility. (**A**) Motor behavior of the intestinal wall during propulsive motility in a segment of guinea-pig ileum in response to distension by infusion of physiological saline through a catheter tied into the oral end. (*a*) Time = 0×. (*b*) With time advanced to 1×, shortening of the segment due to contraction of the longitudinal muscle coat and inhibition of the circular muscle coat expands the lumen into a receiving segment. (*c*) With time advanced to 2×, reduction in the diameter and lengthening of the propulsive segment occurs as the circular muscle coat contracts. Also at time = 2× expansion of the lumen of the receiving segment occurs as the longitudinal muscle coat contracts and the circular muscle is relaxed by firing of inhibitory musculomotor neurons. (*d–e*) Propulsive motility continues to empty the segment at time = 3–4×. Redrawn from video images kindly provided my Prof. Miyako Takaki. (**B**) In a heuristic model for propulsive motility, the circumferential and longitudinal muscle layers of the intestine behave in a stereotypical pattern to propel luminal contents. A "hardwired" polysynaptic circuit in the ENS determines the pattern of behavior of the two muscle layers. During propulsion, the longitudinal muscle coat in the segment ahead of the advancing intraluminal contents contracts while the circumferential muscle layer relaxes simultaneously. Simultaneous shortening of the longitudinal intestinal axis and relaxation of the circumferential muscle in the same segment

direction during emesis in the small intestine. For emetic propulsion, the circuit is "wired" such that it evokes relaxation of the circumferentially oriented muscle layer and contraction of the longitudinal muscle in the direction of the stomach and contraction of the circumferentially oriented muscle layer as the trailing event. Like spinal motor reflexes, sequencing of the behavior of the intestinal longitudinal and circular muscles is hardwired into the circuitry, which ensures that the propulsive motor behavior is repeated stereotypically in every occurrence (Fig. 17.4B). Propulsion of the luminal contents is determined by the sequence in which the hardwired circuit activates excitatory and inhibitory musculomotor neurons to the longitudinal and circular muscle layers. During propulsion, the longitudinal muscle coat in the segment ahead of the advancing intraluminal contents contracts in response to activation of its excitatory motor innervation, while at the same time, the circular muscle layer relaxes in response to activation of its NANC inhibitory motor innervation (Fig. 17.4A). The esophagus and intestine behave geometrically like a cylinder with constant surface area. Therefore, a reduction in radius of the cylinder during contraction of the circular muscle is accompanied by a lengthening of the segment as can be seen in Fig. 17.4A (Wood and Perkins 1970). On the other hand, shortening of the longitudinal axis of the segment, during contraction of the longitudinal muscle coat, is accompanied by a widening of the cross-sectional diameter as seen in Fig. 17.4A. Simultaneous shortening of the longitudinal axis in concert with inhibitory relaxation of the circular muscle coat results in expansion of the lumen, which prepares a receiving segment for the forward-moving intraluminal contents during activation of the hardwired propulsive motor circuit. A propulsive segment is formed behind the receiving segment when synaptic connections in the circuit "turn off" the inhibitory musculomotor innervation to the circular muscle. Silencing of the inhibitory innervation permits electrotonic spread of electrical slow wave current into the circular muscle, depolarization of the muscle fibers to action potential threshold and action potential-evoked contraction of the circular muscle in the propulsive segment.

Contractions recorded by sensing devices implanted on the bowel during digestive and interdigestive small intestinal motor behavior reflect the formation of propulsive segments (see Fig. 17.5). They occur at the frequency of the electrical slow waves because removal of inhibition from the circular muscle allows it to respond to the electrical current flowing from networks of interstitial cells of Cajal during

Fig. 17.4 (continued) results in expansion of the lumen, which becomes a receiving segment for the forward moving contents. The allied function in the circuit is contraction of the circular muscle in the segment behind the advancing intraluminal contents. The longitudinal muscle layer in the same segment relaxes simultaneously with contraction of the circular muscle, which results in conversion of this region to a propulsive segment that propels the luminal contents ahead into the receiving segment. (**C**) Heuristic model for a "hardwired" polysynaptic propulsive motor circuit in the ENS. When the circuit is active, excitatory motor neurons to the longitudinal muscle coat and inhibitory motor neurons to the circular muscle coat are firing to form the receiving segment below the point of stimulation. At the same time firing of excitatory motor neurons to the circular muscle coat and inactivation of inhibitory motor neurons to the circular muscle coat occurs in the propulsive segment above the point of initiation

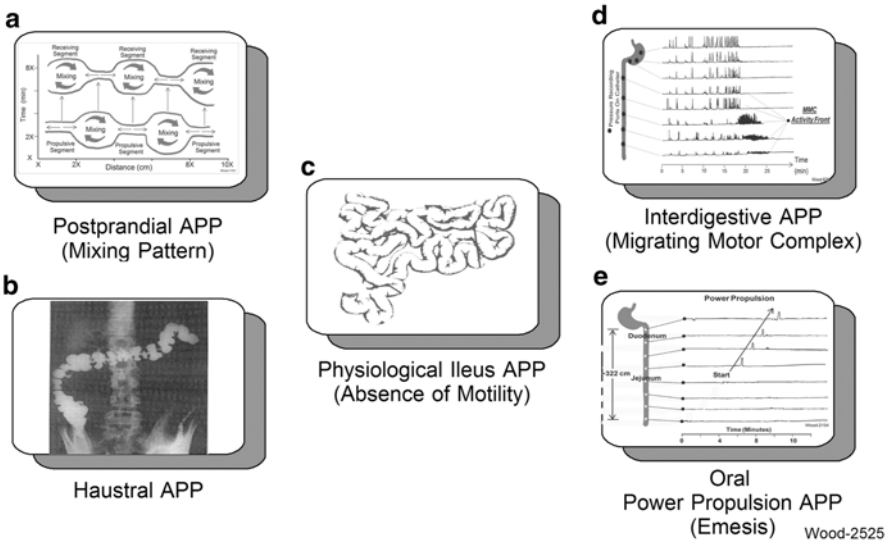

Fig. 17.5 The enteric nervous system can be viewed, heuristically, as a "minibrain" with a library of applications, like digitally programed "apps", for multiple patterns of small or large intestinal behavior. (**a**) A specific app determines motor behavior in the postprandial state. (**b**) A haustral app programs for formation of haustra in the colon. (**c**) Running of a physiological ileus app programs for a quiescent bowel. (**d**) Another app establishes the pattern of intestinal motility, called the migrating motor complex, which characterizes the fasting state. (**e**) The specialized motility pattern that occurs in the upper one-third of the small intestine during emesis reflects output of another of the apps in the library. During emesis, peristaltic propulsion in the upper one-third of the small intestine is reversed for rapid movement of the luminal contents toward the stomach

each slow wave cycle. On the other hand, formation of the propulsive segment during power propulsion occurs unrelated to the frequency of the electrical slow waves and involves much stronger contraction of the circular muscle in the propulsive segment than occurs during peristaltic propulsion in the digestive and interdigestive motor patterns (see Fig. 17.5).

Central Pattern Generators

As might be expected for an independent integrative nervous, such as the ENS, evidence has accumulated to suggest that central pattern generators are expressed at higher levels of neurophysiological organization in the ENS. Central pattern generators (CPGs) are circuits in nervous systems that generate repetitive patterns of motor behavior independent of sensory input. CPGs underlie rhythmic motor behaviors such as walking, chewing, swimming, feeding, flying and respiration in vertebrates and invertebrates (Lomax et al. 2007; Mawe et al. 2009; Gao et al. 2002; Nemeth et al. 1984; Starodub and Wood 2000a, b; Bucher et al. 2006; Marder 2000,

2001; Marder and Bucher 2001; Marder et al. 2005; Marder and Rehm 2005). Rhythmic and cyclical motor and secretory behaviors in the intestinal tract are most likely reflections of the output one or more CPGs at higher levels of ENS organization.

CPGs have three properties in common: (1) motor output patterns consist of rhythmically timed bursts of action potentials that arise either from an ensemble of neurons that can't be traced to any individual neuron in the system or are generated by endogenous firing of a single neuron; (2) stereotypic sequences of repetitive behavior (e.g., walking) are initiated by activation of single "command neurons" or by an overlay of a paracrine neuromodulator on the CPG circuit; (3) motor behavior can be initiated and modified by sensory feedback, but the stereotyped sequence of motor events continues in the absence of sensory input.

Electrophysiological results suggest that CPGs in the ENS have endogenous neuronal oscillators that determine the timing of recurrent firing of ENS musculomotor and secretomotor neurons (Wood 1970; Wood and Mayer 1973). This is evident, in single unit records, for a subset of neurons in stretched intestinal whole-mount preparations from guinea pig, dog and cat small intestine in vitro. The putative CPG neurons fire bursts of action potentials continuously over long time periods with the spike bursts fired precisely at 6-s intervals or at exact multiples of 6 s (i.e., 12, 18, or 24 s) for the cat small intestine. Blockade of synaptic transmission does not change the timing of the discharge, which is expected if the CPGs are endogenous "clocks" (Wood 1975, 2008; Athey et al. 1981).

A response to paracrine release of histamine from inflammatory/immune cells is an example of how a neuromodulator influences a circuit with a CPG in the ENS to generate a rhythmic pattern of glandular secretion linked with motility. Application of histamine to simulate degranulation of enteric mast cells or actual degranulation of mast cells in the intestine of antigen-sensitized animal models in vitro evokes rhythmic cycles of chloride secretion with the peak of each cycle linked to a contraction of the circular muscle coat (Frieling et al. 1993, 1994a, b; Wang and Cooke 1990). The secretory cycles occur at about 1 per min in the guinea pig colon. As each secretory cycle peaks, contraction of the muscularis externa occurs (Cooke et al. 1993). Activation of the CPG in this case is independent of the concentration of the neuromodulator with the exception of requirement for a threshold concentration. The integrated neural network at this level of organization behaves like it incorporates a "switch" that activates the neural program, including the CPG, in on-off manner.

Neuromodulatory action of histamine to activate an ENS behavioral program that incorporates a CPG reminds of a similar neuromodulatory action of the enteroendocrine hormone, cholecystokinin in the small intestine. Application of cholecystokinin, to mimic enteroendocrine release in the cat small intestine, activates in all-or-none manner a neural program for repetitive cycles of propulsive motility (Weems et al. 1985). A comparable repetitive patterning of activation of the "hard-wired" ENS propulsive reflex circuit at a CPG-level of organization continues when flat sheet preparations from guinea pig colon remain tightly stretched for periods of minutes in vitro (Spencer et al. 2001; Spencer and Smith 2004).

Program Library

The ENS can be viewed as a "minibrain", which at a higher level of neurophysiological organization expresses a library of programs for multiple patterns of small or large intestinal behavior that call to mind the digitally programed "apps" in the Apple® Inc. app store. In this context, a specific app determines adaptive musculomotor behavior in the postprandial state and another establishes an adaptive pattern of intestinal motility that characterizes the fasting state. The specialized motility pattern that occurs in the upper one-third of the small intestine, during emesis, appears to reflect output of another of the Apps in the library. During emesis, propulsion in the upper one-third of the small intestine is reversed for rapid movement of the luminal contents over extended distances toward the stomach. The "emetic app" can be "called-up" from the library either by commands from the brain (e.g., emetic action of apomorphine) or by local sensory detection of threatening substances in the lumen (e.g., cupric sulfate). Still another app, referred to as *power propulsion* is "called-up" for defense against infectious invaders, enterotoxins or food allergins, as well as being the app for reverse propulsion during emesis. The ENS library in the small intestine behaves like it might have apps for at least 6 different patterns of motility (see Fig. 17.5). These motility apps are: (1) an interdigestive App; (2) a postprandial App; (3) an aboral power propulsion App; (4) emetic oral power propulsion App; (5) haustral app in the colon; (6) physiological ileus app (Fig. 17.5).

The postprandial app, in the small bowel, starts to run with the intake of a meal (Fig. 17.5a). It programs for a mixing pattern of motor behavior. Repetitive propulsive contractions of the circular muscle, which propagate only short distances, account for the segmentation appearance when the app is running (Fig. 17.5a). Circular muscle contractions in short propulsive segments are separated on either side by receiving chambers with relaxed circular muscle and contracting longitudinal muscle, each of which reflect activation of short "blocks" of the hardwired polysynaptic propulsive circuit in Fig. 17.4B. This mixing activity continues at closely spaced sites along most of the length of the small intestine so long as nutrients are present in the lumen.

The interdigestive app programs the migrating motor complex (MMC), which is the small intestinal motility pattern of the interdigestive state. It replaces the postprandial app after digestion and absorption of nutrients is complete 2–3 h after a meal in humans. Sensors attached to the stomach show the MMC starting as large amplitude contractions at 3 per minute in human distal stomach. Activity starts in the antrum and then migrates in the aboral direction into the duodenum and on through the small intestine to the ileum. When this app is "running" the MMC occupies a limited length of intestine called the activity front, which has an upper and a lower boundary (Fig. 17.5d). The activity front slowly advances, "migrates", down the intestine at a rate that progressively slows as it approaches the ileum. Propulsion of luminal contents in the aboral direction occurs, at electrical slow wave frequency, between the oral and aboral boundaries of the activity front. The circular muscle

contractions seen in the activity front are a reflection of the formation of the propulsive component of the hard-wired propulsive reflex circuit (Fig. 17.4A, B). Each propulsive wave traveling downward in the activity front consists of a propulsive and receiving segment (Fig. 17.4A). Successive propulsive waves start at the oral boundary and propagate to the aboral boundary of the activity front where they stop. Successive propulsive complexes start on average a short distance further in the aboral direction and propagate on average slightly beyond the boundary where the previous one stopped. Thus, the entire activity front slowly migrates down the intestine, "sweeping" the lumen clean as it goes. Running of this app inspired C.F. Code of the Mayo Clinic to name the MMC "the bowel's housekeeper" (Code 1979; Code and Marlett 1975). Physiological ileus is in effect along the bowel oral and aboral to the upper and lower boundaries of the migrating activity front. Neither MMCs nor physiological ileus can be seen in the small intestine in the absence of the ENS.

The power propulsion app programs rapid emptying of the contents of long segments of bowel. Power propulsive motility is characterized by strong, long-lasting contractions of the circular muscle that can travel over long distances along the small or large intestine. The contractions, when recorded by sensors, reflect formation of the propulsive segment of the hard-wired polysynaptic circuit in Fig. 17.4B and are sometimes referred to as "giant migrating contractions" because they are considerably stronger than the phasic contractions of the circular muscle coat that appear in the activity front of the MMC and in the mixing motility pattern (Sarna 1987). Giant migrating contractions have long durations of 18–20 s. They constitute the propulsive segment of the hardwired propulsive circuit in Fig. 17.4A and underlie efficient propulsive motility that strips the lumen clean as they travel at about 1 cm/s over long lengths of intestine. Motility programed by power propulsion apps differs from propulsive motility in the activity front of the MMC app and in the short-segment mixing seen when the postprandial app is running. The circular contractions in the propulsive segment are much stronger, occur independent of electrical slow waves and propagation takes place over much longer stretches of intestine.

Running of the power propulsion app rapidly moves the luminal contents of the distal small intestine or the large intestine in the anal direction. Noxious mucosal stimulation is the trigger for calling-up this app and closing any other app that might be running. Cramping pain, fecal urgency and diarrhea are associated with this motor behavior (Kamath et al. 1990). Exposure of the mucosa to irritants, introduction of luminal parasites, enterotoxins from pathogenic bacteria, allergic reactions, and exposure to ionizing radiation can start the aboral power propulsion app. Characteristics of this nature suggest that this app is a defensive adaptation for rapid clearance of threats from the intestinal lumen (Wood 2004).

The aboral power propulsion app appears as if it integrates as part of a more highly organized defensive app that is called-up by exposure to neuromodulators released during degranulation of enteric mast cells (Wood 2007a, 2012). Reconfiguration of the synaptic networks by an overlay of mast mediators integrates output of a secretomotor app with output of the power propulsion app in a timed

sequence. The secretomotor app runs first and evokes massive mucosal secretion of electrolytes and H_2O that "flushes" threats (e.g., microorganisms, allergins, noxious substances, etc.) into the lumen and maintains them in suspension in the lumen. The secretomotor response is followed immediately by running of power propulsion in the anal direction. Power propulsion rapidly propels the large volume of luminal liquid toward the anus. Arrival of the large volume in the recto-sigmoid region causes rapid distension, which triggers the ENS recto-anal reflex and relaxation of the smooth muscle of the internal anal sphincter. Opening of the internal sphincter, in this situation, underlies sensations of fecal urgency and emotional anxiety because the only remaining protection against incontinence is spinal-evoked contraction of the skeletal muscle of the puborectalis and external anal sphincter. Cramping abdominal pain is a hallmark of the large amplitude propulsive contractions evoked during power propulsion (Kamath et al. 1990). Symptoms of acute, explosive watery diarrhea are self-explanatory at this point.

The emesis app programs the specialized motility pattern that occurs in the upper one-third of the small intestine during emesis (Fig. 17.5e). During emesis, the direction of propulsive motility in the upper one-third of the small intestine is reversed for rapid movement of the luminal contents toward the stomach. As mentioned earlier, the emetic app can be called-up from the library either by commands from the brain or by local sensory detection of threatening substances in the lumen. Like the power propulsion app in the distal small intestine and in the large intestine, the adaptive significance of the emesis app is rapid removal of a threat from the lumen of the upper small bowel.

The physiological ileus app programs for quiescence of the autogenic intestinal smooth muscle (Fig. 17.5c). In clinical terms, ileus refers to forms of mechanical bowl obstruction that can be "dynamic" or "adynamic". Adynamic ileus is pathological obstruction of the bowel due to failure of the smooth muscle to contract. Dynamic ileus is pathological intestinal obstruction due to spastic contraction (i.e., failure to relax) of the circular musculature in a segment of bowel. Mechanical obstruction and adynamic and dynamic ileus are accompanied by severe colicky pain, abdominal distension, vomiting and constipation (Glasgow and Mulvihill 2002). Physiological ileus was suggested as a useful term in reference to non-pathological absence of motility in the small and large intestine (Grundy et al. 2006). It is a normal state of the bowel in which an app in the ENS programs quiescence of motor function. Physiological ileus disappears in the aganglionic segment of Hirschsprung's disease and after destruction or neuronal blockade in the ENS (e.g., treatment with nerve-blocking agents, such as tetrodotoxin). Disorganized and non-propulsive contractile behavior occur continuously (i.e., dynamic ileus) due to the autogenic contractile properties of unitary type smooth muscle when NANC inhibitory musculomotor neurons are blocked or destroyed by pathological processes, such as occur in neuropathic chronic intestinal pseudoobstruction or autoimmune neuropathy in functional gastrointestinal disorders (Wood et al. 2012; Stanghellini et al. 1987).

Looking Ahead

The ENS, like all other independent integrative nervous systems, whether in vertebrates or invertebrates is organized in a hierarchy of structures and functions ranging from the cellular-molecular at the lower tiers to plasticity in programmable synaptic networks ("apps") at the uppermost tiers. ENS neurobiology of the future is likely to continue its forward momentum with research oriented to opening the doors into the higher order functions in integrated synaptic microcircuits that include central pattern generators, program libraries reminiscent of Apple® apps and neuromodulatory reconfiguration of the outputs of hardwired synaptic circuits. Behavioral output properties will emerge at the uppermost tiers that will be unpredicted by complete knowledge of lower tiers. Understanding the uppermost tiers is a challenging but not formidable "brick wall" for the present generation's ENS research. Present and future generations of investigators must confront the need for innovation and application of advanced technologies in order to continue the efforts of past generations to break further through the "brick" wall.

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Chapter 18
Advanced 3D Optical Microscopy in ENS Research

Pieter Vanden Berghe

Introduction

Microscopic techniques are among the few approaches that have survived the test of time. Being invented half way the seventeenth century by Antonie van Leeuwenhoek and Robert Hooke, these techniques are still essential in modern biomedical labs. One of the most important aspects in microscopy is the search to improve resolution as well as the contrast between the item of interest and the background. Different contrast techniques have been invented (phase contrast, differential interference contrast, Hoffmann modulation,…) to make sure that (sub)cellular structures could be identified using light. These techniques have been essential in ENS research since all sharp electrode recordings were made on setups with this type of microscopy approach (Hirst and McKirdy 1975; Wood 1989). Apart from its use in electrical recordings, microscopy has been instrumental in the identification of subpopulations of cells in the ENS, using a variety of staining methods: silver impregnation, neurobiotin injections and antibody labeling.

A significant step forward in the use of microscopy was the introduction of fluorescence approaches. Due to the fact that the intense excitation light is now filtered away from the longer wavelength emission light, the contrast can be improved drastically. The development of different color fluorescent probes attached to selective antibodies has made it possible to identify subpopulations of enteric neurons in a variety of species (Costa et al. 1996; Furness 2000).

Another important impetus to the use of microscopy was the discovery and isolation of the green fluorescent protein (GFP) from the jellyfish Aequorea Victoria

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(Prasher 1995). In the last two decades different variants of this and other coral based fluorescent molecules have complemented the toolkit of the biomedical researcher. By analogy with the classic chemical ion sensors (Fura2, Fluo4 et al.), fluorescent proteins have been mutated and fused to become functional sensors to report pH (e.g. synaptopHluorin) or cytosolic Ca^{2+} concentrations (e.g. GCaMP). This approach has the advantage that, rather than having all cell layers contribute to the signal, only specific enteric glia or neurons will express the reporter, which expands the accuracy with which activity can be measured in a single cell (Boesmans et al. 2013; for review see Boesmans et al. 2015).

Apart from these biological developments, also optical, electronic and computational methodology has improved significantly, which permits to achieve higher resolution, more sensitive and faster recordings. In this paper a number of current developments in microscopy are highlighted in the context of ongoing enteric nervous system research.

3-Dimensional Imaging and Volume Reconstructions

Confocal imaging is an established technique that can be used to optically section fluorescently labeled samples. It can be used to create high quality two dimensional (2D) images because out of focus light is efficiently removed. Apart from generating higher quality 2D images, the three dimensional (3D) image stacks can also be used to accurately determine cellular shape and perform volume rendering. This however requires computation intense deconvolution algorithms, which has been facilitated by improved software developments and ever increasing computing power.

In combination with genetic expression of markers we were able to determine the 3D shape of different groups of glial cells in the enteric nervous system of the mouse (Boesmans et al. 2014). Especially the structure of the type II glial cell is intriguing as it suggests that glial processes from one single cell are in close contact with most of the neuronal fibers that connect two ganglia in the myenteric plexus. Apart from its use in mouse tissue, the computational and reconstruction approach can also be applied to samples labeled with antibodies. One promising example of this quantification method applies to submucous ganglia in biopsies from the human intestine, as these have been shown to contain a small amount of neurons. On the one hand these neurons can be used to investigate marker expression with immunohistochemistry (Lebouvier et al. 2010) but also to record from while tissues are still alive (Cirillo et al. 2013). Volume reconstructions (Fig. 18.1) to determine abnormalities in neuronal processes or glial cell shapes may be useful to understand their association with diseases (Cirillo et al. 2015).

Apart from fixed tissues, also live tissues can be visualized using confocal technology. With one such approach, spinning disk confocal microscopy it is possible to record image stacks (~10 slices volume per second), which generates a 3D cube of information at a temporal resolution (1 Hz) comparable to what is available in 2D CCD camera based systems. The speed at which the recordings can be made

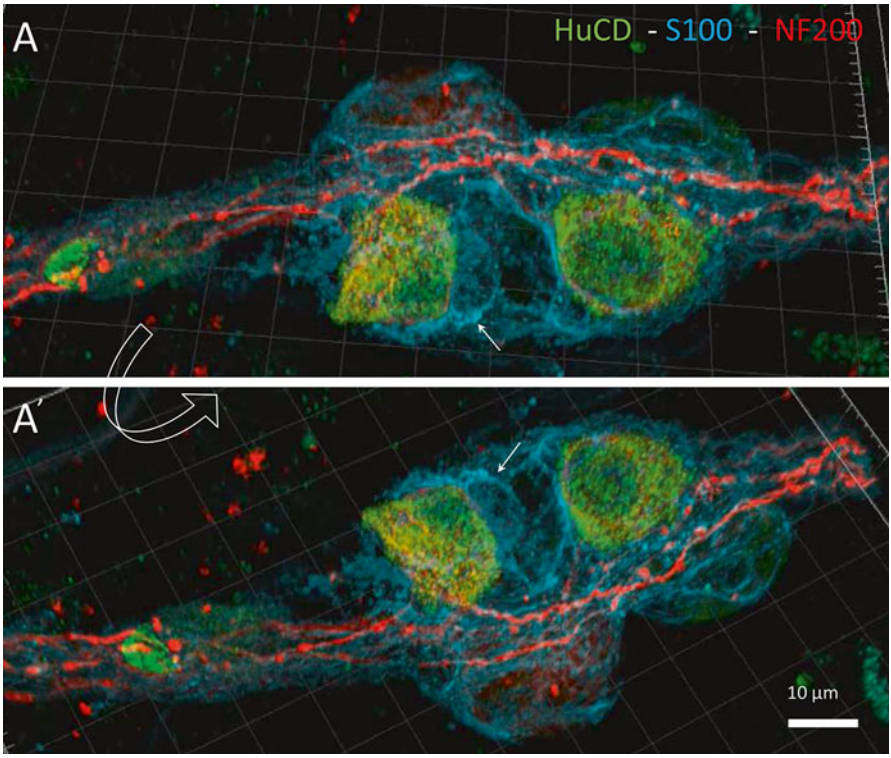

Fig. 18.1 Triple labeling of a human submucous ganglion present in a routine duodenal biopsy. (**A**) and (**A′**) are snapshots taken from a 3D confocal stack of images recorded on a Zeiss confocal microscope and deconvolved using Huygens (SVI). The *white arrow* indicates a glial cell (as identified by S100 antibody labeling) in close apposition to a nerve cell (HuCD) and neuronal fibers (NF200)

obviously depends on the hardware of the recording system. However this is often not the limiting factor, as recording speed may well be determined more importantly by the signal to noise (S/N) ratios that can be obtained. Using genetically encoded Ca^{2+} sensors like GCaMP (Zariwala et al. 2012; Boesmans et al. 2013) high signal to noise ratios can be obtained much more easily than with bulk dye loading techniques (e.g. Fluo4).

Fast Imaging

Virtually all knowledge about action potential generation and propagation is based on micro-electrode and patch-clamp techniques, which measure electrical signals directly. These methods have generated invaluable and crucial information about the nature of the underlying ionic conductances. Although electrical recordings

cannot be beaten in terms of temporal resolution, the spatial aspect of electrode recordings is very poor, as data can only be collected from a limited number of sites, mostly only one, being the postsynaptic cell.

Although voltage sensitive dyes (V_m dyes: di-4-ANEPPS, RH 484) have been around for several decades, their relatively poor quantum yield—compared to some Ca^{2+} indicators—has prevented widespread use of these dyes. Given the improved stability of later generation V_m dyes (di-8-ANEPPS) and enhanced sensitivity of the recording equipment (Obaid et al. 1999; Neunlist et al. 1999), V_m dye recordings have become a lot more efficient. In order to record these fast events dedicated cameras are necessary to collect a sufficiently large signal at kHz frequency. Maximum temporal resolution can only be obtained using widefield fluorescence microscopy in combination with high speed (EMCCD's and sCMOS) cameras designed for fast acquisition (e.g. Obaid et al. 1999, 2005; Neunlist et al. 1999; Buhner et al. 2009). In the enteric nervous system di-8-ANEPPS recordings have been used successfully to monitor action potential discharge and fast excitatory post synaptic potentials elicited by different mediators (e.g. histamine) but also by extracts from mucosal cells obtained from patients with inflammatory bowel diseases (Buhner et al. 2009).

As an alternative to voltage sensors, also Ca^{2+} indicators can be used to monitor neuronal activity. These bright fluorescent molecules have been used as a surrogate for recording nerve activity because of the tight correlation between action potential firing and cytosolic Ca^{2+} events, of which the amplitude is related to the number of action potentials (Hillsley et al. 2000; Vanden Berghe et al. 2002). Although Ca^{2+} imaging has provided invaluable information, with classic image recording speeds, Ca^{2+} transients appear as relatively uniform events. However, the initial rise in cytosolic Ca^{2+} rise involves many different processes such as Ca^{2+} channel opening, release and uptake from intracellular stores and mitochondria. We found that the upstroke in cytosolic Ca^{2+} proceeds stepwise (Michel et al. 2011), reflecting the contribution of individual channels or channel clusters. Ca^{2+} recordings with this level of detail are only possible with cameras that are fast (kHz frequency) and sensitive enough to collect the limited number of photons generated in a millisecond time period. In a recent paper we describe such a microscope configuration, in which speed is combined with relatively high resolution (512×512 pixel CMOS camera chip). This setup makes it possible to record fast Ca^{2+} transients in individual varicosities. This technique allows one to discriminate between pre- and postsynaptic activity in an all optical way (Martens et al. 2014) and is a promising tool to investigate synaptic circuits in the enteric nervous system.

Non-Linear Optical Approaches

When light hits matter it is either absorbed or scattered (reflection, refraction or diffraction), which renders objects color, make them transparent, reflective or opaque. These daily life optical interactions obey a linear law, in that the effect

produced bears a linear relationship with the intensity of the incident light. However this linearity does no longer hold true for high light intensities as generated by high power pulsed lasers. The ensemble of effects produced by high intensity light sources is commonly termed non-linear optics (NLO) and because NLO effects depend quadratically on the incident light intensity they have the advantage to only occur in a small focal volume. Probably the best known phenomenon is 2-photon (2P) absorption, which can be used to excite fluorescent molecules with photons double the wavelength than normally used for single photon excitation. This specific excitation method has a number of important advantages. First, excitation only occurs at a confined (confocal) spot, which reduces phototoxicity as molecules above or below focus are not excited. Not only is 2P excitation intrinsically confocal, infrared light also penetrates significantly deeper into tissues due to reduced scattering, which makes it possible to excite molecules deep into living tissues. Penetration depth is definitely not infinite but at the moment sufficient to image through the entire intestinal wall of small rodents (Fig. 18.2).

A second imaging technique that is more recent and also relies on a NLO effect is second harmonic (SH) imaging. Due to the high intensity of the incident light, frequency doubling can occur, whereby a small amount of light is generated with

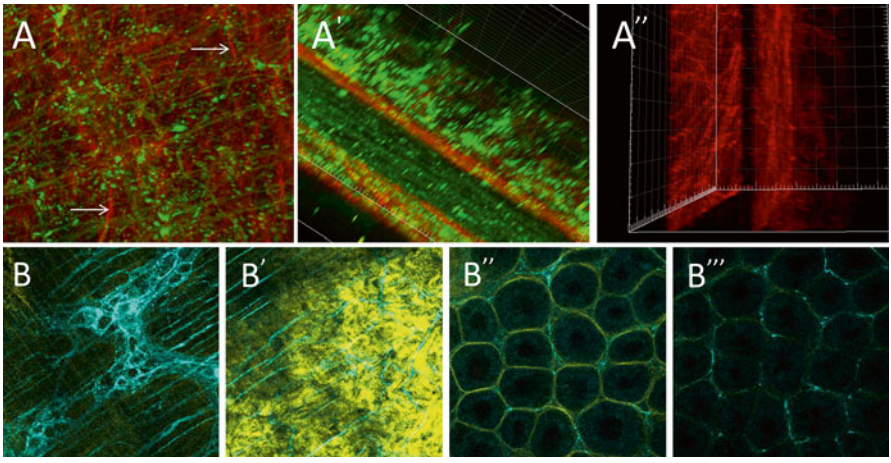

Fig. 18.2 Non-linear optical imaging of the mouse intestinal wall of the synaptopHluorin mouse (Li et al. 2005). This mouse expresses a green fluorescent protein in the fibers of some enteric neurons. Using 2-photon excitation (820 nm) it becomes possible to penetrate and image individual fibers as they extend into the mucosal layers in an intact and live intestinal preparation (*green* in **A**; *cyan* in **B**). Simultaneously with 2P excitation, frequency doubling occurs in the collagen layers (*red* in **A**; *yellow* in **B**). **A**, **A′**, **A″**: Shows snapshots of a 3D reconstruction of the synaptopHluorin as well as the collagen layers (*red*) in the mouse colon. Note that the collagen is present only in two very distinct layers (**A″**). (**B**): Confocal images taken at different depths in the synaptopHluorin mouse intestine. Fluorescence in *cyan*, second harmonic signals from collagen in *yellow*. Note that the fluorescence is readily excitable in myenteric and submucous plexus layers and remains detectable even several hundreds of μm into the crypt and mucosal layer of the intestinal wall (**B″** and **B‴**)

exactly double the frequency (half the wavelength) of the incident light. As this is intrinsically a scattering phenomenon, no absorption and therefore no photodestruction of a given molecule can occur. However not all molecules are capable of generating SH, only non-centrosymmetric molecules that are highly ordered display this effect. A number of endogenous biomolecules have this property including collagen, elastin and tubulin. Especially collagen generates SH very efficiently and can be used to image structural aspects of the intestinal wall (Fig. 18.2). Collagen and other extracellular matrix proteins, secreted by mesenchymal cells, cross link and arrange leading to altered physical fiber properties, which can be detected with these advanced optical techniques. We anticipate that this imaging technology will be useful to investigate structural changes that occur in inflammatory diseases like Crohn's disease (CD), which is characterized by relentless transmural inflammation of the intestine, leading to severe complications like fibrotic stenoses. Transmural strictures arise from extracellular matrix deposition (including collagen) and smooth muscle and myofibroblast hyperplasia. Several extracellular proteins secreted by mesenchymal cells (collagen 1-3, fibronectin) have been found in resection segments of patients with Crohn's strictures.

In conclusion, development of microscopy techniques is not at a standstill. Implementing novel microscopy strategies is of utmost importance to understand not only the cellular interactions in the planar ENS but even more so to investigate how information flows in three dimensions from the mucosa to the nerve layers, how this is influencing the control of blood flow and how that might depend on structural changes like collagen deposition.

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Chapter 19
Excitability and Synaptic Transmission in the Enteric Nervous System: Does Diet Play a Role?

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Abbreviations

5-HT	Serotonin
CCK	Cholecystokinin
P2X	Purinergic receptor

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Introduction

Dramatic changes in diet macronutrient composition can present many challenges to the gastrointestinal (GI) tract. The proportion of protein, sugar and fat may change due to personal decisions or in response to medical advice. The intestine must continue to process nutrients present in the changed diet, but to do so it must update its processing mechanisms (Neunlist and Schemann 2014). For example, the epithelium of the intestine responds quickly to nutrients by up- or down-regulating transporters and enzymes. The intestine responds to a state of over-nutrition (and indeed obesity) by increasing its absorptive capacity and, in times of scarcity, by down-regulating unused transporters. For example, a high sugar meal can rapidly cause new glucose transporters to be brought into play, allowing more efficient uptake—and this process may be exaggerated in diabetic patients (Young et al. 2013). The same is true for the levels of proteolytic enzymes released by the pancreas. A diet high in protein, fat or carbohydrate will lead to a progressively larger number of enzymes produced (Brannon 1990) to facilitate more efficient digestion. Indeed, the standard animal model of obesity using the high fat diet is associated with an increase in pancreatic transcripts for lipases (Birk et al. 2014), though levels return to baseline after several months. In addition to these direct effects, diet may affect the gut microbiome, which may have indirect effects on the ENS. For example, the level of non-digestible fibre or oligosaccharides in the diet can alter the relative proportions of bacteria in the gut (Halmos et al. 2015) and alter bowel behaviour (Halmos et al. 2014); although the clinical implications are unknown (Camilleri and Acosta 2014). An altered gut microbiome (Arrieta et al. 2014) is believed to be an independent regulator of intestinal function and has also been associated with obesity and other diseases such as a low grade inflammation of the GI tract. This was exemplified in a study where low dose administration of antibiotics to juvenile mice resulted not only in a shift in the microbiota populations detected, but also in an increase in adiposity in the animals (Cho et al. 2012).

The links between diet, inflammation and ENS function are complex, but one intriguing connection is through the multiple effects of the hormone leptin (Ob). In addition to its central effects on satiety in the hypothalamus, leptin activates submucous and myenteric neurons (Reichardt et al. 2011; Florian et al. 2013), and has inflammatory activity as an adipocytokine. Leptin has been recognised for some time as a pro-inflammatory cytokine (La Cava and Matarese 2004) and exerts its effects on immune function via expression of various isoforms of its receptor (ObR) expressed on a number of immune cell populations. For example, mice with diet-induced obesity exhibited decreased T-cell activation via ObR/STAT3 signalling (Papathanassoglou et al. 2006) providing the first clear evidence for a direct link between diet, leptin, and the immune response. It reasonable to speculate that this mechanism also has the potential to link change in ENS function with change in diet and/or obesity.

Changes in nutrient type and load are probably also dealt with in the ENS by adapting its motor and secretory programs (Neunlist and Schemann 2014).

For instance, the osmolality of the chyme is maintained between 300 and 400 mOsm/kg in the face of a large increase in solutes as digestive enzymes break down nutrients (Kalantzi et al. 2006). Secretion is under the control of submucosal secretomotor (and vasodilator) neurons which are themselves under control from other enteric neurons that link their activity with motor patterns (Furness 2006). Similarly, the ability of the ENS to switch from a mixing program to a propulsive program will decrease the time that luminal contents are present in the small intestine (thus increasing transit speed). This will also reduce the mixing of contents with enzymes and reduce the surface area of epithelium to which the contents are exposed—if taken to extremes, this will cause a reduced absorption of nutrients as can happen with chronic diarrhoea.

Recording from the ENS

The nerve circuits in the ENS responsible for motor and secretory programs are complex (Furness 2006). In order to understand how these programs operate, researchers need to understand how the excitability of an individual neuron is regulated and how synaptic transmission between functional classes of neurons changes with disease. In order to gain this type of detailed information research methods such as intracellular electrophysiological approaches have traditionally been used. While electrophysiological techniques have provided an excellent insight into ENS function in animal models of disease, unfortunately they are difficult, time-consuming and prone to sampling errors when dealing with large numbers of neurons.

These drawbacks become more significant when looking to uncover potentially more subtle diet-induced changes in ENS behaviour. For example, while acute inflammation causes clear pathophysiological changes to gut behaviour—and an increase in the excitability of enteric neurons (Linden et al. 2003; Lomax et al. 2005b; Nurgali et al. 2007)—over-eating and the subsequent development of an obese and/or a pre-diabetic state in humans is often associated with inconsistent changes in gut behaviour (Mushref and Srinivasan 2013). Whether there are subtle changes to enteric neuronal excitability or ENS function in these patients is even less clear (see below).

This review will focus on recent advances in fast imaging techniques that, using voltage- and calcium-sensitive dyes, allow recordings from many neurons on a scale previously unheard of (Vanden Berghe et al. 2001). These techniques promise to overcome many of the drawbacks listed above by allowing access to a wide range of ENS phenotypes simultaneously. However, do these techniques provide the same quality of information as do the traditional electrophysiological methods? We will explore the sorts of excitability changes in enteric neurons during dietary challenges that have been uncovered using these fast imaging techniques (Neunlist and Schemann 2014) and will compare these with electrophysiological recordings made from animal models of inflammation.

To directly record excitability changes in enteric neurons, the action potential (AP) and the fast excitatory post-synaptic potential (EPSP) are primarily of interest. In enteric neurons, the AP has a duration of ~1 ms and an amplitude of ~100 mV while the fast EPSP is longer at ~40 ms but with a smaller amplitude of ~10 mV). Thus to record both, the recording technique requires both acquisition speed and dynamic range (resolution). To record the occurrence of an AP reliably, an acquisition frequency of at least 500 Hz is needed. The amplitude of the AP will be lost due to undersampling, but counts of the number of APs will be accurate. With an electrophysiological approach, the speed of acquisition is really only limited by the high serial resistance of the sharp electrode—in practice, rates of >10 kHz yield usable data. For an imaging approach, the speed of acquisition has to be balanced against the amount of light gathered. The faster the acquisition rate, the less light is gathered which means only large signals can be seen. Current imaging cameras such as the popular Redshirt CCD system allow a 1 kHz acquisition rate with usable signal levels and full spatial resolution of 80×80 pixels. Because the AP has such a large amplitude, the relative change in fluorescence (ΔF/F) required is only ~1 %/100 mV. For the longer fast EPSPs, a slower acquisition rate is acceptable, perhaps 100 Hz, but the low amplitude means that a better signal to noise ratio is required such as ΔF/F of ~10 %/100 mV. Worse, if changes in fast EPSP amplitude are being investigated then 1 mV resolution would be ideal. Electrophysiological approaches have both the resolution and the high signal to noise ratio required to see 1 mV changes in membrane potential reliably. With the current imaging techniques, a 5 mV change is probably at the limit of detection.

For the remainder of this mini-review, we will cover what traditional methods have told us about cell body excitability and synaptic transmission in enteric neurons, particularly within the context of animal models of inflammation. We will then look at diet-induced changes in enteric neurophysiology revealed by studies using fast-imaging techniques and critically review whether the lessons learnt from inflammatory changes are relevant and sufficient for understanding diet-induced reprogramming.

Changes in ENS Excitability During Inflammation

Most work on disease-driven changes to the ENS has been done using animal models of intestinal inflammation with a view to replicating some aspects of human Inflammatory Bowel Diseases (IBD) (Lomax et al. 2005a). These are the same types of changes—though perhaps less extreme—that we might expect to find during obesity or a pre-diabetic state, each of which have been associated with a low grade inflammation in the GI tract. Thus, it is worth reviewing the kinds of changes seen in enteric neurons during a severe inflammation as seen in animal models. There are a variety of animal models of GI inflammation including chemically induced or post-infection (Strober et al. 2002; Goyal et al. 2014). However, of all

the models commonly used, the majority of electrophysiological recordings have been obtained from the 2,4,6-trinitrobenzene sulfonic acid (TNBS) model of colitis in guinea pigs.

Cell Body Excitability of Enteric Neurons During Inflammation

The cell body of single enteric neurons can be recorded from within in vitro preparations that have much of the local circuitry intact. Sharp conventional electrophysiological techniques can be used to record from the enteric cell body at rest, during current injection, agonist ejection, and stimulation of incoming nerve fibres to elicit synaptic potentials.

The properties of the enteric neuronal soma include the resting membrane potential (RMP), input resistance (R_{in}) at rest and the numbers of APs elicited by stepped current injection. The RMP and the R_{in} at rest can be compared with similar neurons across preparations or between models of disease. However, damage during impalement can contribute to a depolarised RMP and generally a lower R_{in}. If the tissue is from an inflamed gut, then difficulties with dissection and impalement can cause more damage than in control tissues making comparisons difficult. To help control for these issues, many studies also use another measure of excitability. When current is injected via the recording electrode, how readily the cell body fires an AP can be determined. Some neurons may fire an AP at rest (without current injection), but generally a step increase of injected current is used to allow the number and frequency of AP firing to be recorded. In order to establish a stable comparison between different neurons in control versus an inflamed state, many studies use rheobase and the number of APs at 2x rheobase as a measurement. Rheobase is the minimum current required to elicit a single AP and 2x rheobase is an injection of twice that current. This allows differences in excitability to be recorded that complement alterations in RMP and resting R_{in}, but that are less susceptible to the condition of the tissue. For example, most AH neurons in the TNBS model of inflammation had more APs at 2x rheobase, while other membrane measures were unaffected (Lomax et al. 2005b) though a subset of AH neurons also had a depolarised RMP and increased R_{in} (Nurgali et al. 2007). S neurons in the TNBS model likewise showed no changes in resting properties but a subset of orally projecting neurons had an increased number of APs at 2x rheobase (Linden et al. 2003).

Another measure of cell body excitability examines the after-hyperpolarising potential (AHP) following an AP. Many neurons in the ENS have a rapid and/or sustained AHP that, when active, can severely reduce the number and frequency of APs elicited by depolarisation. Thus, the presence or absence of the AHP in the AH/sensory neurons is another measure of excitability (Linden et al. 2003) which computer modelling suggests is the key to the overall state of the circuitry (Thomas et al. 2000). In myenteric and submucosal AH neurons from TNBS treated animals, the AHP was substantially reduced (Linden et al. 2003; Lomax et al. 2005b; Nurgali et al. 2007).

Fast Synaptic Transmission in the ENS During Inflammation

Most enteric neurons respond to electrical stimulation of interganglionic fibre tracts with the generation of a fast EPSP. Enteric neurons in the electrophysiological 'S' class have fast EPSPs by definition, while under some conditions AH neurons (those with the sustained AHP) have occasionally been seen to have a fast EPSP. In S neurons, at least three ligand-gated ion channels contribute to fast EPSPs, with the nicotinic receptor sub-types accounting for the majority in myenteric (Galligan 2002) and submucous (Monro et al. 2004) neurons. There are also P2X-mediated fast EPSPs (Galligan and Bertrand 1994), especially between particular sub-types of enteric neurons (e.g., descending interneurons to inhibitory motor neurons (Bian et al. 2000)), and a relatively minor contribution from 5-HT_3 receptors (e.g., in descending motor pathways (Monro et al. 2002) and secretory pathways (Monro et al. 2008)). The P2X and 5-HT_3 receptor subtypes involved are not clear, but there is evidence that P2X2 and P2X3 receptors are major players (Ren et al. 2003) while mRNA has been found for a variety of 5-HT_3 receptor sub-types (Kapeller et al. 2011).

Although nicotinic fast synaptic transmission between enteric neurons is the most common in all species studied (including humans), the proportion of nicotinic neurotransmission can change in animal models of inflammation. In particular, Lomax et al. showed that the larger fast EPSPs in the TNBS-inflamed submucosal neurons were due to an increase in non-nicotinic transmission via 5-HT_3 and P2X receptors (Lomax et al. 2005b). As mentioned above, non-nicotinic transmission may be more important at particular functional types of synapse, thus, inflammation induced changes may have differential effects on these pathways. Finally, while the S neurons have fast EPSPs that are altered during inflammation, Linden et al. found the myenteric AH neurons that normally do not have a fast EPSP were more likely to have a nicotinic fast EPSP in TNBS-inflamed distal colon (Linden et al. 2003).

Most if not all enteric neurons also respond to trains of electrical stimuli at interganglionic fibre tracts with a slow EPSP mediated by G-protein coupled receptors. Slow EPSPs are also associated with increases in cell body excitability and, thus, it is not surprising that in neurons from inflamed tissues, the duration of the slow EPSP is often longer lasting (Nurgali et al. 2007).

Studies of Enteric Neurons During Obesity and/or a High Fat Diet

Because of the short-comings of traditional electrophysiological methods discussed previously, recent studies have opted to use fast imaging techniques (Vignali et al. 2010) using voltage or calcium sensors to examine diet-induced changes to ENS programming.

One of the first studies to look at changes to the ENS that might accompany alterations in diet was Reichardt et al. (2011) where the anorexigenic hormone

leptin was examined. Leptin is released from adipocytes and supresses central appetite signalling and their hypothesis was that peripheral effects of leptin at the level of the ENS were present. They used a fast voltage-sensitive dye (di-8-ANEPPS acquired at 1000 Hz) to show that microejection of leptin onto an enteric ganglion excited a small population of both myenteric and submucosal neurons from the guinea pig colon. The excitation consisted of a low frequency train of APs with a time course similar to that seen when nicotine was used. In the case of pressure ejected nicotine, the APs are generated by a large depolarisation mediated by the nicotinic receptor; as characterised in electrophysiological studies (e.g., Galligan and Bertrand 1994). In the case of leptin, the constraints of high pass filtering of the imaging data do not allow a slow depolarisation to be seen directly, but the data are consistent with leptin causing a low amplitude depolarisation leading to AP firing. It is well accepted that in high-fat diet induced obesity, there is a high level of blood leptin due to increased adiposity while leptin resistance is also developed subsequently which can compromise the action of leptin in the brain. Whether the same leptin resistance is developed in the obese ENS is currently unclear.

The first study to examine changes to the ENS as a result of altered diet was Baudry et al. (2011). They examined the antrum of mice fed a Western diet, that was a modified chow which was high in fat and simple sugars, for 12 weeks. 'Fast' calcium imaging (Fluo-4 acquired at 40 Hz) recorded calcium transients and absolute levels following a train of electrical stimuli to an interganglionic fibre tract. The calcium transients are likely to represent a fast EPSP with an AP riding on top (Shuttleworth and Smith 1999; Vanden Berghe et al. 2000; Michel et al. 2011), while an increase in the absolute level of intracellular calcium may represent a depolarisation of the membrane potential similar to a slow EPSP (Vanden Berghe et al. 2000). It was shown that the Western diet was not associated with a change in the number of calcium transients (i.e., APs) following nerve stimulation, but that the slow increase in baseline calcium was >50 % larger. The slow increase could relate to a larger individual fast EPSP with a greater accumulation of calcium, and/or there may be a larger slow EPSP that also contributes to the increased baseline.

Roosen et al. (2012) investigated how enteric neurons from guinea pig respond to the neurotransmitter/enteroendocrine transmitter 5-HT, the enteroendocrine transmitter cholecystokinin (CCK), and the orexigenic hormone ghrelin. Ghrelin is released from enteroendocrine cells in the stomach and intestine to increase central appetite signalling while on the other hand CCK and 5-HT are likely to cause suppression of appetite signalling. 'Fast' calcium imaging (Fluo-4, probably acquired at 40 Hz) showed that enteric neurons from fasted guinea pigs were more sensitive to ghrelin while re-fed animals were more sensitive to CCK. Although not truly fast, the calcium signals have been shown to correlate well with AP firing (Martens et al. 2014). Electrical stimulation of fibre tracts caused more neurons to respond with larger responses in re-fed animals versus fasted. In contrast, ghrelin produced larger calcium transients in neurons from fasted animals. Moreover CCK caused a greater number of neurons to respond in the re-fed state, while 5-HT responses were unaltered. A solution with high potassium (75 mM) was used to depolarise the myenteric neurons in a way similar to current injection. There was an

increase in the amplitude of the calcium transient following high potassium in re-fed animals suggesting that the membrane potential may already have been partially depolarised, and thus more excitable, in the re-fed state.

Most recently, Reichardt et al. (2013) used mice fed a Western diet to examine changes in myenteric neuron excitability in the mouse distal colon. A fast voltage-sensitive dye (di-8-ANEPPS acquired at 1000 Hz) enabled exploration of changes in response to microejection of the neurotransmitter analogues nicotine and 2-methyl 5-HT that activate receptors that normally mediate enteric fast EPSPs. The main finding from this research was that 12 weeks of the Western diet increased the number of neurons responding to nicotinic and serotonergic activation from 5 to 10 % while the frequency of AP discharge was unaffected. The correlation between weight and magnitude of response was significant with the heaviest Western diet fed mice having the most responsive neurons to both agonists. The increased responsiveness to nicotine and 2-methyl 5-HT suggests that fast EPSPs in a population of neurons would also be larger. Unfortunately, the types of neurons activated were not revealed by immunohistochemical processing.

Together, these types of studies show that diet can change how the ENS processes information. However, the measures of excitability used by these studies are quite different from those used in the intracellular electrophysiology studies examining inflammation. Rather than using depolarising current to look at action potential discharge, imaging studies have relied mainly on exogenous application of agonists such as compounds thought to be involved in the regulation of food intake or those that are involved in fast synaptic transmission. To date, no imaging studies have looked at fast EPSP amplitude or composition during changes in diet. However, such studies are technically possible as the voltage sensitive dyes have the resolution to detect fast EPSP amplitude directly (Bertrand et al. 2009); such that the proportions of nicotinic versus other fast transmitters can be assessed in diet-induced obesity.

Key Methodological Issues for Fast Imaging of Enteric Neuronal Excitability

Do these fast imaging techniques replace or complement traditional electrophysiological approaches? Several key issues and potential short-comings remain with the imaging studies such as: can individual neurons be uniquely identified by their neurochemical code (or projection pattern); if fast and slow synaptic transmission can be assessed quantitatively; whether signals can be uniquely related to single neurons, dendrites or fibres of passage; and can changes in neuronal activity equate to a difference in organ behaviour.

The functional identity of the neurons recorded from is crucial to understanding their role in the ENS circuitry. The functional class is generally determined by post-experimental immunohistochemical processing to reveal key neuronal markers. While individual neurons can be uniquely identified following intracellular recording (and injection of a marker such as neurobiotin), fast imaging studies must rely on

the position of the neuron within the ganglia to match images of activity versus images of neuronal markers. To complicate matters, initial images are in fresh tissue, while latter images are of fixed tissues with sometimes distorted tissue dimensions relative to that in situ. One step forward in this area is the use of genetically encoded calcium indicators (Boesmans et al. 2013). Currently these indicators are expressed in all enteric neurons and thus give much the same data as exogenously applied indicators, but directed expression in a particular class of neuron (e.g., NOS expressing neurons) would provide much needed data on how a population of such neurons behaves within the ENS circuitry.

Typically, different classes of enteric neuron are dispersed within a ganglion; though there are some rules of thumb (Smith et al. 1999). In practice, this means that dissimilar neurons are often immediate neighbours. With intracellular recordings from the cell body, electrical signals are restricted to a single compartment (i.e., the neuronal soma) with changes in electrically distant parts of the neuron (such as the dendrites) contributing less to signals than those at the cell body. With imaging, all parts of the neuron can theoretically contribute to the signal, but in practice, the dendrites of one neuron may be too spatially distant, or indistinct to be able to attribute to a particular cell body. Fibres of passage also contribute to the electrical signal in voltage sensitive dye experiments, and care must be taken to restrict regions of interest to the main soma of neurons. In small ganglia with only a single layer of neurons this is less of a problem, while large myenteric ganglia with two layers of neurons and fibres can present more significant technical issues.

Whether the changes seen in individual neurons equate to differences in whole organ behaviour has been a perennial problem when examining enteric neuron activity. Sharp electrophysiological recordings suffer greatly in this regard as only a few neurons of a particular class may be recorded from during a series of experiments. Changes seen in a few neurons may not equate into changes in a large number of neurons, and with gut behaviour most responses are likely to be due to large numbers of neurons (e.g., the migrating motor complex—MMC (Thomas et al. 2004)). Fast imaging can record from many more neurons increasing the likelihood that any changes seen in a particular class of neuron are typical, and thus may translate into a change in organ physiology.

Conclusions

Under normal conditions, the gut is well-equipped to extract nutrients from a meal. In times of food scarcity this is a benefit, but in times of plenty this system may extract too many nutrients, contributing to obesity. In this situation, the ENS needs to learn to accommodate and potentially ignore large nutrient loads. Conversely, diseased or shortened intestine may struggle to avoid malnutrition and perhaps there is a way in which the ENS can be fine-tuned to allow greater absorption. Recent studies have shown that diet and/or obesity can change how the ENS processes information. Lessons learnt from inflammatory changes in the ENS provide a good starting point in trying to understand diet-induced reprogramming.

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Chapter 20
Recording In Vivo Human Colonic Motility: What Have We Learnt Over the Past 100 Years?

Phil G. Dinning

Introduction

The human colon is one of the least understood organs of the human body. The conspicuous lack of understanding about its day-to-day functioning is particularly evident in our relatively simplistic understanding of how it fills and empties its content. Many disorders arise from suspected abnormalities in colonic contractions yet, due largely to technical constraints, investigation of human colonic motor function still remains relatively primitive. A look through the majority of the current literature will reveal that studies of colonic motility show (1) anally propagating high amplitude propagating sequences/contractions; (2) low amplitude propagating sequences; (3) a large amount of non-propagating contractions; (4) period rectal and/or colonic complexes; and (5) the occasional episodes of orally (retrograde) propagating pressure waves. These descriptions are based largely upon colonic manometry recordings, with recording sites spaced at least 7 cm apart (the majority >12 cm; (Dinning and Di Lorenzo 2011; Dinning et al. 2010a; Scott 2003)). With the recent developed of high-resolution manometry catheters with sensors spaced between 1 and 2.5 cm the classification colonic motility patterns has been re-investigated and these new studies are highlighting the inaccuracies of low-resolution recording (Dinning et al. 2015). By examining studies of the past as well the current literature this paper will provide a summary of our current understanding of human colonic motility.

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Measurement of Colonic Motility

Themotor activity of the human colon stores, mixes and propels content. These motor patterns arise from interactions between; (1) spontaneous myogenic activity driven by pacemaker cells; and (2) enteric neural circuits, which are modulated by the chemical and physical composition of colonic contents. Enteric neuronal activity is also influenced byextrinsic parasympathetic and sympathetic pathways driven from the central nervous system. The measurement of colonic motility that arise from these interactions is achieved via two primary means; measurement of transit or measurement of the colonic contractility.

Measurement of Transit

After Wilhelm Conrad Röntgen discovery ofX-radiation in 1895 (Röntgen Ray or X-rays) (Underwood 1946), researchers were provided with a tool that allowed them to directly view radio-opaque solutions moving through the gut. With continued real-time X-ray recording, a swallowed bismuth meal could be tracked through the esophagus, stomach, small bowel and colon. One of the first proponents of this technique, Arthur Hertz published a paper in 1907 in which detailed the timing and movements of content through the entire digestive tract (Hertz 1907). This paper included one of the first descriptions of the movement of colonic content during defecation; with all content below thesplenic flexure evacuated and at the same time the contents of the ascending colon would move into the transverse colon.Retropulsion was also documented with Hertz observing that bismuth injected into the rectum later being seen in the transverse colon or caecum (Hertz 1907). A few years later Guido Holzknechtg provided a detailed description of the colonic mass movement on the basis of direct observation under fluoroscopy "*the haustral segmentation evident in the ascending and transverse colon, disappeared as the colonic content shifted to the descending colon. Once the movement was complete the haustral indentations soon reappeared*" (Holzknechtg 1909). In 1913 Hertz published another paper of observations in which he noted that a chief stimulus for the mass movements of colonic content appeared to be the ingestion of food, a process he coined the "gastrocolic reflex" (Hertz and Newton 1913).

With the exception of these "large" observed events, the early studies of colonic transit concluded that the colon was inactive for most of the day and then in response to certain stimuli a series of coordinated contractions would occur that moved the stool towards the rectum. In patients with constipation similarX-ray images of a barium meal or barium enema indicated that this normal degree of coordination of colonic movements was diminished or absent with "incoordination of muscular motor function" being proposed as the basis of non-obstructive constipation (Kruse 1933). This observation was in stark contrast to the prevailing view at the time (and still today) that the colon in constipated patients is lazy or static.

As the dangers of radiation exposure become known prolonged, continuous X-ray recording of colonic motility ceased and were replaced by a series of static images taken at regular intervals. Using such techniques, in addition to description of the previously described mass movements, Ritchie et al. published details of "peristaltic ripples" (Ritchie et al. 1971). These were defined as a series of progressive contractions following one another along the bowel. Unlike the mass peristaltic events, that always travelled towards the rectum, the peristaltic ripples could moved in either an anal or oral direction at speeds of around ~1 cm/min. The studies by Ritchie also demonstrated retrograde movement of content from the sigmoid to descending colon (Ritchie et al. 1971) and an increase indistal colonic retrograde propulsion after a meal (Ritchie 1968). Additional studies utilizing radio-opaque disks or scintigraphy confirmed earlier findings that 50–100 % of colonic contents can be emptied during defecation (Halls 1965; Lubowski et al. 1995), while also showing that if defecation was withheld the contents in the rectosigmoid could undergo retropropulsion back to the transverse colon (Halls 1965). Other observational studies report abarium enema traveling from the rectum to the stomach in as little as 15 min (Alvarez 1967). More recently the real-time monitoring of an ingested magnet with the Magnet Tracking System (Hiroz et al. 2009) indicated "*retrograde displacement was clearly demonstrated as part of the colonic motility pattern in every colonic segment*".

On the basis of these transit studies while "mass movements" where associated with defecation and the movement of large quantities of content along the colon, retrograde or oral propulsion is also an important component of colonic motility.

Measurement of Colonic Contractility

While the studies above described the movement of content, they provided limited information on the real time contractility of the bowel wall. Such measurements have primarily been recorded through colonic manometry; techniques that measure intraluminal force/pressure. The initial manometry studies utilized water or air filled balloons attached to pressure transducers. These recording provided a single pressure trace, usually from the sigmoid colon, that indicated the colon was rarely inactive, as suggested by the earlier fluoroscopic studies, but actually consisted of almost continual pressure waves, that could increase in amplitude and frequency in response to a meal (Welch and Plant 1926). With recording largely confined to the sigmoid or distal colon, many studies in the 1940s and 1950s concentrated on sigmoid motor activity in relation to defecation. Kern et al. administered Acetyl-Beta-Methylcholine Chloride subcutaneously in healthy human subjects and demonstrated that the induced diarrhea was associated with diminished or inhibited motility in the sigmoid colon (Kern et al. 1949). In another patient with a transverse colectomy, the authors were able to record motility from the caecum, transverse colon and sigmoid colon. Using the same drug they demonstrated an in increase in proximal colonic motility and a completed inhibition of sigmoid motility, in association with stool expulsion (Kern et al. 1949).

This study supported earlier studies in dogs that suggested the "wave like" activity in the sigmoid colon played a role in preventing stool from reaching the rectum and that such activity was inhibited when defecation occurred (Galapeaun and Templeton 1938). Further studies in humans showed that sigmoid hypomotility was associated with patients with an "irritable colon" and diarrhea (Almy et al. 1950) and in patients with colitis the number of stool passed was inversely proportional to the amount of activity in the sigmoid colon; the less the activity the greater the stool frequency (Kern et al. 1951).

This sigmoid activity was shown to increase rapidly after a high calorie meal and could be inhibited by the anticholinergic drug, clidinium bromide, suggesting that the previously described "gastro-colonic reflect" was neurally mediated (Snape et al. 1979). More recent manometry studies using multiple channel manometry catheters recorded similar distal colonic motor patterns, that became know rectal motor complexes (Kumar et al. 1989; Rao et al. 2001). These motor patterns were thought to originate in response to the arrival of stool or gas, and thus act as a brake to untimely retard the flow of colonic contents and so keep the rectum empty (Rao and Welcher 1996).

In other regions of the colon balloon manometry catheters, introduced through colostomies were used to capture peristaltic events induced by laxatives or balloon distension (Hardcastle and Mann 1968; Ritchie et al. 1962; Torsoli et al. 1971). Through such stimulation these studies induced "colonic peristalsis", a likely equivalent of the mass movements described in the earlier radiological work (see above) and importantly showed that these peristaltic events were likely to be neurally mediated because their initiation could be blocked by pre-mucosal application of Lidocaine (Hardcastle and Mann 1968).

In the late 1980s the first of the prolonged recordings of spontaneous colonic activity were reported. Using water perfused manometry catheters colonoscopically placed into the transverse or ascending colon, motor patterns were recorded over a 24 h period (Narducci et al. 1987; Bassotti and Gaburri 1988). One of the most readily apparent motor patterns recorded in the healthy controls consisted of an array of large amplitude pressure waves recorded in adjacent channels. This motor pattern became known as the high amplitude propagating contraction (Narducci et al. 1987) and was believed to be the manometric equivalent of the previously described mass movement. These events were infrequent, occurring between 6 and 20 times per 24 h and were more prevalent after morning waking and in response to a high calorie meal. These motor patterns have been associated with spontaneous (Bampton et al. 2000) and stimulated (Kamm et al. 1992) defecation and while their initiation is incompletely understood, distension is likely to play a role (Bharucha 2012) as is intraluminal chemical stimulation (including chenodeoxycholic acid and short-chain fatty acids) (Torsoli et al. 1971; Kamm et al. 1992; Cook et al. 2000; Bampton et al. 2001). In patients with low transit constipation the frequency of these motor patterns is reduced or absent, suggesting that a potential neuropathy may exist (Bharucha 2012; Singh et al. 2013).

While the high amplitude events, became the primary focus of most colonic manometry studies, in reality they made up only a small percentage of the total

colonic motility patterns. The majority of the colonic motility consisted of lower amplitude pressure events, described as segmental activity, consisting of single pressure events or bursts of rhythmic/arrhythmic pressure events, most of which has been classified as non propagating (Bassotti et al. 2005). In health this activity increases throughout the colon (not just the rectosigmoid, as described earlier) after a meal or morning waking (Bassotti and Gaburri 1988; Bampton et al. 2001; Dinning et al. 2010b; Rao et al. 2004), whilst in constipation the colonic response to these stimuli is diminished or absent (Bassotti and Gaburri 1988; Bampton et al. 2001; Dinning et al. 2010b; Rao et al. 2004).

Associating Luminal Transit with Pressure Events

In 1971, using two to four microballoons and combined cinefluroscopy Torsoli et al., temporally associated laxative induced high amplitude propagating events with the movement of content in the transverse colon (Torsoli et al. 1971). Correlating scintigraphic images (one frame per minute) of spontaneous movements of content in the transverse, descending and sigmoid colon with manometry recorded from three recording port separated by 15 cm, Moreno-Osset et al. indicated a degree of association between pressure events and flow (Moreno-Osset et al. 1989). Whilst the manometry traces in that study were relatively simplistic there appeared to be an association between the direction of movement and the levels of motor activity in adjacent sites; content always moving from regions of high activity to low activity (Moreno-Osset et al. 1989). That same paper also suggested that up to 80 % of antegrade movements of content in the descending colon refluxed back into the transverse colon.

In patients with functional diarrhea a combined scintigraphic and manometry study indicated, in comparison to healthy controls, that a lack of distal colonic non-propagating pressure waves appeared to be associated with rapid transport of a radio-opaque tracer into the rectosigmoid after a meal (Bazzocchi et al. 1991). This study supported the finding from the manometric studies described above, which suggested that contractile activity in the sigmoid colon plays a role in preventing rectal filling.

By combining scintigraphic recordings (one frame per 15 s) with colonic manometry incorporating 12 sensors spaced at 10 cm intervals, Cook et al. found a temporal association between spontaneous high amplitude propagating sequences and luminal propulsion (Cook et al. 2000). However, only a third of antegradely propagating events were associated with bolus flow and the effectiveness of the bolus transport was dependent upon site of origin amplitude and velocity of the propagation (Cook et al. 2000). That study also found that a third of isotope movements were associated with apparent non-propagating motor patterns and a further third of movements were associated with no discernible motor pattern. Subsequent studies utilizing a higher scintigraphic frame rate (1 per 10 s) and a manometry catheter with 16 recording sites spaced at 7.5 cm intervals showed that 93 % of all identified

propagating motor patterns were associated with flow, however ~50 % of antegrade flow episodes still occurred with no apparent propagating activity and only 10 % of retrograde flow was associated with retrograde propagating motor patterns (Dinning et al. 2008). Indeed despite the fact that studies of colonic transit all report retrograde flow, very few studies report retrogradely propagating motor patterns, a fact that is likely to reflect the technical limitations of the manometric techniques used.

Future Direction

While the expect ion of some minor changes, for the most part colonic manometry has remained unchanged for the past 20 years. In contrast, during this period, esophageal manometry has seen some major technical advances, the most critical of which is the application of high-resolution manometry (Clouse et al. 1998; Williams et al. 2001). High resolution manometry catheters can contain up to 36 sensors, spaced at 1 cm intervals, providing a detailed profile of pressures along the entire studied region. On the basis of the spatiotemporal topographic maps produced from these data an entire diagnostic classification system, has been devised which aids in the treatment of esophageal and motility disorders (Bredenoord et al. 2012).

Until recently high resolution catheters of sufficient length to record pressures throughout the entire colon simply had not existed. However, over the past few years a few new catheter designs have begun to emerge. By using an area-under the curve analysis of data, high-resolution water perfused catheters (20 sensors at 2.5 cm spacing) have been used to help predict potential neuromuscular pathological phenotypes in children with slow transit constipation (Giorgio et al. 2013). While conversion of data recorded by a 36 sensor catheter (spacing between 4 and 2 cm) into a color topographical map, has been used to more clearly identify motor patterns that have been described previously, such as high amplitude propagating events (El-Chammas et al. 2014).

In both of these papers the data acquired through the higher resolution recording, failed to report any new motor patterns and it is notable that while the colonic motility was recorded with more sensors than had previously been used, the catheters failed to match the spatial resolution of catheters used in the esophagus. To achieve 1 cm spacing over length of the colon, an entirely new form of manometry catheter was developed. Utilizing fiber-optic sensor technology manometry catheters were developed with unto 144 sensors paced at 1 cm intervals (Arkwright et al. 2009). Utilizing these catheters, studies have shown that number of propagating events detected is very much dependent upon the sensor spacing used (Fig. 20.1). Doubling the sensor spacing from 1 to 2 cm nearly halves the number of propagating motor patterns detected, while moving from 1 to 3 cm spacing results in a 30 % chance of mislabeling propagating events (Dinning et al. 2013b). At 10 cm spacing ~60 % of all propagating events identified could be incorrectly labeled.

Of particular interest from the high-resolution recordings in healthy controls was the dramatic increase in the identification of retrogradely propagating motor patterns.

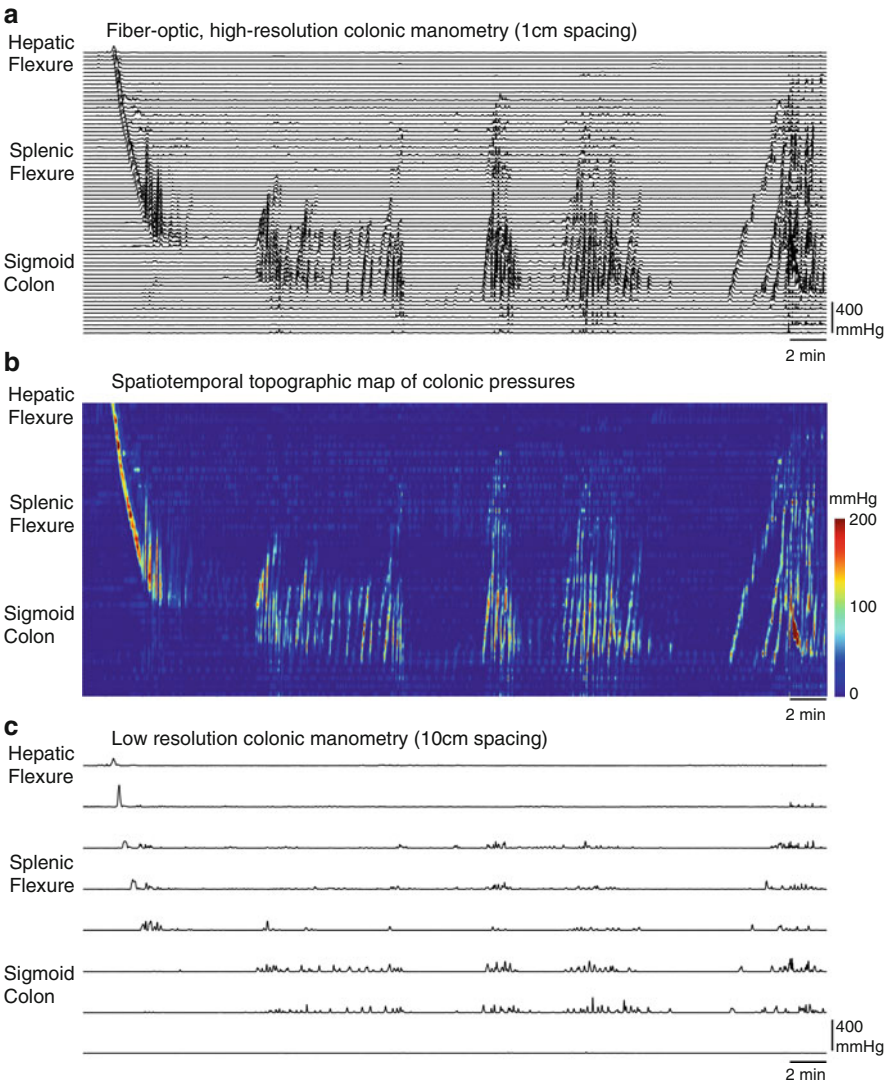

Fig. 20.1 Fiber-optic high resolution manometry trace record from a health adult colon. In (**a**) the entire colonic trace is shown. The same data in displayed as a spatiotemporal topographic map in (**b**). In (**c**) the same data is shown from every tenth sensor, essentially replicating a low-resolution colonic recording. Note that in (**a**) and (**b**) a series of retrograde propagating pressure events can be seen originating in the sigmoid colon. In the low resolution recording these motor patterns can not be identified

As in the previous section, in most studies of colonic manometry retrograde motor patterns were either not identified or were identified in small such small numbers that they hardly rated a mention. At one 1 cm spacing the retrograde motor patterns are by far the most prominent motor pattern identified (Dinning et al. 2013b, 2014).

These motor patterns were most commonly identified in the sigmoid and descending colon occurred in clusters of between 2 and 6 per minute (Fig. 20.1). It is likely that such motor patterns would impede anally directed flow and as such these data support the hypothesis proposed by previous studies that the sigmoid motor activity prevents contents from reaching the rectum until defecation is due to occur (see section "Measurement of Colonic Contractility").

Quantification of motor patterns recorded with these catheters in the healthy colon is only just beginning, however applying discriminant, logistic and cluster analysis of the shapes (gradient, duration, amplitude) of pressure events belonging to different motor patterns suggests that the motor appear to be generated by two independent sources, potentially indicating their neurogenic or myogenic origin (Dinning et al. 2014). The clinical worth and the potential diagnostic value of such recordings in patient groups is still to be determined.

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Letters

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January 29, 2014

Dear Marcello,

I very much regret that I am not with my colleagues and you at the ENS II 2014 meeting. Prior to your invitation, I had made a commitment to a symposium at the Salk Institute that is being held on exactly the same days.

I have especially warm memories of Australia and in particular of Monash University in Melbourne. I arrived in 1969 as a postdoctoral fellow to work with Mollie Holman. We agreed before I arrived that I would learn to use sharp microelectrodes to record from guinea pig taenia coli smooth muscle. Mollie told me on my arrival that she changed her mind. Instead of recording from taenia coli smooth muscle, I was to develop a method for recording intracellularly from the myenteric NANC neurons. Peter Crowcroft, a graduate student in Mollie's lab, and I worked for a month. We got frustrated because we had difficulty holding the impalements. We did manage to hold recordings from two neurons on January 28, 1969. For another three weeks, no success. Peter needed a successful research project for his Ph.D. thesis and I needed to generate new data to publish papers to justify a scholarship as a Fulbright Fellow. We turned our efforts to characterizing the biophysical properties of abdominal sympathetic ganglion neurons leaving the task of recording from enteric neurons to others including David Hirst, Alan North, S. Nishi and Jackie Wood. Jackie Wood, in fact, was already successfully recording from enteric

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neurons in 1969 back in the United States. As it turned out, they were far better than we were and each made seminal contributions to the literature.

Hooked on neurons, Peter Crowcroft and I choose to use sharp microelectrodes to determine the biophysical properties and synaptic transmission in peripheral sympathetic ganglion neurons. We recorded from hypogastric and pelvic ganglia but found it boring. We moved to recording from the inferior mesenteric ganglion. To our surprise, we serendipitously obtained evidence for cholinergic excitatory synaptic input to sympathetic ganglion neurons from mechanosensitive myenteric ganglion neurons in the colon. We had evidence at the cellular level for the existence of a peripheral reflex arc. The idea that peripheral sympathetic prevertebral ganglia function as integrating centers rather than as a relay station as proposed by J. N. Langley was novel and controversial and met considerable resistance from colleagues in the United States, Europe and Australia. The counter argument to our hypothesis was that the input was from *en passant* fibers. Mollie advised by saying that the soundness of our data would prevail. It did and it is now accepted that mechanosensitive myenteric ganglion neurons in the GI tract and sympathetic prevertebral ganglion neurons constitute a negative feedback loop that controls motility, secretion and absorption and that the prevertebral ganglia functionally link different regions of the gastrointestinal tract.

Mollie Holman, Archie McIntyre, Ian Mackenzie, a brief encounter with John Eccles, and time spent sharing an office with Sir Linder Brown who was on a sabbatical from Oxford inspired me to continue to investigate nerves and smooth muscle of the GI tract. My experience in Mollie's lab fueled my interest in neural and muscle control of gastrointestinal motility and the regulating function of neuropeptides. This interest continues to the present day and is now focused on those fleeting ephemeral messenger molecules NO, CO and H_2S.

When I look at the individuals listed in the three generations who will discuss their work, there can be no doubt that our field of enteric biology was and is in safe hands and will continue to be and that everybody working together will move our field to new heights.

Warm regards,

J.H. Szurszewski, Ph.D.
Professor of Physiology
Mayo Clinic Distinguished Investigator

Hi Marcello

I guess you slowly getting excited to be one of the patrons for the next two days. I wish I could be part of it and meet again a lot of friends. I always found it a privilege to be friends with the members of our little ENS community. I remember that there were some animosities among different labs in the older days. This may have been fruitful in some sense but I think it was good for the field that the next generation (I view myself as part of this) was much more collaborative and "friendly" although the discussions remained tough. I am convinced that this friendly atmosphere has also helped to advance the field and I hope that the current generation will proceed like this. Collaborations will be important to advance basic science.

Our field is in a perfect and may be unique position to also tackle the very important translational aspects because clinicians and basic scientist talk to each other and actually most of the time also listen to and understand each other.

I wish you all the success for the meeting. I am convinced it will turn out to be a great event, scientifically and socially. You have to remind some of the participants that they reached an age where the social events may be too tough on their liver.

I will certainly miss to talk to you again. I always enjoyed our chats on science and the other non-science related things in life. I still remember the first time we met at a meeting in Munich organised by Prof. Holle. After I gave my presentation on the circuitry in the gastric myenteric plexus we met in the restroom. While standing in front of the urinals you all the sudden turned around (fortunately only your head) and said: "*well this was a nice talk Michael, but the circuit at the end was it a God that told you this or do you have any data?*". Till today I tell my students this story every time they present too speculative conclusions. Of course, needless to say that I was right with the circuit after all.

All the best also for the "little-brain gang" you gathered in Adelaide

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