The Significance of Interstitial Cells in Neurogastroenterology

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Smooth muscle layers of the gastrointestinal tract consist of a heterogeneous population of cells that include enteric neurons, several classes of interstitial cells of mesenchymal origin, a variety of immune cells and smooth muscle cells (SMCs). Over the last number of years the complexity of the interactions between these cell types has begun to emerge. For example, interstitial cells, consisting of both interstitial cells of Cajal (ICC) and platelet-derived growth factor receptor alpha-positive (PDGFR α ⁺) cells generate pacemaker activity throughout the gastrointestinal (GI) tract and also transduce enteric motor nerve signals and mechanosensitivity to adjacent SMCs. ICC and PDGFR α ⁺ cells are electrically coupled to SMCs possibly via gap junctions forming a multicellular functional syncytium termed the SIP syncytium. Cells that make up the SIP syncytium are highly specialized containing unique receptors, ion channels and intracellular signaling pathways that regulate the excitability of GI muscles. The unique role of these cells in coordinating GI motility is evident by the altered motility patterns in animal models where interstitial cell networks are disrupted. Although considerable advances have been made in recent years on our understanding of the roles of these cells within the SIP syncytium, the full physiological functions of these cells and the consequences of their disruption in GI muscles have not been clearly defined. This review gives a synopsis of the history of interstitial cell discovery and highlights recent advances in structural, molecular expression and functional roles of these cells in the GI tract.

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Key Words

Enteric nervous system; Receptor, platelet-derived growth factor alpha; SIP syncytium; Slow waves; TMEM16A protein, mouse

Introduction and Background

Interstitial cells of Cajal (ICC) were first described by the Spanish neuroanatomist Santiago Ramón y Cajal. Cajal identified these cells using histological staining techniques available at the

time, specifically Golgi's silver impregnation method and Ehrlich's vital methylene blue method.¹⁻⁴ However, due to the fact that these methods were widely accepted to be superior stains for neurons, he arrived at the logical but erroneous conclusion that these cells were primitive, accessory neurons.^{1,3,4} Despite this initial description, he later correctly hypothesized that these cells were act-

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ed upon by “principal” components of the nervous system and they in turn influenced the contractile activity of neighboring smooth muscle cells (SMCs).⁵ Soon after, Sir Arthur Keith, the anatomist who first described the sino-atrial pacemaker system in the heart, postulated that the cells identified by Cajal may act as a pacemaker system for the intestinal musculature.^{6,7} It should also be mentioned that during these early years of ICC research the cells were also misidentified as connective tissue cells^{8,9} and Schwann cells.^{10,11}

In the 1950s and 1960s, the expanding use of electron microscopy (EM) facilitated more detailed investigations into the nature of ICC. Unfortunately, some of the initial EM studies seemed to reinforce the idea that ICC were neurons.¹² Further confusion stemmed from the inability of investigators to effectively correlate “light- and electron microscopy of tissue sites containing both ICC and fibroblasts.”¹³

Jacques Taxi sought to clarify the situation by employing Cajal’s original staining methods in combination with both light and EM.^{14–16} His skill and experience with these techniques led him to reach conclusions different from previous EM studies, namely that ICC being distinct from neurons, Schwann cells, SMCs, fibroblasts and macrophages. He concluded that ICC are neuron-like cells but only due to their propensity to co-stain with neurons.^{14–16}

Seminal studies by Imaizumi and Hama,¹⁷ and Yamamoto¹⁸ ushered in the modern era of ICC research when they used new tissue preparation techniques and concluded, from ultrastructural evidence, that ICC may be involved in transmitting stimuli between neurons and smooth muscle. Faussone-Pellegrini et al¹⁹ also suggested that ICC may act as intermediates in neurotransmission because they observed close contacts of ICC with nerve endings and with SMCs in human esophagus and stomach. They further concluded that ICC were specialized SMCs, rather than neurons or fibroblasts, due to the presence of smooth endoplasmic reticulum (ER), scarcity of rough ER and the presence of filaments. The fact that ICC were observed to be rich in mitochondria and poor in contractile filaments, similar to pacemaker cells in the heart, led them to suggest that ICC may be pacemakers of the gastrointestinal (GI) tract.¹⁹

Over the past 120 years the cells originally identified by Cajal have been classified in many ways and interest in them has waxed and waned as a consequence. This was best expressed by the German neurohistologist Stach²⁰ as quoted by Thuneberg¹³:

“As nerve cells or as part of the vegetative endformation, the ICC have played a very important part in the discussions of in-

nervation of effector tissue... As Schwann cells their importance undoubtedly became reduced. In the role of connective tissue cells (fibroblasts, macrophages) they almost lost importance. As specific innervated cells with demonstrated unequivocal relations to smooth muscle cells of the gastrointestinal tract the interest in them should increase again.”

Stach was correct, and research on ICC has exploded during the past 30 years. Technological advances, such as genetically modified mouse models and immunohistochemical identification of ICC, have resulted in a multitude of studies confirming that ICC are indeed the pacemakers of the GI tract and that they are also mediators of enteric neurotransmission. More recent studies will be reviewed in later sections in this article.

Until recently most discussion of interstitial cells referred to ICC, however it is now recognized that a second population of interstitial cells shares common anatomical spaces and imparts additional regulatory control in neurogastroenterology. The second class of cells, formerly referred to as fibroblast-like, is now known as platelet-derived growth factor receptor alpha-positive (PDGFR α^+) cells, due to identification of these cells by the prominent expression of this receptor tyrosine kinase. Smooth muscle cells are electrically coupled to ICC and PDGFR α^+ cells, forming a syncytium referred to as the SIP syncytium. The SIP syncytium is responsible for the behaviors of GI muscles classically referred to in the literature as “myogenic.” We now understand that the “myogenic” factors regulating GI motility result from the integrated behavior of the SIP syncytium. Thus, it seems clear that “*SIPgenic*” should supplant the term myogenic to include behaviors such as the setting of muscle membrane potential, regulation of basal excitability and generation of the pacemaker activity responsible for electrical slow waves, segmentation and gastric peristalsis. Tools are now available for rigorous cellular studies of SIP cells, and progress is being made to determine the role of these cells in GI physiology and pathophysiology. This review summarizes classic descriptions of interstitial cells and recent progress to understand the important roles these cells have in neurogastroenterology.

Subpopulations of Interstitial Cells of Cajal

The discovery that ICC could be specifically and reliably identified using antibodies against the tyrosine kinase receptor, KIT,^{21–25} has facilitated detailed examination of ICC distributions throughout the GI tract. As a result, several subpopulations

Figure 1. Illustration depicting the various subpopulations of interstitial cells of Cajal (ICC) and their relative locations in the gut wall from several organs. Subserosal ICC (ICC-SS), located between the serosa and longitudinal muscle layer, are present in the small intestine and colon of various species. ICC-MY are located within the intermuscular plane between the circular and longitudinal layers throughout the gastrointestinal (GI) tract of all species studied. ICC-IM represent intramuscular ICC located within the circular and longitudinal muscle layers and are a prominent population of ICC in the stomach and colon. ICC-DMP represent a population of cells within the specialized deep muscular plexus region of the small intestine. In the small intestine of primates ICC-IM are also found within the circular muscle layer. Submucosal ICC (ICC-SM) are found on the inner aspect of the circular muscle layer and are prominent in the gastric antrum and colon.

of ICC have been identified (based on their anatomical location in the muscle wall) and a short description of each class of ICC is provided in this section. They are listed in order of their location from the serosa to the mucosa of the walls of GI organs (Fig. 1).

Subserosal Interstitial Cells of Cajal

Stellate interstitial cells have been observed in the subserosa of various GI organs and species.²⁶ Methylene blue stained subserosal ICC (ICC-SS) in the mouse small intestine,²⁷ and KIT immunohistochemistry made their visualization possible in the mouse²⁸ and guinea-pig colon.^{29,30} ICC-SS have also been identified by EM and their ultrastructural features described.³⁰ In several studies the authors performed double label immunohistochemistry with antibodies to PGP9.5 (protein gene product 9.5), a pan neuronal marker. Their results showed no close relationship between ICC-SS and enteric nerve fibers.^{29,30}

In contrast to these studies, subpopulations of ICC-SS in the primate GI tract were closely apposed to enteric nerve fibers.³¹ A possible reason for this disparity is that the relationship between nerves and ICC-SS was found exclusively in taenia coli, thick

bands of longitudinal muscle that are found in primate colon but not in the colons of mice and guinea-pigs. Relatively few nerve fibers were observed in non-taeniated regions, and they were not closely associated with ICC-SS.³¹ A functional role for ICC-SS in the colon remains to be elucidated. Aranishi et al³⁰ have suggested that ICC-SS may be stretch receptors while the close apposition with enteric nerve fibers described by Blair et al³¹ suggests that a sub-population of these cells could be involved in neuroeffector transmission. Note that the morphology of ICC-SS depicted in Figure 1 most closely resembles that of ICC-SS found in the non-taeniated regions of the primate colon.

Myenteric Interstitial Cells of Cajal

Several terms have been used to denote the ICC located in the region between the circular and longitudinal muscle (i.e., region of the myenteric plexus). Originally, this type of ICC was referred to as ICC-AP,³² after Auerbach's plexus (Leopold Auerbach, 1828-1897). Later, when the term myenteric plexus replaced Auerbach's plexus in common usage, the groups of Daniel, Huizinga and Komuro used the term ICC-MP (myenteric plexus),³³⁻³⁵ while we prefer to employ the term ICC-MY (myenteric). ICC between the muscle layers do not penetrate into the myenteric plexus or the interganglionic tracts, but rather surround the neural structures, as can be seen from double label immunohistochemical images of gut wall cross sections.^{31,35} Thus, it seems misleading to call these cells ICC-MP, which implies they are part of or integrated into the myenteric plexus, as are glial cells. We believe the term ICC-MY properly describes the class of ICC that lies within the myenteric plexus region between the circular and longitudinal muscle layers but are not intrinsic to either muscle layer.^{36,37}

ICC-MY are found in most regions of the GI tract in all mammalian species studied. ICC-MY are multipolar and possess multiple primary processes which branch further contact and connect electrically with neighboring cells to produce a complex 3-dimensional network.²⁶ The ICC-MY network has been implicated as the primary pacemaker region in the stomach and small intestine and to be involved in generating higher frequency activity in the colon.^{21,23,24,38-41} Evidence suggesting that ICC-MY are the primary pacemakers of the GI tract is discussed later.

Intramuscular Interstitial Cells of Cajal

Intramuscular ICC (ICC-IM) are bipolar cells that are found throughout the circular and longitudinal muscle layers of organs in the GI tract and run parallel to the surrounding SMCs.

ICC-IM have been suggested to be critical mediators of enteric neurotransmission⁴²⁻⁴⁶; evidence for and against this will be discussed in later sections. ICC-IM also act as mechanosensitive regulators of pacemaker activity^{47,48} and play a role in the guidance of vagal afferent nerves that form intramuscular arrays in the stomach.⁴⁹⁻⁵¹

It should also be noted that ICC-IM may be capable of generating pacemaker activity in some regions. For example in the corpus of the stomach, primary pacemaker activity was attributed to ICC-IM.⁵² In the antrum ICC-IM have been suggested to be a means of propagation of slow waves from the greater to the lesser curvature,^{53,54} and in the distal antrum, ICC-IM are suggested as a means of generation and propagation of slow waves.⁵⁵

Interstitial Cells of Cajal of the Deep Muscular Plexus

An additional subpopulation of ICC-IM is found in the small intestine at the level of the deep muscular plexus (DMP; a non-ganglionated nerve plexus) and are referred to as ICC-DMP. The DMP is located close to the submucosal surface of the circular muscle and is separated from the submucosa by a thin layer of specialized SMCs.⁵⁶ Unlike ICC-MY at the myenteric plexus, ICC-DMP are heavily incorporated into the DMP, intertwined with the processes of motor neurons, and forming very close associations (<20 nm) with nerve varicosities.⁵⁷ Nerve processes and processes of ICC-DMP run in parallel with the long axes of SMCs, similar to ICC-IM.²⁶ Data have suggested that ICC-DMP are also involved in enteric motor neurotransmission.⁵⁸⁻⁶²

Septal Interstitial Cells of Cajal

ICC have also been observed in the septal regions (ICC-SEP located between and surrounding muscle bundles) of larger animals, including dogs,⁶³⁻⁶⁶ monkeys⁶⁷ and humans.⁶⁸⁻⁷² In larger animals the muscle layers are much thicker than in rodents and it has been suggested that the function of septal ICC is to facilitate propagation of slow waves through the muscle layer.^{65,68} Thus, ICC-SEP may provide a Purkinje cell-like function to facilitate slow wave propagation and coordinate excitation-contraction coupling through the entire thickness of the muscle. In the canine colon slow wave activity persisted in regions near septa after removal of the primary pacemaker region, along the submucosal border of the circular muscle layer, providing evidence that ICC-SEP are capable of generating pacemaker activity.⁶⁵ In the stomach ICC-SEP are capable of regenerative propagation of

slow waves over distances of at least 20 mm.⁶³ More recently, Ca^{2+} imaging experiments have demonstrated that in human small intestine, pacemaker activity originates in ICC-MY and is subsequently propagated along ICC-SEP into circular muscle bundles.⁶⁸

Submucosal Interstitial Cells of Cajal

Submucosal ICC (ICC-SM) have been identified in stomach and colon in several species.²⁶ They have been identified in the stomach of dogs,⁶³ mice⁷³ and rats⁷⁴ and in the colon of dogs,^{75,76} mice,⁷⁷ rats⁷⁸ and guinea-pigs.^{79,80} The axis of ICC-SM lies parallel to the underlying circular SMCs. However, they also have smaller processes that appear to make contacts with neighboring ICC-SM, forming a loose network.⁸¹ Studies of several animal species have suggested that ICC-SM are involved in pacemaker activity. For example, the submucosal surface of the circular muscle layer is the primary site for generation of slow waves in the canine colon,^{82,83} although pacemaker activity is also provided by ICC-MY that generate higher frequency oscillations known as myenteric potential oscillations (MPOs).⁸⁴ A similar hypothesis including 2 independent pacemakers has also been suggested for pacemaker activity in the rat⁸⁵ and mouse⁸⁶ colons.

Interstitial Cells of Cajal as Pacemakers of the Gastrointestinal Tract

After the first suggestion that the cells noted by Cajal might be pacemakers in the GI tract,^{6,7} others came to similar conclusions. For example, Ambache, who was the first to show that slow waves determined the frequency of intestinal contractions, suggested that ICC may be responsible for generation of slow waves.⁸⁷ During the past 30 years much progress has been made on the pacemaker role for ICC and this hypothesis is now widely accepted. The following sections detail the major points supporting the pacemaker function of ICC.

Interstitial Cells of Cajal Are Found in Pacemaker Regions

Electrophysiological studies on isolated portions of the GI tract wall showed specific regions within GI muscles in which pacemaker activity originates⁸⁸ and ICC populate each of these regions. In the stomach (corpus and antrum) and small intestine the pacemaker activity is generated in the myenteric plexus region,^{63,89-92} while in colon, pacemaker activity originates along the submucosal surface of the circular muscle layer and in the myen-

teric region between the circular and longitudinal muscle layers.^{75,77,82-86,93} It should be noted that there are other types of cells present in the pacemaker regions, such as enteric neurons, glia, immune cells and PDGFR α ⁺ cells. However, slow waves persist in the presence of tetrodotoxin⁹⁴ and in the GI tracts of mice when enteric neurons are absent.⁹⁵ Suzuki et al⁸⁹ also established that when circular and longitudinal muscle layers were separated slow waves were recorded only from muscle strips that still had ICC attached (identified by methylene blue staining), suggesting that slow waves are generated by the ICC in the myenteric plexus region.

Morphological Features of Interstitial Cells of Cajal Suggest They Are Involved in Pacemaker Activity

Several morphological and ultrastructural characteristics of ICC support the hypothesis that these cells are pacemakers in the GI tract. Gap junctions are present between neighboring ICC,^{63,64,96-100} which is important because ICC need to communicate in a network via low resistance connections to coordinate the spread of slow waves. ICC also form gap junctions with neighboring SMCs,^{63,64,97-100} an essential feature required to convey slow wave activity to SMCs (see later for a thorough discussion of gap junctions).

Another morphological observation with implications for ICC function is the presence of caveolae, which are known to have important functions in signal transduction. Caveolin proteins (caveolin-1, -2 and -3) act as scaffolds to recruit various proteins that work together in signaling cascades.¹⁰¹ The importance of caveolae in ICC and pacemaking was illustrated by Cho and Daniel¹⁰² who disrupted caveolae by either depleting or augmenting membrane cholesterol, and found that the frequency of phasic mechanical activity was reduced. They also demonstrated that several proteins involved in calcium handling (e.g., L-type Ca²⁺ channels and the Na²⁺/Ca²⁺ exchanger) were associated with caveolin-1 in ICC.¹⁰² Close associations occur between caveolae and peripheral ER, another prominent morphological feature of ICC.^{103,104} These authors suggest that proteins found within caveolae and peripheral ER act as a functional unit to control the local concentration of Ca²⁺, an important signal for generation of pacemaker activity by ICC.^{103,104} More recently, it has been shown that ICC are anoctamin-1 (ANO1) negative in mice that lack caveolae (caveolin-1 knockout mice).¹⁰⁵ ANO1 is a calcium activated chloride channel that is fundamental to the generation of slow waves,^{106,107} again suggesting an important role for cav-

eolae in pacemaker activity.

An abundance of mitochondria has been observed in ICC and these organelles are in close apposition to the ER and the plasma membrane (PM). Together these components form a "pacemaker unit" that contains the various ion channels, transporters and energy production capacity fundamental to the generation of pacemaker activity. Pacemaker units are subcompartments of ICC that have a restricted cytoplasmic volume formed between the PM, the ER and mitochondria. Tiny fluxes of ions within the volume of the pacemaker unit can achieve significant changes in ionic concentrations that are necessary to activate ion-sensitive ion channels and transporters. For example, small influxes of Ca²⁺ are thought to activate Ca²⁺-induced Ca²⁺ release and activation of Ca²⁺-activated Cl⁻ channels.¹⁰⁷ Pacemaker activity runs continuously, and restoration of ionic gradients within pacemaker units requires production of ATP to power ion pumps, such as the Na⁺/K⁺ ATPase (Na⁺ pump) and Ca²⁺ ATPase (SERCA pump). Thus, mitochondria also play an important role in maintaining pacemaker activity.

Slow Waves Are Absent in Animals Lacking Interstitial Cells of Cajal

In order to conclusively establish their importance in GI physiology several strategies have been employed to remove ICC from GI muscles. The first experiments removed areas rich in ICC by dissection.^{82,89,92} However, this approach left questions regarding specificity because it was impossible to remove only ICC without some damage to surrounding SMCs. Chemical lesioning with toxic substances that might accumulate in ICC was also used,¹⁰⁸⁻¹¹⁰ however this approach could also be criticized on the basis of non-specificity. For example, methylene blue is a substance that has been shown to be taken up by ICC in some regions of the GI tract in several species. In the mouse intestine proper handling allows fairly selective uptake of methylene blue in ICC and then exposure to light causes photo toxicity and loss of slow wave activity.¹¹⁰ Unfortunately tissues treated in this manner depolarize to potentials at which slow waves undergo depolarization block and therefore it was uncertain whether the inhibition of slow waves was due to the damage to ICC or to depolarization.¹¹¹

The discovery that ICC were selectively labeled by antibodies against the tyrosine kinase receptor, KIT, was a huge advance that allowed visualization of ICC in many different animal species.^{23,112} The role of KIT in development of ICC offered a novel opportunity to observe the functions of GI muscles in the

Figure 2. Loss of pacemaker activity in mice with disrupted interstitial cells of Cajal (ICC) networks. (A) Wild type mice with normal slow waves (a) lose slow wave activity (b) in the small intestine after intraperitoneal injection with the KIT neutralizing antibody ACK2. (B) Slow waves present in wild type controls (a) are lost in small intestines of mice (b) that have a mutation in the KIT receptor (W/W^V). Signaling via the KIT receptor (i.e., stem cell factor receptor) in ICC is essential for their survival. (C) SI/SI^d mice that have a mutation in stem cell factor, the ligand for the KIT receptor, lack slow waves in the small intestine (b) compared to wild type controls (a). Recordings in panels A, B and C were performed in the presence of L-type calcium channel blocker, nifedipine, in order to block muscle contraction and thus facilitate cell impalement. (D) Recordings from wild type and SI/SI^d mice in the absence of nifedipine: (a) Calcium action potentials are visible on the peaks of most regular slow waves in wild type mice. (b) In SI/SI^d mice irregular clusters of Ca^{2+} action potentials are observed in the absence of slow waves, demonstrating that the smooth muscle tissue is still capable of producing action potentials in the absence of ICC. Adapted from Torihashi et al (A),²³ Ward et al (B)²¹ and Ward et al (C and D).²²

absence of ICC. Studies of this type provided the first compelling evidence that ICC are pacemaker cells,^{21,23,24} mediate enteric motor neurotransmission^{42,44} and provide mechanosensitive regulation of pacemaker activity.^{47,48}

In 1992, Maeda et al¹¹² used a neutralizing antibody (ACK-2) to block KIT receptors in vivo. Newborn mice were injected with ACK-2 and the results revealed that the animals developed abnormal gut motility and distension of the gut wall, eventually producing lethal paralytic ileus in BALB/c mice. These mice displayed reduced KIT^+ cells in the affected gut regions.¹¹² However, Maeda and coworkers failed to recognize the KIT^+ cells as ICC because the cells were not able to be labeled with methylene blue and cell identity was not definitive from immunocytochemistry. Experiments of this sort were re-

peated by Torihashi et al,²³ and these studies confirmed the findings that mice treated with neutralizing antibodies from birth displayed significant reductions in ICC (ICC were identified by both methylene immunocytochemistry and EM). These studies also showed that ICC loss was accompanied by loss of slow wave activity in the small intestine (Fig. 2A).²³ Later studies showed that blocking KIT in organotypic culture also caused loss of slow wave activity in the intestine and stomach.^{113,114}

The KIT receptor is encoded by the W (dominant white spotting) locus in mice and the utilization of W/W^V mutants was another key step in confirming ICC as the pacemakers of the GI tract. W/W^V mutants are compound heterozygotes that have been used often as an experimental model because the W mutation, a complete ablation of the tyrosine kinase segment of the KIT re-

ceptor, is usually embryonic lethal.¹¹⁵ The W^V mutation is a point mutation that preserves partial function of the tyrosine kinase.¹¹⁶ Thus, W/W^V mice exhibit heterogeneous losses in ICC populations: ICC-MY of the small intestine are mostly lost, as are ICC-IM of the stomach, lower esophageal sphincter (LES) and pyloric sphincter.^{42,43} W/W^V mice lack pacemaker activity in the small intestine (Fig. 2B).^{21,24,117} Furthermore, similar observations have been made in steel-Dickie (Sl/Sl^d) mice, that have mutations in stem cell factor, which is the natural ligand for the KIT receptor (Fig. 2C and 2D).²² In addition to mice, *Kit* mutant rats (Ws/Ws) have also been shown to have lesions in ICC-MY in the small intestine^{97,118} and ICC-IM in the stomach.¹¹⁹ Intestinal motility was shown to be disrupted in these rats.¹²⁰ Smooth muscle tissues and cells appear to be unaffected in W mutants and are capable of producing Ca^{2+} action potentials, responses to agonists and contractile responses.^{21,22,42,44} The results of studies using neutralizing antibodies and genetic studies showed that subpopulations of ICC (i.e., ICC-MY in the small intestine and stomach) are responsible for the generation of pacemaker activity. Moreover, experimental models of obstruction, postsurgical inflammation and pathological conditions, such as diabetes, have also been shown to lead to decreased numbers of ICC and disruption of pacemaker activity.^{39,121-123}

Mechanisms Responsible for Pacemaker Activity and Slow Waves

Several mechanisms have been proposed to underlie the generation of pacemaker activity in ICC. Earlier studies were performed on intact muscle layers, but such studies are complicated by the fact that ICC are electrically coupled into a network and also coupled to SMCs and PDGFR α^+ cells. Drugs and ionic changes thought once to have selective effects on SMCs can have contradictory effects on different cells, making the interpretation of experiments quite difficult. Experiments on isolated cells identified voltage-dependent inward and outward currents⁴⁰ and a non-selective cation current¹²⁴ in cells identified as ICC. Numerous conductances have been reported in studies of cultured ICC, but (1) it is not always clear that ICC are actually the subjects of these studies because cells are not routinely identified unequivocally and (2) the phenotype of ICC appears to change rapidly in cell culture conditions. Due to the variable conditions of cell cultures, we will not spend much time discussing mechanisms derived from these cells in the present review.

In 2009, freshly dispersed ICC from murine small intestine were shown to express a Ca^{2+} -activated Cl^- conductance that ap-

peared to be the product of *Tmem16a* (now officially named *Ano1*).¹⁰⁷ Knockouts of *Ano1* failed to develop electrical rhythmicity in spite of the presence of normal numbers and appearance of ICC.¹⁰⁶ The disparity with earlier studies can be explained by the significant differences in the methods used. The most important difference was that by Zhu et al¹⁰⁷ who performed experiments on freshly isolated ICC, making use of mice with selective expression of a bright green fluorescent reporter (copGFP) in ICC. Goto et al¹²⁴ also used freshly dispersed ICC in their study, however these investigators failed to identify a Cl^- conductance in the cells studied.

Expression of ANO1 was first identified in ICC and cells of GI stromal tumors which were also KIT $^+$.^{125,126} Furthermore, a microarray study demonstrated that *Ano1* is one of the most highly expressed genes in ICC.¹²⁷ However, at the time of these studies the function of the protein encoded by *Ano1* was unknown. In 2008, 3 laboratories independently confirmed that *Ano1* encodes a Ca^{2+} -activated Cl^- channel (CaCC)¹²⁸⁻¹³⁰ and after these discoveries ANO1 protein was found in ICC throughout the GI tracts of several species including humans.^{31,67,106,131} Recordings from freshly isolated ICC demonstrated a CaCC that was activated by depolarization and sensitive to intracellular Ca^{2+} , extracellular Ca^{2+} and blockers of T-type Ca^{2+} channels.¹⁰⁷ The CaCC currents reversed at the Cl^- equilibrium potential and the single channels had a conductance of (7.8 pS), which was consistent with the conductance produced by expression of ANO1 in HEK293 cells (i.e., 8 pS).¹³⁰ The kinetics of the currents in ICC, however, differed dramatically from the kinetics of ANO1 channels expressed heterologously in model cells.¹³² These differences seem to be due to the fact that localized Ca^{2+} dynamics are responsible for activation of ANO1 in ICC and it appears to be difficult to regulate Ca^{2+} in the cellular compartment from which ANO1 channels are activated. A subcellular structure (pacemaker unit) that might be created by very close apposition of ER to the PM could create conditions needed for Ca^{2+} activation of ANO1 channels in these cells. Thus the slow wave currents activated in ICC have a voltage-dependent step, most likely voltage-dependent activation of Ca^{2+} entry superimposed upon the kinetics of CaCC activation.¹³³

It is also worthy to note that several splice variants of ANO1 are expressed, referred to as a, b, c and d.^{128,134,135} Ferrera et al¹³⁵ conducted patch clamp experiments on HEK293 cells expressing specific splice variants and discovered that the resulting channels displayed different properties. For instance, variant *b* was 4-fold more sensitive to calcium, whereas the time-dependence of acti-

vation at positive membrane potentials was attenuated in variant *c*.¹³⁵ Murine stomach and small intestine express variants *b*, *c* and *d*.¹⁰⁶ The consequences of the expression of different splice variants in ICC from different regions of the GI tract or at different times during development are not currently understood. A recent study documented altered splice variant expression in gastric muscles of diabetic patients,¹³⁴ and it was suggested that channel remodeling might participate in the aberrant pacemaker activity noted in diabetic gastroparesis.

Spontaneous Transient Depolarizations

Intracellular recordings from small bundles of GI smooth muscle have revealed a continuous discharge of spontaneous transient depolarizations (STDs; also known as unitary potentials).^{42,136-140} STDs result from spontaneous transient inward currents (STICs) that are generated in ICC and spread passively into electrically coupled muscle cells.¹⁴¹⁻¹⁴³ Within a small strip of smooth muscle there are many ICC, all of which might generate STICs. The stochastic nature of this discharge accounts for the noisy membrane potentials in these muscle strips.¹⁴⁴ STDs have important functions in pacemaker activity, including: (1) STDs are the basic electrical event initiating pacemaker activity in ICC. During the period between slow waves, STDs in ICC-MY increase in frequency and begin to summate until a threshold for activation of voltage-dependent Ca^{2+} channels is reached. Ca^{2+} entry coordinates the Ca^{2+} release events and facilitates active regeneration of other cells within the ICC network.¹⁴⁵ (2) In ICC-IM STDs may summate to enhance the plateau phase of the slow wave.^{53,137,144,146,147} Figure 3 depicts the events underlying generation of pacemaker potentials and slow waves in GI muscles.

Unlike the antrum, slow waves are not recorded in the fundus of many species. STDs cause the resting potentials of the SIP syncytium of the fundus to be somewhat more depolarized and may therefore contribute to the basal tone of this region of the stomach.¹⁴¹ Although STDs are generated in the ICC-IM of the fundus they do not summate to generate slow waves, possibly due to the lack of voltage-dependent Ca^{2+} channels in ICC-IM of this region.¹⁴⁸

Investigators have also postulated a link between STDs in ICC-IM and neural responses. For example, excitatory cholinergic stimulation increases STDs in the gastric antrum, and summation of these events can produce a positive chronotropic effect on slow wave generation.^{45,137,149} Hirst et al¹⁴⁹ concluded that during sustained vagal nerve stimulation summation of

STDs in ICC-IM could cause these cells to become the dominant pacemakers, driving the generally dominant ICC-MY. STDs have also been linked to inhibitory nitergic neurotransmission. Suzuki et al⁴⁵ noted that, in antral muscles, STDs were suppressed in response to neutrally released nitric oxide (NO). In the fundus they found that sodium nitroprusside, an NO donor, caused hyperpolarization of membrane potential, concomitant with a reduction in STDs.¹⁴²

STICs, and therefore STDs, depend upon intracellular Ca^{2+} . This conclusion is supported by the observations that thapsigargin, cyclopiazonic acid (SERCA inhibitors) and the Ca^{2+} chelator (BAPTA-AM) blocked STDs.^{137,146} Caffeine, which causes unloading of intracellular Ca^{2+} stores, also results in the termination of STICs and STDs.^{38,137,142,146} The Ca^{2+} -dependence of slow wave generation has also been demonstrated.¹⁵⁰⁻¹⁵² It is generally accepted that Ca^{2+} release from intracellular stores (most likely inositol 1,4,5-triphosphate [IP_3]-sensitive stores) is the fundamental event responsible for pacemaker activity, and mice lacking IP_3 -Type 1 receptors fail to generate slow waves.¹⁵³

Mechanism of Slow Wave Propagation

Slow waves, generated by ICC, conduct passively into SMCs. Slow waves recorded in ICC-MY have larger amplitudes than slow waves recorded from adjacent SMCs.^{38,154} This indicates that SMCs are incapable of actively regenerating slow waves. However, slow waves have been shown to propagate over long distances in muscle strips and in organs of the GI tract. This capacity for regenerative propagation of slow waves is fundamental to the organization of motility patterns. As regeneration of slow waves does not occur in SMCs, active propagation is most likely a property of ICC networks. This may explain the continuous networks of ICC in all phasic regions of the GI tract rather than concentration of ICC into discrete pacemaker regions, as in heart.¹⁵⁵ For example, slow waves are propagated without decrement in regions of muscle containing ICC, but cannot propagate actively in areas of muscle devoid of ICC.¹¹³

The rate at which slow waves propagate (2-40 mm/sec) far exceeds the rate of diffusion of Ca^{2+} waves in cells.¹⁴⁵ Thus a voltage-dependent mechanism is needed to explain the propagation rates observed. The main conductance responsible for STICs and slow wave depolarization (ANO1) is not sensitive to voltage activation. Therefore, an additional voltage-dependent conductance(s) must coordinate activation of ANO1 channels to accomplish slow wave propagation over distances in ICC networks. The most likely candidate of this coordinating conduc-

tance is a dihydropyridine-resistant Ca^{2+} conductance because ICC-MY express such a conductance which is blocked by Ni^{2+} or mibefradil (blockers of T-type Ca^{2+} channels) and slow wave propagation is unaffected by nifedipine but totally blocked by Ni^{2+} or mibefradil.^{139,156-159} Propagation of pacemaker activity, as observed by Ca^{2+} imaging, was also blocked by Ni^{2+} and mibefradil.¹⁶⁰ Further, the T-type α_{1H} -subunit is expressed in ICC-MY.¹²⁷ Taken together there are substantial data supporting the role of a T-type Ca^{2+} conductance in slow wave propagation. Calcium entry is thought to initiate Ca^{2+} -induced Ca^{2+} release, which in turn activates ANO1 channels locally. Depolari-

zation ensuing from Cl^- efflux would, in turn, activate voltage-dependent Ca^{2+} entry in the next cell to regenerate slow waves cell-to-cell.

Other investigators have proposed an alternative idea for voltage-dependent propagation of slow waves. These authors have considered the possibility that there may be voltage-dependent synthesis of IP_3 or possibly voltage-dependent sensitization of IP_3 receptors.^{146,152,161} Evidence has been presented to suggest such a mechanism in SMCs^{162,163} and megakaryocytes.¹⁶⁴ However to date, this mechanism has not been demonstrated in ICC.

Figure 3. The intracellular mechanism underlying generation of pacemaker activity in interstitial cells of Cajal (ICC). (A) Stochastic release of Ca^{2+} from intracellular stores in ICC results in transient activation of anoctamin-1 (ANO1) channels (B), producing spontaneous transient inward currents (STICs) in primary pacemaker cells. The Na-K-Cl cotransporter (NKCC1) replenishes intracellular chloride ions. (C) The depolarization of ICC activates voltage-dependent, dihydropyridine-resistant Ca^{2+} channels (VDCC) in the plasma membrane (PM). Ca^{2+} entry causes additional Ca^{2+} release from IP_3 -dependent Ca^{2+} channels ($\text{IP}_3\text{R1}$) and summation of ANO1 currents, resulting in slow wave generation. Slow waves depolarize nearby ICC (horizontal arrow) triggering activation of voltage-dependent Ca^{2+} channels in adjacent secondary pacemaker cells. Ca^{2+} entry in adjacent cells activates ANO1 channels and regenerates slow waves. Spread of events in this fashion occurs throughout ICC networks. Termination of slow waves occurs by reuptake of Ca^{2+} into the endoplasmic reticulum (ER) via the sarco/ER Ca^{2+} -ATPase pump (SERCA).

Summary of the Events Underlying Generation and Propagation of Slow Waves

Overall the best current idea for the processes of slow wave activation and propagation is summarized below. See Figure 3 for diagrammatic representation of the components and apparatus involved in slow wave generation and Figure 4 for the different phases of a representative slow wave from the human gastric antrum.

1. Stochastic release of Ca^{2+} from intracellular Ca^{2+} stores in ICC (Fig. 3A) results in transient activation of ANO1 channels, producing STICs. STICs are highly localized inward currents that produce STDs in ICC (Fig. 3B and Fig. 4).

2. Depolarization of ICC (STDs) activates voltage-dependent, dihydropyridine-resistant Ca^{2+} channels (Fig. 3B and Fig. 4).

3. Ca^{2+} entry causes additional Ca^{2+} release from IP_3 -dependent Ca^{2+} channels and summation of ANO1 currents, resulting in a slow wave (Fig. 3B and Fig. 4).

4. Slow waves depolarize nearby ICC triggering activation of voltage-dependent Ca^{2+} channels in adjacent cells (Fig. 3C).

5. Ca^{2+} entry in adjacent cells activates ANO1 channels and

Figure 4. Representative electrical slow wave from the human gastric antrum. Slow waves consist of several discrete phases. An initial upstroke phase (1) rises from a diastolic membrane potential of -70 mV (0). Phase 1 is thought to be due to release of intracellular calcium from the endoplasmic reticulum and activation of anoctamin-1 and voltage-dependent, dihydropyridine-resistant Ca^{2+} channels. The transient depolarization to -26 mV (2) is followed by a partial repolarization, likely due to activation of an A-type potassium conductance in smooth muscle cells, to a plateau phase (3) that is sustained for several seconds. Phase 3 is likely to be caused by a balance between inward and outward conductances (i.e., calcium ion entry, chloride ion efflux versus potassium ion efflux). The membrane potential then returns to the resting diastolic potential during phase 4.

regenerates slow waves. Spread of events in this fashion occurs throughout ICC networks (Fig. 3C).

6. Slow waves conduct passively into neighboring SMCs, and depolarization of these cells activates voltage-dependent, L-type Ca^{2+} channels. Ca^{2+} influx into SMCs triggers excitation-contraction coupling.

Interstitial Cells of Cajal as Mediators of Enteric Neurotransmission

Visceral smooth muscle tissues are innervated by several types of neurons: sympathetic, parasympathetic and enteric. Excitatory and inhibitory enteric motor neurons innervate the muscles of the GI tract. Morphological studies show these neurons have frequent varicose swellings along their axons,^{165,166} which are considered to be the locations of neurotransmitter release. Neurotransmitters are released en passage (i.e., they are released as an action potential travels along the neuron and invades each varicosity).¹⁶⁷ Some investigators have suggested that neurotransmission in the gut occurs by “volume transmission,” in which neurotransmitters are released into the interstitial space and then diffuse through the interstitium to receptors that can be some distance from the sites of release.^{168,169} This “volume transmission” concept has been challenged in recent years. For example, careful ultrastructural studies of varicosities have revealed the presence of distinct junctions with electron dense areas along the membrane of the varicosity and the effector cell.¹⁷⁰ Furthermore, it has been suggested that neurotransmission does not simply occur between neurons and SMCs in the GI tract and intramuscular interstitial cells might be intermediaries. The evidence for and against this idea is reviewed in the following sections.

Close Associations Between Interstitial Cells of Cajal and Enteric Neurons

A possible role for ICC in neurotransmission was suggested by Cajal,⁵ but there was little evidence supporting this until ultrastructural studies revealed very close contacts (< 20 nm) between varicosities and ICC. For example, Imaizumi and Hama¹⁷ observed close associations in the gizzard of the love bird and reiterated Cajal’s theory that ICC might be involved in transmitting stimuli from neurons to smooth muscle. Similar findings were obtained by Yamamoto¹⁸ in studies of the small intestine of the mouse and bat and by Faussone-Pellegrini et al¹⁹ in studies of human LES and proximal stomach. Further evidence for the idea of innervation of ICC came from ultrastructural studies that identi-

Figure 5. Close associations and synaptic-like specializations between enteric neurons and interstitial cells of Cajal (ICC). (A) Transmission electron micrograph showing areas of increased electron density at sites where enteric neurons (N) and intramuscular ICC (ICC-IM) are closely apposed in the murine gastric antrum (arrow). Electron densifications were observed on both pre- and post-synaptic membranes. (B) The site indicated by the arrow in A is shown at higher magnification in B. The arrow in B also indicates areas of increased electron density as in A. (C and D) Double label immunohistochemistry images that show close structural relationships (arrows) between nerve fibers (green) and ICC (KIT; red) in the taenia coli of primate colon. (C) Close apposition between the pan-neuronal label, PGP9.5 (protein gene product 9.5) and ICC. (D) shows the relationship between inhibitory *nNOS*⁺ nerve fibers and ICC. (E) Double labeling of the pre-synaptic soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein, synaptotagmin (green) and ICC (red) in murine fundus. The image demonstrates that pre-synaptic proteins are closely apposed to ICC, suggesting that ICC are likely the innervated post-synaptic cell. Area in (E) outlined by white box is shown at higher magnification in panel (F). Scale bars: A, 0.5 μ m; B, 0.1 μ m; C, 25 μ m; D, 25 μ m; E, 10 μ m; and F, 5 μ m. Adapted from Beckett et al (A, B, E and F)¹⁷⁴ and Blair et al (C and D).³¹

fied synapse-like specializations at regions of close association between nerve fibers and ICC (Fig. 5A and 5B).^{58,64,170-174} It appeared that these specializations are similar to synaptic structures in the CNS¹⁷⁵ and skeletal neuromuscular junction synapses.^{176,177} Close associations between nerve fibers and ICC have also been observed in multiple species using double label immunohistochemistry (Fig. 5C and 5D).^{31,35,43,44,46,58,64,98,171,178-185} Immunohistochemical studies have also provided evidence for synapse-like specializations between enteric nerves and ICC. Beckett et al¹⁷⁴ utilized antibodies against pre-synaptic soluble N-ethylmaleimide-sensitive factor attachment protein receptor

(SNARE) proteins (proteins that mediate vesicle fusion), synaptotagmin and synaptosomal-associated protein-25 (SNAP-25), and observed that they were localized to varicosities which were found in close association with ICC-IM and were not commonly apposed to SMCs (Fig. 5E and 5F). Post-synaptic density proteins were found in ICC-IM using an antibody directed against the post-synaptic density-95 (PSD-95) family (PSD-95, PSD-93 and synapse-associated protein 97 [SAP97]). Quantitative RT-PCR experiments demonstrated that expression levels of PSDs-93 and -95 were reduced in *W/W^v* mice with reduced ICC-IM.¹⁷⁴

For ICC-IM to convey signals from nerve fibers to SMCs, it

is logical that ICC and their neighboring SMCs must be connected in some way. Ultrastructural studies revealed gap junctions between ICC-IM and SMCs.^{17,18,44,64,173} This was especially apparent in the case of ICC-DMP, which have an abundance of large gap junctions.^{27,57,99,186-190}

Functional Evidence Demonstrating the Importance of Interstitial Cells of Cajal in Enteric Neurotransmission

If ICC are involved in neurotransmission they must also express appropriate receptors and effectors to bind neurotransmitter and transduce signals into post-junctional responses. ICC express receptors for neurotransmitters such as neurokinins (NK1 and NK3 receptors), vasoactive intestinal polypeptide (VIP-1 receptors) and acetylcholine (muscarinic M₂ and M₃ receptors).^{62,127,191-197} Numerous studies have shown responses to NO in the GI tract depend upon the production of cyclic GMP (cGMP) by soluble guanylate cyclase.¹⁹⁸⁻²⁰⁰ Guanylate cyclase is a heterodimer comprised of 2 subunits, α and β . Using immunohistochemistry Iino et al^{182,201} showed that both subunits are strongly expressed in ICC but not resolved in smooth muscle. Neural stimulation or application of NO donors increases cGMP in ICC, suggesting that ICC are primary targets for NO in GI muscles.²⁰¹⁻²⁰³ Furthermore, NK1 receptors are internalized in ICC in response to application of substance P and nerve stimulation, signifying that ICC are also targets for excitatory peptides.^{61,62} Wang et al²⁰⁴ found that protein kinase C ϵ (PKC ϵ) translocates from the cytoplasm to the membrane of ICC-DMP in response to cholinergic stimulation, implying that a functional response to cholinergic stimulation also occurs in ICC. A more recent study showed that Ca²⁺-activated chloride channels are activated in ICC in response to cholinergic neurotransmission.²⁰⁵ The channel involved is likely to be ANO1 expressed exclusively in ICC in the GI tract.^{67,106,107,131} Additionally, inhibition of a basally active Ca²⁺-activated chloride channel has been suggested to account for part of the response to nitrergic stimulation.²⁰⁶⁻²⁰⁸ It has yet to be determined whether ANO1 in ICC is the Cl⁻ conductance involved in nitrergic neurotransmission.

Similar to ICC and pacemaking, the most compelling evidence that ICC are important factors in mediating enteric neurotransmission came from studies on *Kit* mutants. Stomachs of *W/W^v* mice lack most ICC-IM and intracellular recording combined with electrical field stimulation (EFS) showed that excitatory cholinergic and inhibitory nitrergic post-junctional re-

sponses were greatly reduced in circular muscle responses of fundus and antrum^{42,44-46} and longitudinal muscle responses of the antrum.¹⁷⁹ Neural responses are also reduced in the LES and pyloric sphincter⁴³ and in the fundus of *Sl/Sl^v* mice.¹⁷⁸ It has proven the role of ICC-DMP in neurotransmission because they remain in the small intestine of *W/W^v* mice. Neutralizing KIT antibody reduces ICC-DMP and causes reduction in nitrergic and cholinergic responses to neurotransmission.⁶⁰

The enteric nervous system appears normal in *W/W^v* mice, and it was noted that the smooth muscle was capable of normal responses to bath applied neurotransmitters. These data suggest that the defects in neurotransmission of *W/W^v* mice are due to the absence of ICC-IM.^{42,44} Although neurotransmission is severely disrupted in *W/W^v* mice, some components of post-junctional responses remain. In the antrum, the post-junctional response consists of fast inhibitory junction potential (fIJP; purinergic), a slow inhibitory junction potential (nitrergic) and an excitatory junction potential (cholinergic). When sustained stimulation is applied a non-cholinergic excitatory component is revealed, likely mediated by peptide neurotransmitters such as neurokinins.²⁰⁹ The fIJP and the non-cholinergic excitatory component are unaffected in the antrums of *W/W^v* mice.^{42,209,210} The persistence of these components was vexing for several years until a new class of interstitial cell, which is thought to mediate purinergic transmission, was recently discovered.^{31,181,211-214} These interstitial cells were previously known as fibroblast-like cells (FLCs), but they are now identified by expression of PDGFR α ⁺ and are therefore referred to as PDGFR α ⁺ cells. It is currently uncertain if PDGFR α ⁺ cells are involved in mediating excitatory neurotransmission and thus the reason for the persistence of the non-cholinergic component remain to be elucidated.

The results of several new studies utilizing modern genetic techniques have contributed to the idea that ICC are involved in mediating enteric neurotransmission. Mice with genetic deactivation of soluble NO-sensitive guanylyl cyclase (NO-GC; the NO receptor) were found to die prematurely due to GI obstruction.²¹⁵ Mice with NO-GC knocked-down specifically in SMCs (SM-GCKO) retained nitrergic neurotransmission, as NO-mediated relaxation was evident in SM-GCKO mice.²¹⁶ Little effect was noted with NO-GC knocked-down specifically in ICC, but nitrergic relaxation was abolished with NO-GC knocked-down in ICC and SMC. Mice with knockdown in ICC and SMC also displayed increased whole gut transit time. These data suggested that nitrergic responses may be mediated in both ICC and SMC in the murine fundus.

In another recent paper, Bhetwal et al²¹⁷ investigated calcium sensitization of contraction in response to both bath-applied agonists and EFS.²¹⁷ The sensitivity of smooth muscle contraction to calcium is known to be augmented by phosphorylation of PKC-potentiated phosphatase inhibitor protein of 17 kDa (CPI-17) and myosin phosphatase targeting subunit 1 (MYPT1), both of which result in inhibition of myosin light chain phosphatase. The results of this study showed that bath-applied carbachol (a cholinergic agonist) and EFS produced calcium sensitization by different mechanisms: carbachol increased phosphorylation of CPI-17 via the PKC pathway and MYPT1 via the Rho kinase pathway, whereas EFS only increased the phosphorylation of CPI-17. However, it was observed that EFS was capable of increasing phosphorylation of both CPI-17 and MYPT1 when performed in the presence of neostigmine (a cholinesterase inhibitor). Furthermore, in the fundus of *W/W^v* mice, which lack most ICC-IM, EFS alone was found to enhance phosphorylation of both CPI-17 and MYPT1. These data suggest that acetylcholine (ACh) released from nerves likely acts on a select population of muscarinic receptors that are near varicosities (perhaps on ICC-IM), while in contrast, bath applied agonists are able to stimulate receptors throughout the tissues. When neostigmine is added before EFS, ACh overflows post-junctional volumes, activating receptors on SMCs. Similarly, in *W/W^v* tissues nerve-released ACh is not restricted from a greater sphere of influence by ICC-IM and overflows to receptors on SMCs. These studies demonstrate that bath-applied neurotransmitters and neurotransmitters released from neurons activate different effector pathways, most likely due to the activation of different populations of receptors on different types of cells.

Another study examined excitatory and inhibitory neurotransmission in the murine ileum and colon using tamoxifen inducible activation or deactivation of specific genes.²¹⁸ In experiments in which diphtheria toxin was induced in ICC, the ICC population decreased by 50% within 3 days after tamoxifen. Total gut transit time and gastric emptying were decreased in these animals. Mechanical and electrophysiological recordings showed that slow waves were abolished in the small intestine, and excitatory junction potentials were abolished in the colon in response to activation of intrinsic nerves. To more thoroughly investigate whether ICC are involved in inhibitory neurotransmission, cGMP-dependent protein kinase 1 (*Prkg1*) was knocked down in other experiments. *Prkg1* is known to be a critical mediator in NO neurotransmission in GI muscles.²¹⁹ Mice with reduced *Prkg1* displayed reduced NO-dependent smooth muscle

relaxation and GI dysfunction.^{200,220} Tamoxifen-induced knock-down of *Prkg1* in approximately 40% of ICC resulted in severe GI dysfunction, as illustrated by a substantial increase in GI transit time, and the NO-dependent component neural responses was abolished.

Further evidence supporting a role of ICC-IM in nitrergic neurotransmission in the fundus was recently published. In this study it was shown that NO-GC was expressed in several cell types including ICC and SMCs. To determine the role of NO-GC in each cell type, Lies et al²²¹ examined mice with NO-GC knocked-down globally (GCKO) and specifically in SMC (SM-GCKO), ICC (ICC-GCKO) and both SMC/ICC (SM/ICC-GCKO). The nitrergic IJP was abolished in ICC-GCKO and reduced in SM-GCKO fundus,²²¹ supporting the original findings from *W/W^v* mutant animals which demonstrated that ICC-IM are involved in nitrergic inhibitory neuroeffector responses.

Evidence Opposing a Role for Interstitial Cells of Cajal in Enteric Neurotransmission

Some investigators have argued that ICC are not important in enteric motor neurotransmission, citing experiments in which mechanical and electrical responses were retained in mutants lacking or having depressed populations of ICC. Several studies have concluded that ICC-IM are not involved in nitrergic²²²⁻²²⁵ or cholinergic neurotransmission.^{222,226} Zhang et al²²² suggested that the defects in neurotransmission observed in *W/W^v* mice might be due to alterations in the SMCs caused by *Kit* deficiency, rather than a direct consequence of ICC loss. It was also demonstrated by Huizinga and colleagues that cholinergic activity is prominent in *Ws/Ws* rats, leading them to assume that ICC are not involved in cholinergic neurotransmission.²²⁶ However, it should be remembered that persistence of neural responses in animals lacking ICC does not prove that ICC have no role in mediating neurotransmission under normal circumstances. It should also be noted that loss of ICC populations in *W/W^v* mice is often not complete, and this might explain the remaining responses in some experiments.²²⁷ Also, animals with *Kit* deficiency through development may compensate for the imposed loss-of-function by developing or emphasizing alternative regulatory mechanisms. For example, Zhang et al²²⁶ detected a marked increase in muscarinic (M2) receptors on the SMCs of *Ws/Ws* rats. Further, the authors of this study employed long trains of stimulation (as opposed to the single pulses and short trains used in previous studies), which presumably increased neurotransmitter release. This, coupled with the increased sensitivity of smooth muscle, make it

unsurprising that cholinergic responses persist. Increases in purinergic receptors have also been reported in W/W^V tissues²¹⁰ and this may account for the augmented purinergic inhibitory response in these tissues.⁴² In another study Zhang et al²²² reported that ICC were also not required for nitrergic neurotransmission in the LES because a proportion of W/W^V mice had normal responses to nitrergic stimulation, while the responses were diminished in the remainder of animals, as previously reported.⁴³

Other investigators have claimed that if ICC are required for nitrergic neurotransmission then W/W^V animals should have the same phenotype as neuronal nitric oxide synthase knockout mice ($nNOS^{-/-}$). They have reported that the LES of $nNOS^{-/-}$ mice exhibit hypertension, whereas W/W^V LES tissues are hypotensive.²²⁴ Different phenotypes were also observed in the pyloric sphincter of $nNOS^{-/-}$ and W/W^V mice.²²⁵ In accordance with their hypothesis the authors concluded that ICC are not involved in nitrergic neurotransmission. However, previous studies have provided evidence that ICC-IM are mediators of both nitrergic and cholinergic neurotransmission. Thus, there is no reason to expect that the phenotype of W/W^V mice would mirror the phenotype of a mouse in which only nitrergic transmission is compromised. The hypothesis, results and conclusions of these studies appeared to not fully utilize observations of previous studies and may have been short sighted. In a recent study micromanometry was used to investigate LES contractions in wild type, $nNOS^{-/-}$ and W/W^V mice.²²⁸ These authors also found that LES was hypertensive in $nNOS^{-/-}$ mice and hypotensive in W/W^V mice. However, by examining ICC with immunohistochemistry they found that loss of ICC-IM correlated with decreased LES relaxations.

The fact that some W/W^V mutant mice retain a component of the NO-dependent post-junctional neuronal response was explained in a recent study. Examination of W/W^V fundus tissues using KIT immunohistochemistry revealed that ICC-IM persisted in the fundus of many W/W^V animals. The nitrergic inhibitory component was absent when ICC-IM were absent but was still present, albeit reduced in tissues where ICC-IM were present.²²⁹ These data support the hypothesis that ICC-IM mediate nitrergic inhibitory neurotransmission in the fundus and may explain the discrepancies in previous functional studies.

Some investigators have speculated that NO released from neurons would diffuse freely away from the site of release and it would not be possible to confine NO to a defined neuro-effector junction.²³⁰ While this statement may be true, it lacks appreciation for the amount of NO released from each varicosity, whether

every varicosity releases transmitter in response to every action potential, whether concentrations of NO reaching post-junctional cells are sufficient to elicit a response after diffusion, whether NO is deactivated within the interstitium or by post-junctional cells, and whether cells exposed to NO released from neurons express appropriate receptors and effectors. At the present time the precise mechanism for the post-junctional responses and which cells might participate in the integrated post-junctional response to NO are poorly understood.

NO elicits hyperpolarization and relaxation responses in GI muscles, and these responses depend upon generation of cGMP and cGMP dependent protein kinase.^{199,200,220} The proteins that mediate membrane hyperpolarization are debated, and some investigators have viewed the hyperpolarization response to be due to activation of a K^+ conductance²³¹⁻²³³ while others have suggested the response is due to suppression of an inward current due either to a non-selective cation conductance²⁰⁶ or a chloride conductance.²³⁴ Resolution of these questions and determining which of the cells in the SIP syncytium express the specific types of ion channels responsible for responses to NO will require additional experimentation. Final confirmation of the mechanism of nitrergic inhibition and clear descriptions of post-junctional mechanisms responsible for the integrated response in tissues will be aided by the precision of inducible gene deactivation experiments, but knowing which cells and some idea about which proteins are responsible for effects will be necessary before specific cells and genes can be targeted.

Platelet-derived Growth Factor Receptor Alpha Cells

Another class of interstitial cells, with a similar distribution to ICC, has been referred to in the literature as FLCs.²³⁵⁻²³⁷ These cells were first identified and distinguished from ICC by EM. Several ultrastructural features are distinctly different from the ICC that are often found in close proximity. For instance, FLC exhibit a well-developed rough ER, and do not possess caveolae.²³⁶ Ultrastructural studies have also confirmed that FLCs are closely associated with nerve varicosities,^{74,238} however the very close, synapse-like contacts observed between nerve varicosities and ICC have not been reported for FLC. Recently, Iino et al²¹¹ showed that FLCs could be specifically and reliably identified using antibodies directed against the PDGFR α .²¹¹ Thus, FLCs are now referred to as PDGFR α^+ cells.^{31,181,213,214,239} The ability to easily identify PDGFR α^+ cells has facilitated examination of

their distribution and relationships to other cell types. The close associations with nerves observed in ultrastructural studies have been verified numerous times in a variety of species and organs: mouse fundus, corpus, small intestine and colon,²¹¹ mouse colon,²¹³ mouse internal anal sphincter,¹⁸¹ monkey fundus, antrum, small intestine and colon,³¹ human stomach²³⁹ and human colon.²¹⁴ PDGFR α ⁺ cells are found within muscle bundles (distinguished as PDGFR α ⁺-IM) and in the plane of the myenteric plexus (PDGFR α ⁺-MY) to make the terminology for this class of interstitial cells consistent with the common terminology applied to ICC. Localization close to nerve terminals suggested that PDGFR α ⁺ may also have a role in transducing input from enteric motor neurons.

Some years ago it was found that FLCs express small-conductance calcium-activated potassium (SK3) channels.^{212-214,239-242} SK3 channels are blocked by apamin (a peptide neurotoxin found in bee venom) and purinergic fIJs are also known to be apamin-sensitive. Thus, it is possible that PDGFR α ⁺ cells could contribute to the fIJs component of enteric inhibitory responses.

Using a reporter strain of mice that express an enhanced GFP-histone 2B fusion protein driven off the cell-specific promoter for *Pdgfr α* , it was possible to isolate and study the characteristics of PDGFR α ⁺ cells.²¹³ PDGFR α ⁺ cells were found to express all of the genes encoding the proteins that are necessary to bind purine neurotransmitters and transduce purinergic signals into appropriate electrical responses.^{213,243} Other components of the SIP syncytium express far lower levels of the most relevant genes (*P2ry1* and *Kcnn3*).²⁴³ These observations suggest that PDGFR α ⁺ cells, rather than ICC or SMCs, could be an important mediator of purinergic neurotransmission. This is consistent with the observation that the apamin-sensitive fIJP persists in the absence of ICC-IM.^{42,210} The case for PDGFR α ⁺ cells having a functional role in neurotransmission is further strengthened by the observation that they respond to purinergic agonists. Kurahashi et al²¹⁴ showed that a large amplitude outward current is elicited in PDGFR α ⁺ cells when they are exposed to candidate purine neurotransmitters: ATP, β -NAD, ADP-ribose and ADP. Smooth muscle cells responded either with small inward currents or no response to these agonists.²¹³ These data strongly suggest that PDGFR α ⁺ cells are major targets for purinergic neurotransmission in GI muscles. More definitive proof of this hypothesis would be to examine neural responses in animals lacking PDGFR α ⁺ cells or having specific genetic deactivation of key genes. Treatment of mice with crenolani, a specific inhibitor of PDGFR α caused these cells to fail to

develop. Loss of PDGFR α ⁺ cells was found to result in a parallel loss of the purinergic fIJP in GI muscles.²⁴⁴ It is possible that PDGFR α ⁺ cells could also play a role in nitergic neurotransmission. These cells have been shown to express NO-GC^{182,216} and residual relaxation in SM/ICC-GCKO mice has been detected in response to high levels of NO.²⁴⁵

Gap Junctions Between Interstitial Cells of Cajal and Smooth Muscle Cells

An essential structural element of the SIP syncytium is electrical connectivity between SMCs and interstitial cells. Electrical responses generated in interstitial cells would have little or no impact on smooth muscle contractility without the presence of low resistance electrical connections between the cells. The current hypothesis regarding interstitial cells and their role in neurotransmission is that activation or suppression of conductances in interstitial cells in response to neurotransmitters modulates the input resistance and excitability of the greater SIP syncytium; inward and outward currents are conducted to SMCs via the low resistance connections between these cells.

Many reports of gap junctions between ICC and SMCs exist.^{63,64,97-100} Multiple studies have also found that ICC express a variety of connexins (primarily connexin 43) which are the transmembrane proteins that assemble to form gap junctions.^{73,246,247} Electrical coupling between ICC and smooth muscle was verified by physiological experiments, in which injection of currents into circular muscle cells caused electrotonic potentials in ICC-MY.¹⁵⁴ Park et al¹⁶⁰ demonstrated that Ca²⁺ transients propagating through ICC-MY and smooth muscle were disrupted after the application of 18 β -glycyrrhetic acid, a gap junction uncoupler, providing functional evidence that gap junctions are involved in ICC-smooth muscle coupling. Analogous to the sinoatrial node of the heart,^{248,249} relatively weak coupling may be an important factor in avoiding rapid dissipation of pacemaker current into the large volume of the smooth muscle syncytium before it has reached the threshold to entrain pacemaker currents within the ICC network.^{99,145} Gap junctions have also been observed between SMCs and PDGFR α ⁺ cells.^{236,242} Thus, it appears that the structural requirements for connectivity between interstitial cells is present, as necessary for interstitial cells to perform as pacemakers, regulators of electrical excitability and mediators of responses to motor neurotransmitters.

Some authors have observed that gap junctions between ICC-MY and SMCs are small, few in number or impossible to

resolve with transmission electron microscopy.^{27,33,99,250} In addition, results from functional experiments using 18 β -glycyrrhetic acid have been questioned because non-specific effects can be elicited by this compound and other compounds used to uncouple gap junctions.²⁵¹⁻²⁵³ The gap junction uncouplers, octanol and carbenoxolone, failed to disrupt pacemaker activity in various species,^{99,254} however no tests to measure the degree of electrical uncoupling have accompanied these studies. More recently, Daniel et al²⁵⁵ used peptide analogs of epitopes in the extracellular domain of connexins because these are considered to be specific gap junction uncouplers. Application of these peptide analogs had no effect on paced contractions and nerve transmissions in murine small intestine, but again no tests of electrical coupling were included in the results. Daniel has suggested that gap junctions are not required for propagation of slow waves from ICC to SMCs, and has suggested instead that coupling might occur through electrical field potentials in the narrow spaces between neighboring cells.³³ Evidence that this type of coupling can occur between cells comes mainly from theoretical studies²⁵⁶⁻²⁶⁰ and there has been no verification of this concept in GI muscles.

As discussed previously gap junctions between ICC-IM and SMCs are widely accepted. Connexin 43 is robustly expressed in ICC-IM and ICC-DMP,^{102,246,247} and Kito et al¹⁴¹ demonstrated the spread of fluorescent dye between ICC-IM and SMCs in the rat fundus. Kobil et al²⁶¹ also showed that Lucifer yellow, injected into ICC-DMP, spread to surrounding SMCs, and dye spread was blocked by gap-junction uncouplers. Despite the

structural basis for coupling in morphological studies, functional evidence for the involvement of gap junctions and the role of coupling between SMCs and ICC-IM in neurotransmission is lacking. Recent studies have failed to show block of junction potentials in murine small intestine and colon after application of dominant negative gap junction peptides and uncouplers.^{255,262} It should be noted, however, that low degree of electrical coupling may be sufficient for spread of electrical potentials between cells with very high input resistances and no rigorous tests of electrical coupling have accompanied studies of gap junction uncouplers. Nevertheless, one must conclude, at present that the importance of gap junctions in pacemaker activity and enteric motor responses is still controversial.

Conclusions

Over the past few decades an explosion of data supporting roles for interstitial cells in GI pacemaker activity and enteric motor neurotransmission have emerged. There are at least 2 distinct classes of interstitial cells involved in the regulation of electrical excitability in GI muscles. Interstitial cells are electrically coupled to SMCs, forming a multicellular SIP syncytium, which is illustrated in Figure 6. Changes in conductance, brought about spontaneously as in the case of pacemaker activity, or in response to neurotransmitters, paracrine substances, hormones, or immune factors, in any of the SIP cells affects the input resistance and electrical excitability of the greater SIP syncytium. Thus, rig-

Figure 6. Diagrammatic representation of the SIP syncytium. The multicellular electrical syncytium consists of at least three distinct cell types. Kit⁺ interstitial cells of Cajal (ICC; red) and PDGFR α ⁺ interstitial cells (green) form low-resistance gap junctions with each other and neighboring smooth muscle cells (SMCs; arrows). SMCs form gap junctions with each other (red lines). Enteric motor nerves (light blue) are closely apposed to both classes of interstitial cells and make synapse-like contacts with ICC (dark blue lines) at vesicle-laden varicosities.

orous analysis of excitation-contraction coupling in GI muscles must include detailed evaluation of the membrane conductances and responsiveness to bioagonists of each type of SIP cell. Investigations utilizing animals such as W/W^V mice have led to ICC being accepted as the pacemakers of the GI tract. Much has been deduced about the pacemaker mechanism through use of genetic models and by direct recording from ICC freshly isolated from animals. A model of a current concept of pacemaker activity is shown in Figures 3 and 4.

In contrast to the role of ICC in pacemaking, the role of ICC and PDGFR α^+ cells as mediators of motor neurotransmission is still controversial. Progress on this topic in the future will depend upon genetic models engineered for inducible knockout of key receptors and effectors in a cell-specific manner in adult animals. The next era of interstitial cell research may provide these experiments and the results will greatly enhance our understanding and appreciation of this class of cells in GI motility and motor diseases.

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