SYMPOSIUM REPORT

Involvement of intramuscular interstitial cells of Cajal in neuroeffector transmission in the gastrointestinal tract

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Specialized cells known as interstitial cells of Cajal (ICC) are distributed in specific locations within the tunica muscularis of the gastrointestinal (GI) tract. ICC serve as electrical pacemakers, provide pathways for the active propagation of slow waves, are mediators of enteric motor neurotransmission and play a role in afferent neural signalling. Morphological studies have provided evidence that motor neurotransmission in the GI tract does not occur through poorly defined structures between nerves and smooth muscle, but rather via specialized synapses that exist between enteric nerve terminals and intramuscular ICC or ICC-IM. ICC-IM are coupled to smooth muscle cells via gap junctions and post-junctional responses elicited in ICC-IM are conducted to neighbouring smooth muscle cells. Electrophysiological studies from the stomachs and sphincters of wild-type and mutant animals that lack ICC-IM have provided functional evidence for the importance of ICC in cholinergic excitatory and nitrergic inhibitory motor neurotransmission. Intraperitoneal injection of animals with Kit neutralizing antibody or organ culture of gastrointestinal tissues in the presence of neutralizing antibody, which blocks the development and maintenance of ICC, has provided further evidence for the role of ICC in enteric motor transmission. ICC-IM also generate an ongoing discharge of unitary potentials in the gastric fundus and antrum that contributes to the overall excitability of the stomach.

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At least three distinct functional classes of interstitial cells of Cajal (ICC) exist within the tunica muscularis of the gastrointestinal (GI) tract. In phasically contracting regions, a network of ICC lies between the circular and longitudinal muscle layers at the level of the myenteric plexus and are termed ICC-MY. ICC-MY function as electrical pacemakers generating slow waves which control the frequency of phasic contractions of the tunica muscularis (Ward *et al.* 1994; Huizinga *et al.* 1995; Torihashi *et al.* 1995) (Fig. 1). ICC-MY also provide an active propagation pathway through which pacemaker activity spreads, ensuring the co-ordinated spread of slow waves within the GI tract (Horiguchi *et al.* 2001). In the colon, ICC located along the submucosal surface of the circular muscle layer (ICC-SM) also provide pacemaker function in this organ (Smith *et al.* 1987).

A second population of ICC, known as intramuscular ICC or ICC-IM, are located within the smooth muscle layers of the GI tract and are scattered amongst the smooth muscle cells. In the stomach ICC-IM serve two primary functions: throughout the circular muscle layers of the gastric fundus, corpus and antrum, and within the longitudinal muscle layers of the fundus and proximal corpus, ICC-IM form an intimate relationship with enteric nerve terminals (Fig. 2) and are essential for a functional cholinergic excitatory and nitrergic inhibitory motor innervation of the smooth muscle in these tissues (Burns *et al.* 1996; Ward *et al.* 2000; Beckett *et al.* 2002, 2003; Suzuki *et al.* 2003; Song *et al.* 2005*a*). Similar structural relationships also exist between enteric nerves and ICC-IM in several sphincters of the GI tract and loss of ICC-IM attenuates motor neurotransmission in these regions (Ward *et al.* 1998). In the gastric antrum but not the fundus, ICC-IM also act as tension transducers to the smooth muscle and contribute to the generation of slow wave activity (Suzuki & Hirst, 1999; Won *et al.* 2005). In the small intestine, ICC-IM rather than being dispersed within the muscle layers, are concentrated along the inner

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surface of the circular layer in the region known as the deep muscular plexus, and are termed ICC-DMP (Torihashi *et al.* 1993). ICC-DMP provide a similar functional role as ICC-IM in the stomach and are critical for the transmission of neural information to the circular smooth muscle cells of this tissue (Ward *et al.* 2006). Additional ICC-IM are also found within the circular and longitudinal muscles layers of the intestine of larger animals, including primates. A final population of ICC are found distributed over the surface of muscle bundles and within septae that separate muscle bundles and are termed ICC-SEP. These ICC are found in the stomach, small intestine, colon and recto-anal regions of the GI tract. The physiological role of ICC-SEP has been investigated in the stomach where they serve much like Pukinje fibres in the heart, conveying and co-ordinating the spread of pacemaker activity deep into and between muscle bundles and may also be involved in enteric motor neurotransmission (Horiguchi *et al.* 2001).

This symposium review will give an overview perspective of the evidence that supports the role of ICC-IM in enteric motor neurotransmission and will highlight more recent structural, molecular and functional evidence that endorses the importance of ICC-IM as critical mediators in enteric motor neurotransmission.

Involvement of ICC in motor neurotransmission

Morphological studies have identified varicose swellings along the lengths of autonomic motor nerves including fibres within the enteric nervous system (Gabella, 1979) and it is generally well accepted that these are the sites of release for most neurotransmitters. There is, however, considerable controversy as to how neurotransmitters that are released during activation of motor nerves are capable of producing post-junctional responses in neuroeffector cells. Many neurophysiologists that study the enteric nervous system envision the release of neurotransmitters as an *en passage* process, occurring as action potentials conduct down nerve fibres into nerve varicosities and functional innervation being defined as the volume through which a neurotransmitter can diffuse from the varicosity and reach post-junctional receptors in sufficient concentrations to produce a physiological response in the neuroefffector cell (Burnstock, 1981). This perception occurs in spite of several recent ultrastructural studies

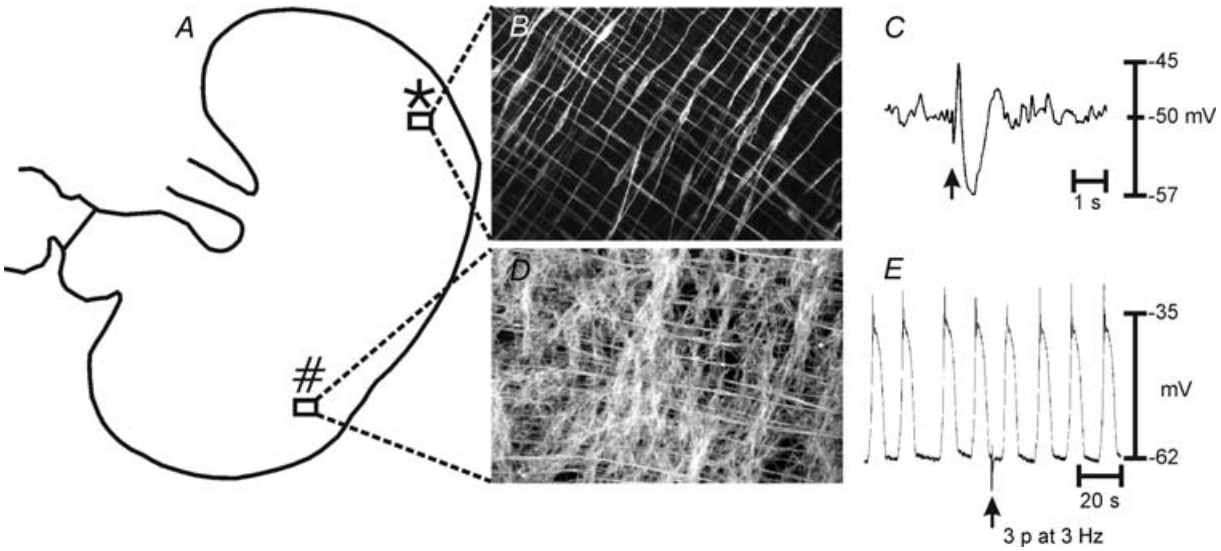

Figure 1. Structural organization and electrical activity of the murine stomach
A is a diagrammatic representation of a cross-section through the murine stomach. *B* shows the structural organization of ICC in the gastric fundus taken from a region in *A* represented by *. Spindle-shaped intramuscular ICC (ICC-IM) are found interposed between the smooth muscle cells in the circular and longitudinal muscle layers running at approximate right angles to each other. *C* shows typical electrical activity and response to electric field stimulation (EFS, single pulse, 0.5 ms in duration). In the gastric fundus, pacemaker activity is not normally present and electrical activity consists of spontaneous unitary potentials. Following EFS (arrow) a bi-phasic, neurally evoked response consisting of a cholinergic excitatory junction potential followed by a nitrergic inhibitory junction potential. In the gastric antrum (#) two distinct populations of ICC exist. ICC-IM are found within the circular muscle layer and a fusiform network of ICC-MY are found at the intermuscular plane between the circular and longitudinal muscle layers (*D*). In *E*, spontaneous pacemaker activity present in the gastric antrum can be observed. EFS (0.1 ms; 3 pulses at 3 Hz, arrow) causes neurally evoked phase advancement in pacemaker activity that is mediated through ICC-IM and is absent in W/W^V mice.

using serial reconstruction that have identified distinct neuromuscular junctions in several visceral tissues with electron densification along the membranes of both nerve varicosity and neuroeffector cells (Luff *et al.* 1987; Gabella, 1995).

Evidence now exists within the gastrointestinal tract that neuroeffector junctions are much more complicated than enteric nerve terminals lying closely apposed to smooth muscle cells. Rather, ultrastructural studies are showing that rather than enteric motor nerves innervating smooth muscle cells directly, junctional specializations exist between enteric nerve terminals and ICC-IM (Fig. 3) and that cholinergic and nitrergic innervation results from the activation of specific receptors and down stream signalling pathways in ICC-IM rather than smooth muscle. Functional neurotransmission cannot occur in the absence of these cells (Burns *et al.* 1996; Ward *et al.* 2000).

Synaptic specializations exist between enteric nerve terminals and ICC-IM

Ultrastructural studies described above have identified membrane densifications between enteric nerve terminals and ICC-IM in different organs of the GI tracts from several species (Roman *et al.* 1975; Daniel & Posey-Daniel, 1984; Wang *et al.* 1999, 2000; Mitsui & Komuro, 2002; Horiguchi *et al.* 2003) (Fig. 3). The ultrastructure of these membrane specializations are not unlike the nerve-to-nerve synapses that exist in the central nervous system (Sanmarti-Vila *et al.* 2000; Kennedy, 2000; Aoki *et al.* 2001) or the structural arrangement of the skeletal neuromuscular junction (Boaro *et al.* 1998; Ruegg, 2001). However, although synaptic specializations have been intensely investigated in the CNS and at the skeletal neuromuscular junction using molecular and functional approaches, little is known about the identity of the molecular apparatus, structural arrangement or the functional roles of proteins that make up the synaptic specializations in the autonomic nervous system. Even less is known about these proteins that exist between nerve terminals and neuroeffector cells in the GI tract.

It has recently been established that enteric motor nerve terminals in the rat oesophagus and small intestine and in the murine stomach contain members of the *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors or SNAREs that are involved in the release of neurotransmitters from these terminals (Nirasawa *et al.* 1997; Aguado *et al.* 1999; Beckett *et al.* 2005). SNAREs are involved in neurovesicle docking to the presynaptic membrane, fusion of the neurovesicle and release of neurotransmitter in the synaptic cleft. Several of the SNARE proteins that have been identified to date in the murine stomach include synaptotagmin, syntaxin and SNAP-25 (Beckett *et al.* 2005). Each of these proteins has a specific role in the neurotransmitter release process (Mehta *et al.* 1996; Sudhof & Rizo, 1996). Varicosities containing

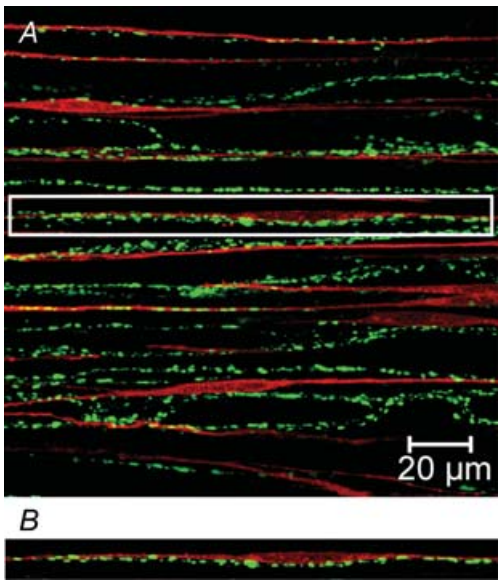

Figure 2. Close structural arrangement between enteric nerves and ICC-IM in the murine stomach
A shows double labelling of excitatory motor nerves with vesicular acetylcholine transporter (vAChT, green) and ICC with Kit (red). Confocal microscopy reveals that enteric nerves track ICC-IM for several hundreds of micrometres within the circular muscle layer of the fundus. A similar structural arrangement occurs in the gastric antrum (not shown). *B* is an isolated section of the image in *A* identified by the white box, highlighting the close anatomical relationship between nerve terminals and ICC-IM. Scale bar in *A* applies to both panels. Courtesy of Dr Satoshi Iino.

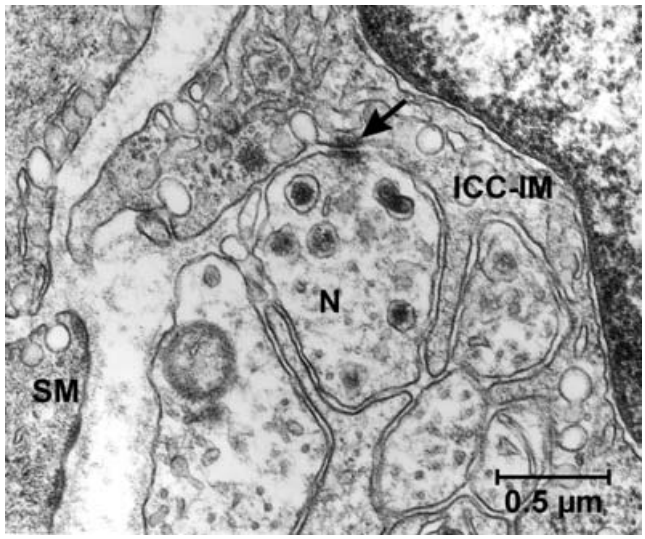

Figure 3. Synapse-like specializations exist between enteric nerve terminals and ICC-IM in the canine gastric antrum
The presynaptic membrane of the enteric nerve terminal (N) displays electron densification at the same site as the postsynaptic membrane on ICC-IM (ICC-IM; arrow). Note that enteric nerves do not form close apposition to smooth muscle cells (SM) or form synaptic specializations. Courtesy of Dr Kazu Horiguchi.

these SNARE proteins were only observed in intimate association with ICC-IM and were not observed in close apposition to smooth muscle cells (Beckett *et al.* 2005). These data support the hypothesis that ICC-IM are directly innervated by active sites where neurotransmitter release occurs.

The electron-dense region that exists on the post-synaptic membranes of ICC-IM opposite the pre-synaptic membrane densifications on enteric nerve terminals are also structurally similar to those observed at nerve-to-nerve synapses in the CNS and are classically termed postsynaptic density proteins or PSD. Transcripts for two postsynaptic scaffolding proteins PSD-93 and PSD-95 have been detected by RT-PCR in the murine stomach. Quantitative RT-PCR revealed that expression of PSD-93 and PSD-95 are decreased in the stomachs of W/W^V mutants that lack ICC-IM. Finally, double-labelling immunohistochemical experiments using antibodies that recognize the PDZ domain of the PSD-95 family members (PSD-95 and PSD-93, and SAP 97) and Kit revealed the expression of PSD proteins on ICC-IM but not neighbouring smooth muscle cells (Beckett *et al.* 2005). These data suggest that ICC-IM express the necessary proteins to form post-synaptic proteins and further support the hypothesis that ICC-IM are directly innervated.

Evidence for the functional innervation of ICC-IM by enteric motor nerves

ICC possess a variety of receptors for neurotransmitters, hormones and paracrine substances. These receptors include receptors for neurotransmitters and hormones, including NK1 receptors (Sternini *et al.* 1995; Grady *et al.* 1996; Portbury *et al.* 1996; Vannucchi *et al.* 1997; Lavin *et al.* 1998), VIP receptors (Epperson *et al.* 2000), somatostatin receptors (Sternini *et al.* 1997), bradykinin B2 receptors (Choi *et al.* 2006) and CCK-A receptors (Patterson *et al.* 2001). ICC-DMP also express gKinase-I (Salmhofer *et al.* 2001), and increases in cGMP in ICC in response to nitric oxide donors or stimulation of enteric motor nerves, suggests that ICC are targets for inhibitory neurotransmission (Shuttleworth *et al.* 1993; Young *et al.* 1993).

More direct functional evidence for the primary role of ICC in enteric motor neurotransmission came from experiments performed on the stomachs of W/W^V mutant mice that lack ICC-IM. In the absence of ICC-IM post-junctional neural responses to cholinergic excitatory and nitrergic inhibitory neurotransmission were absent or greatly attenuated within the circular muscle layers of the gastric fundus and antrum (Burns *et al.* 1996; Ward *et al.* 2000; Beckett *et al.* 2002, 2003; Suzuki *et al.* 2003). Similarly in the longitudinal muscle layer of the gastric antrum that also lacks ICC-IM in wild-type animals there is an absence of cholinergic and nitrergic responses (Song *et al.* 2005*a*). Although there was an absence of excitatory and inhibitory neural responses from the fundus and antrums of W/W^V mutants, enteric motor nerves were present and functional and muscles responded to the exogenous application of neurotransmitters in a manner similar to wild-type controls (Ward *et al.* 2000; Beckett *et al.* 2002).

Although post-junctional cholinergic and nitrergic responses are absent or greatly attenuated in W/W^V mutant mice that lack ICC-IM, neural responses still persist. In the gastric antrum of wild-type animals nerve stimulation evokes a complex series of post-junctional responses, consisting of an initial apamin-sensitive inhibitory junction potential (IJP) and a slower nitrergic IJP; the inhibitory responses are followed by an excitatory response that consists of both atropine-sensitive and at more sustained stimulation frequencies an insensitive excitatory response (Song *et al.* 2005*b*). In antrums of W/W^V mice the initial apamin-sensitive component still persists. Sustained stimulation of W/W^V mutant tissues also reveals a non-cholinergic excitatory response that is probably mediated through neurokinins (Song *et al.* 2005*b*). The presence of these post-junctional responses but the absence of cholinergic and nitrergic responses when ICC-IM is absent is interesting. It should be noted that there is an up-regulation of P2Y receptors on smooth muscle cells of W/W^V mutants, suggesting that there may be a compensatory role for purinergic transmission in the absence of ICC-IM and nitrergic neurotransmission. Alternatively parallel innervation of gastric muscles may occur by neurotransmitters that target different cellular components in the tissue.

In the small intestine ICC-IM are replaced by a dense network of ICC located at the level of the deep muscular plexus. ICC-DMP are intimately associated with enteric nerve terminals (Torihashi *et al.* 1993). Enteric nerve terminals appear to form synapses preferentially with ICC-DMP rather than smooth muscle cells (Wang *et al.* 1999). The functional role of ICC-DMP is difficult to prove since they persist in the small intestines of W/W^V and Sl/Sl^d mutants and post-junctional neural responses appear intact. In the small intestine, ICC-DMP develop later than ICC-MY and fully developed ICC-DMP networks do not exist until approximately 10 days after birth (P10). Cholinergic and nitrergic neural responses are poorly developed at birth but become more robust by P10, after ICC-DMP have developed. Although ICC-DMP are not disrupted in W/W^V mutant animals, they become disrupted when newborn animals are injected with the Kit neutralizing antibody ACK2 (Torihashi *et al.* 1995) or when tissues isolated from P0 mice are placed in organotypic cultures and treated with ACK2 for 10 days (Ward *et al.* 2006). Blocking Kit and the development of ICC-DMP caused loss of cholinergic and nitrergic neural

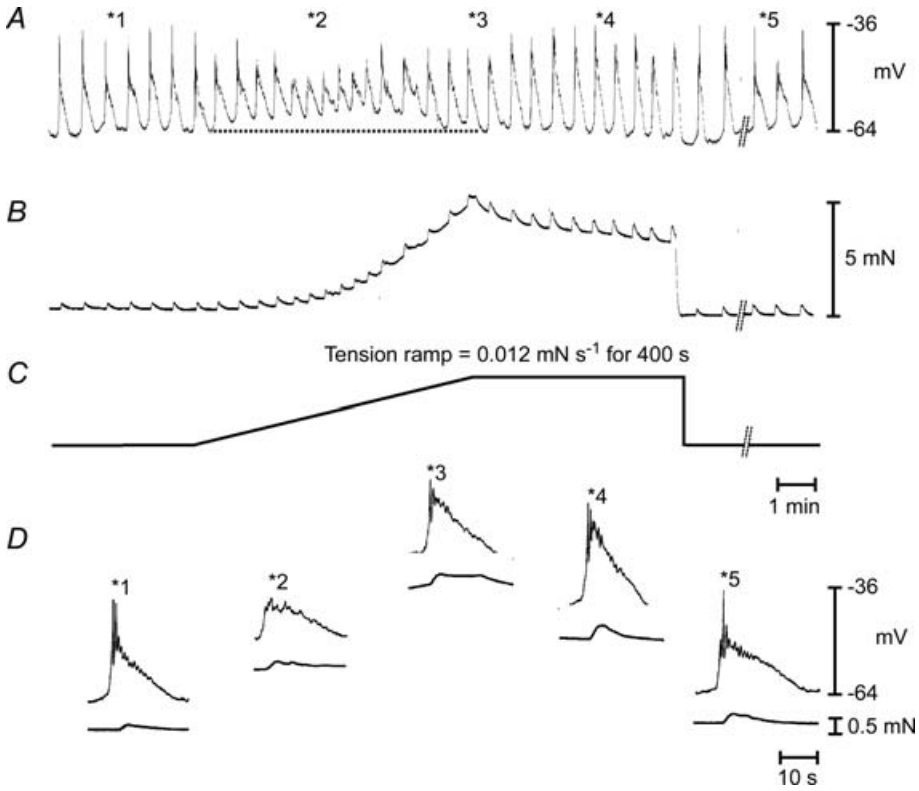

Figure 4. Stretch-dependent responses of the gastric antrum
A and *B* show changes in electrical activity and isometric force in response to stretch of the gastric antrum. Stretching the antrum induced membrane depolarization, an increase in slow wave frequency and increase in the amplitude and frequency of contractions. *C* shows the length ramp. Time bar in *C* applies to panels *A*–*C*. Break in records represents 5 min for recovery. *D* shows examples of slow waves and contractions at an increased sweep speed at different stages of the stretch protocol (times denoted in *A* as *1–5). Note the increase in frequency and contractions during the period of stretch (dotted line under membrane potential trace). During the periods of increasing muscle length, slow wave frequency and membrane potential recovered partially. Courtesy of Dr Kyung-Jung Won.

responses, while non-cholinergic excitatory responses remained after loss of ICC-DMP. These observations are consistent with the hypothesis that cholinergic and nitrergic motor neural inputs in the small intestine are mediated via ICC-DMP and thus ICC-DMP appear to serve a function in the small intestine that is similar to the role of ICC-IM in the stomach.

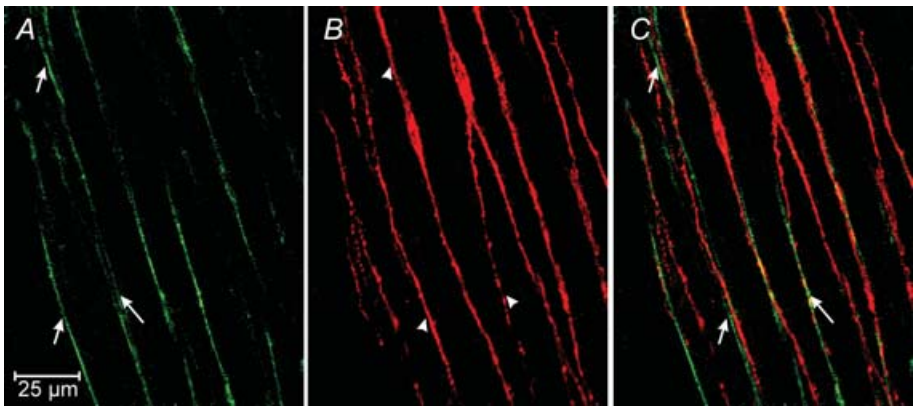

Figure 5. Close anatomical relationship between vagal afferents and ICC-IM in the murine gastric fundus
A shows biotinylated dextran amine–fluorescein anterograde labelling of vagal afferent intramuscular arrays (IMAs; green; arrows) within the circular muscle of the murine gastric fundus. *B* shows ICC-IM (red; arrowheads) within the circular muscle layer and *C* shows an overlay of panels *A* and *B*. Note that vagal IMAs are in close apposition to ICC-IM for hundreds of micrometres (arrows). Scale bar in *A* applies to all panels.

Integrative responses of ICC-IM

From the ultrastructure of ICC, morphologists also proposed that ICC may act as stretch sensors in the GI tract (Thuneberg, 1989). Organs of the GI tract undergo considerable length changes during filling and emptying, and the enteric nervous system generates the appropriate motor patterns to accomplish these tasks. ICC serve critical functions in motility, so it is extremely important to understand whether the functions of these cells are regulated by stretch of the bowel wall.

In the stomach, neural inputs to ICC-IM are superimposed upon intrinsic stretch-dependent responses, and the final influence of ICC on motility is highly length dependent (Won *et al.* 2005). Stretch of antral muscles causes depolarization and a positive chronotropic effect on the generation of slow waves (Fig. 4). Antral stretch responses depend upon the rate of muscle lengthening, and responses to stretch adapt and reset rapidly. Interestingly, it is not the primary pacemaker cells (ICC-MY) that respond to length changes. ICC-IM possess mechano-sensors and transduction mechanisms that convey stretch-dependent responses in the gastric antrum, and these responses are absent in W/W^V mice that lack ICC-IM. Repetitive stretch ramps increased pacemaker frequency and initiated unitary potential discharge on the upstroke of slow waves (Won *et al.* 2005). Stretch of isolated antral muscle bundles caused generation of unitary potentials that summated to produce regenerative potentials (G. Song, K. M. Sanders and S. M. Ward, unpublished observations). Summation of unitary potentials into regenerative potentials in the gastric antrum in response to membrane depolarization or cholinergic stimulation has been shown to conduct to the ICC-MY network and drive this normally dominant group of pacemaker cells (Hirst *et al.* 2002). Stretch has similar, and probably, additive effects to excitatory neural drive on antral pacemaker activity. Thus ICC-IM appear to be a site of convergence for both neural and mechanical inputs that regulate pacemaker activity.

The mechano-sensor that responds to stretch is under investigation. There is evidence that generation of arachidonic acid may be the initial response to stretch. Responses to stretch were inhibited by indomethacin, suggesting that arachidonic acid metabolism is critical for ICC-dependent stretch responses. We have shown that ICC-IM express COX-II (Porcher *et al.* 2002), and stretch-dependent responses were lacking in COX-II-deficient mice. Thus, it appears that products of COX-II may be the effectors of stretch responses in the antrum.

Summary

Morphological and functional studies demonstrate that the neuromuscular junction in gastrointestinal muscles is not as simple as once thought. At least three different cell types make up the enteric neuromuscular junction. Classical excitatory and inhibitory neurotransmitters are concentrated and released from neurovesicles located in enteric nerve terminals or varicose regions of motor nerves, whereas nitric oxide is probably synthesized *de novo* as calcium concentration increases in nerve terminals upon membrane depolarization. Enteric nerve terminals make intimate synapses with ICC-IM, which are situated between the nerve terminals and neighbouring smooth muscle cells. ICC-IM play a critical role in the reception and transduction of cholinergic excitatory and nitrergic inhibitory neurotransmission. ICC-IM form gap junctions with smooth muscle cells and post-junctional electrical responses generated in ICC are conducted to the smooth muscle syncytium. By this contact, ICC can regulate the neuromuscular responses observed throughout the GI tract. Recent morphological evidence using anterograde tracing methods, has shown close apposition between vagal and spinal afferents and ICC-IM within the stomach wall (Fig. 5) and their absence in mutant animals that lack ICC-IM also supports a role for ICC-IM as possible integrators for in-series stretch-dependent changes in this organ.

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