This document is confidential and is proprietary to the American Chemical Society and its authors. Do not copy or disclose without written permission. If you have received this item in error, notify the sender and delete all copies.

No role of interstitial cells of Cajal (ICCs) in genesis of inhibitory junction potentials (IJPs): evidence from a novel mouse model of genomic ICC depletion

Journal:	*ACS Chemical Neuroscience*
Manuscript ID:	cn-2013-001439
Manuscript Type:	Review
Date Submitted by the Author:	26-Jul-2013
Complete List of Authors:	CHAUDHURY, ARUN; VA BOSTON HEALTHCARE SYSTEM, MEDICINE

SCHOLARONE™
Manuscripts

No role of interstitial cells of Cajal (ICCs) in genesis of inhibitory junction potentials (IJPs): evidence from a novel mouse model of genomic ICC depletion

Arun Chaudhury

VA Boston HealthCare System, Boston

No conflict of interest

Address for Correspondence:

Arun Chaudhury, M.D.
VA Medical Center
1400 VFW Parkway
West Roxbury MA 02132
Phone: 6173022613
Email arunchaudhury.boston@gmail.com

Abstract

The passage of food through the gut involves complex motor patterns of the gastrointestinal smooth muscles to facilitate digestion, absorption and egestion. Normally, neuromuscular neurotransmission shall imply direct communication between the motor nerve terminals and the smooth muscle fibers after electrical field stimulation (EFS). Among many resident and transmigratory cells in the stroma interspersed in the neuromuscular bundles, a mesenchymally derived cell, the interstitial cells of Cajal (ICC), has received much attention, especially in relation to its plausible function in neurotransmission. With an aim to address this significant issue, Klein *et al* (2013) demonstrate intact fast and slow IJPs and normal post-EFS colonic mechanical relaxations after acute interstitial cells of Cajal (ICC) depletion in tamoxifen treated *c-Kit*$^{CreERT2/+}$;*LSL-R26*$^{DTA/+}$ mice. The genomic expression of diphtheria toxin specifically in the ICCs to cause cellular death is the investigative group's highly novel approach to selectively deplete the ICCs. The end-point neurophysiological assay of inhibitory neurotransmission involves microelectrode recordings of inhibitory junction potentials (IJPs) after galvanic nerve stimulation of gastrointestinal smooth muscle strips in vitro. Intact IJPs are *sine qua non* of normal enteric inhibitory neuromuscular neurotransmission that result in mechanical relaxation. The demonstration of intact IJPs in tamoxifen treated *c-Kit*$^{CreERT2/+}$;*LSL-R26*$^{DTA/+}$ mice provide paradigm-shifting and unambiguous evidence that ICCs play no role in inhibitory signal transduction and genesis of fast and slow IJPs, the electrophysiological signatures of neurotransmission of inhibitory signals from the stimulated nerve terminals to relax gastrointestinal smooth muscles.

Keywords

IJP, EJP, mechanical relaxation, nitric oxide, ATP, slow wave

Significance of Enteric neuromuscular neurotransmission and participating cellular components: extent of a controversy in smooth muscle neuromuscular neurotransmission

The passage of food through the gastrointestinal tract involves complex motor patterns of the gastrointestinal smooth muscles to facilitate digestion, absorption and egestion.[1-4] Motor nerve terminals form *en passant* like junctions as it courses through the gut muscular bundles.[5] Among many resident and transmigratory cells in the stroma interspersed in the neuromuscular bundles, a mesenchymally derived cell, the interstitial cell of Cajal (ICC), has received much attention, especially in relation to its plausible function in neurotransmission.[6] These arguments have mainly based on reports that ICCs located in proximity to the nerve terminals possess receptors and signal transduction apparati of both excitatory and inhibitory neurotransmitters released from the nerve terminals, the same repertoire of receptors being also present within the smooth muscle cells (Figure 1).[7-10]

The neurotransmitters, mainly acetylcholine and substance P, are excitatory in nature, and causes smooth muscle contraction after depolarization of the smooth muscle membrane, while the inhibitory neurotransmitters, purines like ATP and the gasotransmitter nitric oxide (NO), causes smooth muscle relaxation by hyperpolarization of the smooth muscle membrane.[5, 11-15]

Normally, it may be construed that neuromuscular neurotransmission would imply a linear concept of communication between the motor nerve terminals and the smooth muscle fibers after nerve stimulation. However, the presence of the intercalated ICC within the neuropil, which appears closely apposed to the nerve terminals in two dimensional sections of the gastrointestinal tract,[16] has been the main basis for the hypothesis of their possible participation in transduction of signals from the stimulated nerve terminals to the smooth muscles. Considerable debate exists regarding whether direct communication occurs between nerve terminals and smooth muscles after electrical stimulation, or whether any intermediary cell, like for example, the ICC, transduces the neurotransmitter signals to the smooth muscles during neurotransmission.[6-7,15,17- 20] Inhibitory neuromuscular neurotransmission plays a central role in gut physiology, as failure of the smooth muscles to relax may cause obstruction of oro-aboral passage of gastrointestinal contents.[7,13, 21-25]

Evidence from Klein et al (2013) of recorded intact fast and slow inhibitory junction potentials (f/sIJPs) after genomic depletion of interstitial cells of Cajal

In this review, I will focus on discussion of a paradigm-shifting evidence recently obtained from a novel conditional knockout mice model which provides convincing evidence of the lack of role of ICCs in direct purinergic and nitrergic neurotransmission. Utilizing the exquisite fidelity of the inducible site-specific Cre/loxP recombination system,[26] Klein *et al* (2013) demonstrate intact fast and slow IJPs and normal post-electrical field stimulation (EFS) colonic mechanical relaxations after acute interstitial cells of Cajal (ICC) depletion in tamoxifen treated *c-Kit*$^{CreERT2/+}$;*LSL-R26*$^{DTA/+}$ mice.[25] Klein *et al* (2013) correctly identify the outstanding importance of addressing the role of interstitial cells of Cajal in enteric neurotransmission.[25]

Klein et al (2013) incorporates a novel technological tool to obtain reductionist perspective of this highly significant area of investigation. Namely, the genomic expression of

diphtheria toxin specifically in the ICCs to cause cellular death is a highly novel approach to selectively deplete the ICCs. Until now, mice models were available with only patchy loss of ICCs, for example, loss of ICC-IMs within the neuromuscular bundles in W/Wv mice.[27] Klein et al's (2013) generation of this novel genomic model with only selective depletion of ICCs is a quantum leap that will enhance our understanding of the signaling and other role of ICCs in enteric neuromuscular neurotransmission.

Alternate interpretations to the central conclusions of Klein et al (2013) from the presented key evidence: Inhibitory junction potentials (IJPs), sine qua non of inhibitory neuromuscular neurotransmission, can be recorded in the absence of ICCs

Notably, Klein et al's (2013)[25] results provide opportunity for alternate interpretations of the central conclusions based on the presented evidence. The end-point neurophysiological assay of inhibitory neurotransmission involves microelectrode recordings of inhibitory junction potentials (IJPs) after galvanic nerve stimulation of gastrointestinal smooth muscle strips in vitro.[28] Intact IJPs are *sine qua non* of normal enteric inhibitory neuromuscular neurotransmission that result in mechanical relaxation (Figure 2).[4,11-15, 22-24, 29-39] Hence, the central conclusion of Klein et al (2013) that "*acute TAM-induced depletion of ICC by conditional expression of DTA ……impairs neurotransmission in the GI tract of adult animals*" is in disconcordance with the presented key evidence, viz. intact fast and slow IJPs and normal mechanical relaxation of the gut in *c-Kit*$^{CreERT2/+}$;*LSL-R26*$^{DTA/+}$ mice.[25] In contrary, Klein et al's results (2013) using the specific ICC-depletion model provide paradigm-shifting evidence that ICCs play no role in genesis of fast and slow IJPs (Figure 3), the electrophysiological signatures of neurotransmission of inhibitory signals from the nerve terminals to gastrointestinal smooth muscles after EFS.

Although the P2Y1 receptor involved in transducing purinergic inhibitory neurotransmission in the gut is accepted with consensus,[22-24] there is confusion regarding the cellular location of the purinergic receptor that mediates fast IJP. Suggestions have been made that the purinergic receptor mediating the purinergic IJP is localized on the PDGFRα fibroblast-like cells (FLCs) that mediate purinergic IJP.[40] This may be a possible explanation why intact fast IJP was observed by Klein et al.[25] However, confirmatory evidence regarding the role of these FLCs have yet to come, as apamin sensitive outward currents reported in FLCs are also seen in smooth muscle cells.[40,41] Currently, there is not much technical expertise to image lowering of cytosolic calcium after agonist stimulation, so alteration of calcium signals after P2Y1 agonism may not be easily obtained. Recently, Tango imaging (Barnea et al 2008) of G protein coupled receptors (GPCRs) has been reported.[42] P2Y1 is a GPCR, and thus a reliable anatomical reporter like TANGO may be utilized to obtain confirmatory evidence of P2Y1 receptor activation in gut smooth muscle cells or FLCs after stimulation induced purinergic agonist release. Also, biochemical evidence needs to be provided how the effector of purinergic signaling is translocated from the FLC to the smooth muscles within the millisecond timescale of genesis of the fast IJP. Nevertheless, Klein et al's (2013)[25] novel conditional knockout approach can help address these issues and provide wealth of relevant information in purinergic neurotransmission.[15]

Rationale against the role of interstitial cells of Cajal (ICCs) as a nitric oxide (NO) sink

The authors hypothesize that NO targets only cells in close proximity to its synthesis and that ICCs act as a NO sink.[25] However, there is no morphological evidence that ICCs warp nerve terminals in 3D or blankets muscularis externa from the nerve fibers criss-crossing its bulk.[5] Nitric oxide diffuses in all three dimensions from its site of synthesis, the diffusion distance can be as high as 500 microns and dependent on regional scavengers including protein, lipid, haem or oxygen.[43,44] The diffusion distance during enteric neuromuscular nitrergic neurotransmission has never been estimated, but can be reasonably presumed much less than 500μm, thus the separation distance between the nerve terminal and the smooth muscle probably not being a limiting factor in diffusion.

Qualitative evidence provided by Klein et al (2013)[25] shows that incomplete decrease of Prkg1 (Protein Kinase G isoform 1β) in ICCs decreases sIJP amplitude. These results would imply that ICCs are the major cellular site of Prkg1 upregulation, which is thence transported to smooth muscles. If such is the case, then it is not clear why or how deletion of ICCs (which imperatively resulted in $Prkg1_{ICC}$ loss) [25] produced no effect on sIJP. The maximum size of molecules that can pass through gap junctions is 1 kDa.[45] It is unlikely that Prkg, with approximate MW of 100 kDa, can pass through ICC/smooth muscle gap junctions. The stained whole mounts show numerous areas of absent Prkg1 staining in the smooth muscles,[25] raising the alternate possibility that this decrease of Prkg1 in smooth muscles may have resulted in inhibition of the sIJP amplitude. This important issue merits being resolved in future studies.

A recent study reported that deletion of soluble guanylyl cyclase (sGC) by floxing the sGC exons specifically within the ICCs produced no effect on smooth muscle relaxations.[7] It is unclear how then partial deletion of Prkg1 in ICCs,[25] which acts downstream of sGC, result in diminished amplitude of the nitrergic slow IJP. In this case of floxed Prkg1[25], despite the presence of ICCs, how could NO traverse the long distance to smooth muscles? The key findings from these two recent publications[7,25] are inconsistent , which merits to be addressed in future studies.

The predominant isoform of Prkg1 in ICCs is Prkg1β,[25] whereas the predominant isoform of Prkg1 in enteric smooth muscles is both Prkg1β and Prkg1α.[8] Prkg1α is 10 fold more sensitive to cGMP oscillations than Prkg1β.[46] While Prkg1β binds to IRAG to diminish free calcium, Prkg1α interacts specifically with the myosin binding subunit of myosin phosphatase, the major enzyme involved in molecular regulation of smooth muscle relaxation,[47] thus implying obvious direct role in smooth muscle relaxation.[46] It is likely that Prkg1α, the final mediator of smooth muscle relaxation, is elevated within the sarcoplasm *per se* via direct NO signaling from the nerve terminals to the smooth muscles. This important testable hypothesis may be achieved using the novel genomic model and using imaging probes for cyclic nucleotide signaling.[48]

c-Kit$^{CreERT2/+}$;LSL-R26$^{DTA/+}$ mice may provide detailed insights into excitatory neurotransmission

Diminished off-contractions after ICC depletion are presented as the only evidence for excitatory neurotransmission.[25] Off-contractions have complex pharmacology and not

necessarily cholinergic mediated.[49] Klein et al have not demonstrated atropine-responsiveness of the fast EJPs in wild-type animals.[25] Spontaneous and stimulated on-contractions and fast and slow EJP pharmacology may be examined in this model[25] to obtain more rigorous evidence for integration of excitatory neurotransmission by ICCs.

Mechanical recordings are excellent methods to assay neurotransmission resulting in contraction, as the risk of the expulsion of the recording electrode does not exist. Important information may also be obtained by studying portions of the gastrointestinal tract like the gastric antrum which are known to exhibit spontaneous contractions.[50]

Incomplete depletion of ICCs in TAM-treated c-Kit$^{CreERT2/+}$;LSL-R26$^{DTA/+}$ mice: implications on interpretations of the function of ICCs and slow waves

Early reports have demonstrated rhythmicity in smooth muscles, and its relation to nerve activity.[51] The cellular source of origin of slow waves is a matter of intense controversy due to conflicting observations, with variability based on regions of the gut as well as differences in species, with theories supporting either myogenic site of origin[52] or within the ICCs, and thereafter, electrotonically being conducted to the smooth muscles, and both sources possibly being modulated by neural activity. It has been argued, based on circumstantial evidence, of dual or parallel inhibitory innervation, i.e., a direct pharmacomechanical coupling between the motor nerve terminals and the smooth muscles, as well as a separate innervation of the ICCs by the nerve terminals.[7,16] A full treatise of this important topic is simply beyond the scope of this review and only a fraction with relevance to re-discussion of Klein et al's evidence is only considered here. It has been discussed that the slow waves arise as a result of neurotransmission between the nerve terminals and the ICCs.[53,54]. Very little is known regarding basal states of enteric neuromuscular neurotransmission,[55] or the characteristic of the spectrum of summated responses that result in the release of inhibitory or excitatory neurotransmitters during neuromuscular transmission.[56-58] Slow waves were not abolished after genetic deletion of ICCs but the frequencies and amplitude diminished or were irregular (Klein et al 2013).[25] This may be interpretive of an alternate provocative possibility of either dispensable role of ICCs for genesis of slow waves but a pacesetter like function in amplitude and frequency modulation, or may have resulted from the incomplete deletion of the ICCs after conditional knockout.

TAM-*c-Kit*$^{CreERT2/+}$;*LSL-R26*$^{DTA/+}$ mice, where only 50-60% of ICCs were deleted by incomplete expression of the diphtheria toxin (Klein et al 2013), resembles W/Wv mice, which also has patchy loss of ICCs.[27] Deleter strain using CreERT2 show expression of Cre in only about 70% cells in peripheral tissues.[59] Klein et al's investigative group possess the necessary technical expertise to improvise on this efficiency of the knockout model. Furthermore, future experiments with this animal model will provide us with definitive insights into the role of ICCs in slow wave generation. Because slow wave is a network output,[60,61] the location specificity of the loss of ICCs becomes critical information. This may be easily demonstrated by co-staining gastrointestinal tissues obtained from *c-Kit*$^{CreERT2/+}$;*LSL-R26*$^{DTA/+}$ mice with other reliable ICC markers like TMEM16A.[62,63]

Integration of excitatory and inhibitory neurotransmission with slow waves involves challenging simultaneous recordings of slow waves with superimposed spontaneous or stimulated excitatory and inhibitory junction potentials (EJPs & IJPs)

Klein et al (2013) concludes the study's findings as "integration of enteric excitatory and inhibitory neurotransmission with slow waves by the ICCs". There are only scant previous studies where attempts have been made to obtain the challenging recordings of the merging of slow waves with either spontaneous or stimulated IJP or EJP, based on which any conclusion of integration may be derived.[53,64,65] The current study (Klein et al 2013)[25] has not provided sufficient evidence on the integrative roles of ICCs with junction potentials.

In the rare simultaneous recordings of IJPs superimposed upon slow waves, it has been shown that the amplitude and duration of slow waves was altered after sIJP in the canine colon, or that inhibitory junction potentials recorded with stimulation at the plateau phase of the slow waves caused termination of the slow wave activity.[53]

Slow wave behavior has been demonstrated to model like a linear van der Pol oscillator.[66-68] How post-stimulation events result in desynchronization of the slow wave, and how it regains resynchronization remains outstanding questions, which the current genomic model may provide valued insights upon obtaining these superimposed recordings and other focused studies. With future fine tuning of the genomic manipulation and possibility of generation of a model with global loss of ICCs, greater insights shall be obtained into the cellular source of slow waves, cellular sources of modulation of ion conductance changes and their potential role in gastrointestinal motility.

The contribution of the neural activity in genesis of slow waves may also be examined using mice models of exocytosis failure like Munc18-1 knockout[69] or genetic knockout mice models of nonvesicular neurotransmitter synthetic enzyme, for example, as has been examined in the nNOSα knockout mice.[70] Pharmacologic blockade of nitrergic signaling in *c-Kit*$^{CreERT2/+}$;*LSL-R26*$^{DTA/+}$ mice or creation of crosses with knockout mice of different backgrounds will offer important insights into the genesis of complex phenotypic characteristics like colonic migratory motor complexes.

Phenotypic similarity of c-Kit$^{CreERT2/+}$;LSL-R26$^{DTA/+}$ mice with W/Wv mice

Gastric emptying was delayed in the TAM-treated *c-Kit*$^{CreERT2/+}$;*LSL-R26*$^{DTA/+}$ mice despite the intact nitrergic slow IJP.[25] There is significant variability in nitrergic neurotransmission in the LES of W/W(v) mutant mice.[71,72] This phenotype of the mice generated by Klein et al[25] again indicates similarity with W/Wv mice, wherein duodeno-antral reflux, as reported earlier,[27] may have resulted in residual gastric ICG fluorescence.[25] It seems that this new model may be of relevance to investigating intestinal pseudo-obstruction, the pathophysiological basis of which is mostly unknown.

The *c-Kit*$^{CreERT2/+}$;*LSL-R26*$^{DTA/+}$ mice also show segmented terminal portions of the colon probably resulting from intraluminal fecal pellets, and thus, Klein et al's (2013)[25] model provides a unique opportunity to test the hypothesis whether myokymic movements in the absence of slow oscillations and excitatory neurotransmission are present and adequate for luminal content propulsion. This may provide valuable insights into the functional redundancy that protects

against frank intestinal obstruction in disorders of neurotransmission. Preliminary reports of such ripple-like muscular movements have recently been published.[73]

Translational significance of the differential role of ICCs and inhibitory neurotransmission in gastrointestinal motility: pathophysiological rationale

Despite reports of alterations of numbers of ICCs in motility disorders, it may not directly correlate with specific pathophysiology resulting from defective inhibitory neurotransmission (Sarna 2008).[17] For example, studies in mice models of defective gastric emptying and human tissue biopsies show both reduction in ICCs and nNOS in nerve terminals, and thus are not able to conclusively correlate the disease pathophysiology with just alteration of ICC numbers and actually strongly suggests comorbidities involving both ICCs and defective neurotransmission.[74,75] Deficits in inhibitory neurotransmission may result from deletion of nNOSα,[21,27] or deficiencies of its transcriptional activators[76] or cofactors[77,78], or motor proteins critical for nonvesicular and vesicular neurotransmission,[79-81] or defects in the smooth muscles *per se*.[7,13,22-24,47,82]

The significance of the neuromuscular apparati, its constituent cellular elements and their neurophysiological functions has long been a topic of vigorous investigation.[83-86] The complexity of deficits of neurotransmitter functions now presents with even more tenets. For example, while nNOS enzymatic functional defect has been related with changes in gastric emptying, and correlated as a pathological explanation for diabetic gastroparesis,[87] the current model presents evidence of impaired gastric emptying even with intact slow IJP,[25] which reveals impaired gastric emptying despite normal nitrergic neurotransmission. Studies on a different gastrointestinal model of diabetic complications have reported loss of nitrergic neurotransmission without alteration of ICC numbers.[88] Clearly, we are now equipped with a new innovative tool, the *c-Kit*$^{CreERT2/+}$;*LSL-R26*$^{DTA/+}$ mice,[25] which will tremendously bolster our understanding of the cellular biology and electrophysiological properties of ICCs and contribute to our understanding of the variability of gastrointestinal motor functions in health and disease.

Summary: No role of ICCs in genesis of IJPs, electronic signatures of enteric neuromuscular neurotransmission

In summary, the unequivocal evidence of intact fast and slow IJPs and mechanical relaxations after genomic deletion of ICCs in mice provided by Klein et al (2013)[25] rigorously support the conclusion of the lack of contribution of ICCs in inhibitory signal transduction and genesis of inhibitory junction potentials, the hallmarks of EFS-induced inhibitory neuromuscular neurotransmission.

References

1. Cannon, W.B. (1902) The Movements of the Intestines studied by Means of the Röntgen Rays. *J Med Res.* 7, 72-5.
2. Bornstein, J.C. (2008) Purinergic mechanisms in the control of gastrointestinal motility. *Purinergic Signal.* 4, 197-212.

3. Huizinga, J.D, Lammers, W.J. (2009) Gut peristalsis is governed by a multitude of cooperating mechanisms. *Am J Physiol Gastrointest Liver Physiol.* 296, G1-8.
4. Costa, M., Wiklendt, L., Arkwright, J.W, Spencer, N.J, Omari, T., Brookes, S.J, Dinning, P.G. (2013) An experimental method to identify neurogenic and myogenic active mechanical states of intestinal motility. *Front Syst Neurosci.* 11;7:7. doi: 10.3389/fnsys.2013.00007.
5. Goyal, R.K., Chaudhury, A. (2013) Structure activity relationship of synaptic and junctional neurotransmission. *Auton Neurosci.* 176, 11-31.
6. Sanders, K.M., Hwang, S.J., Ward, S.M. (2010) Neuroeffector apparatus in gastrointestinal smooth muscle organs. *J Physiol.* 588, 4621-39.
7. Groneberg, D., Lies, B., König, P., Jäger, R., Seidler, B., Klein, S., Saur, D., Friebe, A. (2013) Cell-Specific Deletion of Nitric Oxide-Sensitive Guanylyl Cyclase Reveals a Dual Pathway for Nitrergic Neuromuscular Transmission in the Murine Fundus. *Gastroenterology.* 145(1):188-96.
8. Huber, A., Trudrung, P., Storr, M., Franck, H., Schusdziarra, V., Ruth, P., Allescher, H.D. (1998) Protein kinase G expression in the small intestine and functional importance for smooth muscle relaxation. *Am J Physiol.* 275, G629-37.
9. Salmhofer, H., Neuhuber, W.L., Ruth, P., Huber, A., Russwurm, M., Allescher, H.D. (2001) Pivotal role of the interstitial cells of Cajal in the nitric oxide signaling pathway of rat small intestine. Morphological evidence. *Cell Tissue Res.* 305, 331-40.
10. Epperson, A., Hatton, W.J., Callaghan, B., Doherty, P., Walker, R.L., Sanders, K.M., Ward, S.M. and Horowitz, B. (2000) Molecular markers expressed in cultured and freshly isolated interstitial cells of Cajal. *Am J Physiol Cell Physiol.* 279, C529-39.
11. Burnstock, G., Campbell, G., Bennett, M., Holman, M.E. (1963) Inhibition of the smooth muscle on the taenia coli. *Nature*. 200, 581-2.
12. Goyal, R.K., He, X.D. (1998) Evidence for NO. redox form of nitric oxide as nitrergic inhibitory neurotransmitter in gut. *Am J Physiol.* 275, G1185-92.
13. King, B.F. (2012) Resolution and concordance in dissecting the compound inhibitory junction potential. *J Physiol.* 590, 1777-8.
14. Bridgewater, M., Cunnane, T.C., Brading, A.F. (1995) Characteristic features of inhibitory junction potentials evoked by single stimuli in the guinea-pig isolated taenia caeci. *J Physiol.* 485, 145-55.
15. Goyal, R.K., Sullivan, M.P., Chaudhury, A. (2013) Progress in understanding of inhibitory purinergic neuromuscular transmission in the gut. *Neurogastroenterol Motil.* 25, 203-7.
16. Iino, S., Horiguchi, K., Nojyo, Y. (2008) Interstitial cells of Cajal are innervated by nitrergic nerves and express nitric oxide-sensitive guanylate cyclase in the guinea-pig gastrointestinal tract. *Neuroscience.* 152, 437-48.
17. Sarna, S.K. (2008) Are interstitial cells of Cajal plurifunction cells in the gut? *Am. J. Physiol. Gastrointest. Liver. Physiol.* 294, G372-90. Erratum in *Am. J. Physiol. Gastrointest. Liver. Physiol.* 294, G1299 (2008).
18. Goyal, R.K., Chaudhury, A. (2010) Mounting evidence against the role of ICC in neurotransmission to smooth muscle in the gut. *Am J Physiol Gastrointest Liver Physiol.* 298, G10-3.

19. Somlyo AP, Somlyo AV. (1994) Signal transduction and regulation in smooth muscle. *Nature.* 372, 231-6.
20. Sanders, K.M. (1996) A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. *Gastroenterology.* 111, 492-515.
21. Mashimo, H., Goyal, R.K. (1999) Lessons from genetically engineered animal models. IV. Nitric oxide synthase gene knockout mice. *Am. J. Physiol.* 277, G745-50.
22. Gil V, Martínez-Cutillas M, Mañé N, Martín MT, Jiménez M, Gallego D. (2013) P2Y(1) knockout mice lack purinergic neuromuscular transmission in the antrum and cecum. *Neurogastroenterol Motil.* 25(3):e170-82.
23. Gallego D, Gil V, Martínez-Cutillas M, Mañé N, Martín MT, Jiménez M. (2012) Purinergic neuromuscular transmission is absent in the colon of P2Y(1) knocked out mice. *J Physiol.* 590, 1943-56.
24. Hwang SJ, Blair PJ, Durnin L, Mutafova-Yambolieva V, Sanders KM, Ward SM. (2012) P2Y1 purinoreceptors are fundamental to inhibitory motor control of murine colonic excitability and transit. *J Physiol.* 590, 1957-72.
25. Klein, S., Seidler, B., Kettenberger, A., Sibaev, A., Rohn, M., Feil, R., Allescher, H.D., Vanderwinden, J.M., Hofmann, F., Schemann, M., Rad, R., Storr, M.A., Schmid, R.M., Schneider, G., Saur, D. (2013) Interstitial cells of Cajal integrate excitatory and inhibitory neurotransmission with intestinal slow-wave activity. *Nat Commun.* 4, 1630. doi: 10.1038/ncomms2626.
26. Nagy, A. (2000) Cre recombinase: the universal reagent for genome tailoring. *Genesis*.26, 99-109.
27. Sivarao, D.V., Mashimo, H., Goyal, R.K. (2008) Pyloric sphincter dysfunction in nNOS-/- and W/Wv mutant mice: animal models of gastroparesis and duodenogastric reflux. *Gastroenterology.* 135, 1258-66.
28. Bülbring, E., Tomita, T. (1967) Properties of the inhibitory potential of smooth muscle as observed in the response to field stimulation of the guinea-pig taenia coli. *J Physiol.* 189, 299-315.
29. Gillespie, J.S. (1962) The electrical and mechanical responses of intestinal smooth muscle cells to stimulation of their extrinsic parasympathetic nerves. *J Physiol.*162, 76-92.
30. Furness, J.B. (1969) An electrophysiological study of the innervation of the smooth muscle of the colon. *J Physiol.* 205, 549-62.
31. Smith, T.K., Bornstein, J.C., Furness, J.B. (1990) Distension-evoked ascending and descending reflexes in the circular muscle of guinea-pig ileum: an intracellular study. *J Auton Nerv Syst.* 29, 203-17.
32. Huizinga, J.D., Berezin, I., Daniel, E.E., Chow, E. (1990) Inhibitory innervation of colonic smooth muscle cells and interstitial cells of Cajal. *Can J Physiol Pharmacol.* 68, 447-54.
33. Christinck, F., Jury, J., Cayabyab, F., Daniel, E.E. (1991) Nitric oxide may be the final mediator of nonadrenergic, noncholinergic inhibitory junction potentials in the gut. *Can J Physiol Pharmacol.* 69, 1448-58.
34. Stark, M.E., Bauer, A.J., Sarr, M.G., Szurszewski, J.H. (1993) Nitric oxide mediates inhibitory nerve input in human and canine jejunum. *Gastroenterology*. 104, 398-409.

35. Plujà, L., Fernández, E., Jiménez, M. (1999) Neural modulation of the cyclic electrical and mechanical activity in the rat colonic circular muscle: putative role of ATP and NO. *Br J Pharmacol.* 126, 883-92.
36. Hirst, G,D., Bywater, R.A., Teramoto, N., Edwards, F.R. (2004) An analysis of inhibitory junction potentials in the guinea-pig proximal colon. *J Physiol.* 558, 841-55.
37. Vanneste, G., Robberecht, P., Lefebvre, R.A. (2004). Inhibitory pathways in the circular muscle of rat jejunum. *Br J Pharmacol.* 143, 107-18.
38. Gwynne, R.M., Bornstein, J.C. (2007) Mechanisms underlying nutrient-induced segmentation in isolated guinea pig small intestine. *Am J Physiol Gastrointest Liver Physiol.* 292, G1162-G1172.
39. Zhang, Y., Mashimo, H., Paterson, W.G. (2008) Regional differences in nitrergic innervation of the smooth muscle of murine lower oesophageal sphincter. *Br J Pharmacol.* 153, 517-27.
40. Kurahashi, M., Zheng, H., Dwyer, L., Ward, S.M., Don, Koh, S., Sanders, K.M. (2011) A functional role for the 'fibroblast-like cells' in gastrointestinal smooth muscles. *J Physiol.* 589, 697-710.
41. Vogalis, F., Goyal, R.K. (1997) Activation of small conductance Ca(2 +)-dependent K+ channels by purinergic agonists in smooth muscle cells of the mouse ileum. *J Physiol.* 502: 497–508.
42. Barnea, G., Strapps, W., Herrada, G., Berman, Y., Ong, J., Kloss, B., Axel, R., Lee, K.J. (2008) The genetic design of signaling cascades to record receptor activation. *Proc Natl Acad Sci U S A.* 105, 64-9.
43. Porterfield, D. M., Laskin, J.D, Jung, S.K, Malchow, R.P, Billack, B., Smith, P.J, Heck, D.E. (2001) Proteins and lipids define the diffusional field of nitric oxide. *Am. J. Physiol. Lung. Cell. Mol. Physiol.* 281, L904-12.
44. De Backer, O., Colpaert, E.E, Lefebvre, R.A. (2004) Influence of polyethylene-glycol-superoxide dismutase and combined depletion and repletion of antioxidants on nitrergic relaxation in the pig gastric fundus. *Eur J Pharmacol.* 486, 223-32.
45. Yeager, M., Nicholson, B.J. (1996) Structure of gap junction intercellular channels. *Curr Opin Struct Biol*. 6, 183-92.
46. Francis, S.H., Busch, J.L, Corbin, J.D, Sibley, D. (2010) cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacol Rev.* 62, 525-63.
47. Murthy, K.S. (2006) Signaling for contraction and relaxation in smooth muscle of the gut. *Annu Rev Physiol.* 68, 345-74.
48. Nikolaev, V.O., Gambaryan, S., Lohse, M.J. (2006) Fluorescent sensors for rapid monitoring of intracellular cGMP. *Nat Methods.* 3, 23-5.
49. Crist, J., Gidda, J.S., Goyal, R.K. (1984) Characteristics of "on" and "off" contractions in esophageal circular muscle in vitro. *Am J Physiol.* 246, G137-44.
50. Anuras, S., Cooke, A.R., Christensen, J. (1974) An inhibitory innervation at the gastroduodenal junction. *J Clin Invest.* 54, 529-35.
51. Eccles, J.C., Magladery, J.W. (1937) Rhythmic responses of smooth muscle. *J Physiol.* 90, 68-99.
52. Sanders, K.M., Koh, S.D., Ro, S., Ward, S.M. (2012) Regulation of gastrointestinal motility-insights from smooth muscle biology. *Nat Rev Gastroenterol Hepatol.* 9, 633-45.

53. Huizinga, J.D., Berezin, I., Daniel, E.E., Chow, E. (1990) Inhibitory innervation of colonic smooth muscle cells and interstitial cells of Cajal. *Can J Physiol Pharmacol.* 68, 447-54.
54. Forrest, A.S., Ordög, T., Sanders, K.M. (2006) Neural regulation of slow-wave frequency in the murine gastric antrum. *Am J Physiol Gastrointest Liver Physiol.* 290, G486-95.
55. Gil V, Gallego D, Grasa L, Martín MT, Jiménez M. (2010) Purinergic and nitrergic neuromuscular transmission mediates spontaneous neuronal activity in the rat colon. *Am J Physiol Gastrointest Liver Physiol.* 299, G158-69.
56. Agoston, D.V., Whittaker, V.P. (1989) Characterization, by size, density, osmotic fragility, and immunoaffinity, of acetylcholine- and vasoactive intestinal polypeptide-containing storage particles from myenteric neurones of the guinea-pig. *J Neurochem.* 52,1474-80.
57. Monro, R.L., Bornstein, J.C., Bertrand, P.P. (2008) Synaptic transmission from the submucosal plexus to the myenteric plexus in Guinea-pig ileum. *Neurogastroenterol Motil.* 20,1165-73.
58. Dickson, E.J., Spencer, N.J., Hennig, G.W., Bayguinov, P.O., Ren, J., Heredia, D.J., Smith, T.K. (2007) An enteric occult reflex underlies accommodation and slow transit in the distal large bowel. *Gastroenterology.* 132, 1912-24.
59. Hameyer, D., Loonstra, A., Eshkind, L., Schmitt, S., Antunes, C., Groen, A., Bindels, E., Jonkers, J., Krimpenfort, P., Meuwissen, R., Rijswijk, L., Bex, A., Berns, A., Bockamp, E. (2007) Toxicity of ligand-dependent Cre recombinases and generation of a conditional Cre deleter mouse allowing mosaic recombination in peripheral tissues. *Physiol Genomics.* 31, 32-41.
60. Edwards, F.R., Hirst, G.D. (2005) An electrical description of the generation of slow waves in the antrum of the guinea-pig. *J Physiol.* 564, 213-32.
61. Sanders, K.M., Koh, S.D., Ward, S.M. (2006) Interstitial cells of cajal as pacemakers in the gastrointestinal tract. *Annu Rev Physiol.* 68, 307-43.
62. Gomez-Pinilla, P. J., Gibbons, S.J., Bardsley, M.R., Lorincz, A., Pozo, M.J., Pasricha, P.J., Van de Rijn, M., West, R.B., Sarr, M.G., Kendrick, M.L., Cima, R.R., Dozois, E.J., Larson, D.W.,Ordog, T., Farrugia, G. (2009) Ano1 is a selective marker of interstitial cells of Cajal in the human and mouse gastrointestinal tract. *Am. J. Physiol. Gastrointest. Liver. Physiol.* 296, G1370-81.
63. Huang, F., Rock, J.R., Harfe, B.D., Cheng, T., Huang, X., Jan, Y.N., Jan, L.Y. (2009) Studies on expression and function of the TMEM16A calcium-activated chloride channel. *Proc Natl Acad Sci U S A.* 106, 21413-8.
64. Ambache, N. (1947) The electrical activity of isolated mammalian intestines. *J Physiol.* 106, 139-53.
65. Bulbring, E., Burnstock, G., Holman, M.E. (1958) Excitation and conduction in the smooth muscle of the isolated taenia coli of the guinea-pig. *J Physiol.* 142, 420-37.
66. Sarna, S.K., Daniel, E.E., Kingma, Y.J. (1972) Simulation of the electric-control activity of the stomach by an array of relaxation oscillators. *Am J Dig Dis.* 17, 299-310.
67. Aliev, R.R., Richards, W., Wikswo, J.P. (2000) A simple nonlinear model of electrical activity in the intestine. *J Theor Biol.* 204, 21-8.
68. Daniel, E.E. (1992) Developments in the study of smooth muscle research: reflections on 42 years. *Can J Physiol Pharmacol.* 70, 469-80.

69. Verhage, M., Maia, A.S., Plomp, J.J., Brussaard, A.B., Heeroma, J.H., Vermeer, H., Toonen, R.F., Hammer, R.E., van den Berg, T.K., Missler, M., Geuze, H.J., Südhof, T.C. (2000) Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science.* 287, 864-9.
70. Dickson, E.J., Heredia, D.J., McCann, C.J., Hennig, G.W., Smith, T.K. (2010) The mechanisms underlying the generation of the colonic migrating motor complex in both wild-type and nNOS knockout mice. *Am J Physiol Gastrointest Liver Physiol.* 298, G222-32.
71. Zhang, Y., Carmichael, S.A., Wang, X.Y., Huizinga, J.D., Paterson, W.G. (2010) Neurotransmission in lower esophageal sphincter of W/Wv mutant mice. *Am J Physiol Gastrointest Liver Physiol.* 298, G14-24.
72. Suzuki, H., Ward, S.M., Bayguinov, Y.R., Edwards, F.R., Hirst, G.D. (2003) Involvement of intramuscular interstitial cells in nitrergic inhibition in the mouse gastric antrum. *J Physiol.* 546, 751-63.
73. Roberts, R.R., Ellis, M., Gwynne, R.M., Bergner, A.J., Lewis, M.D., Beckett, E.A., Bornstein, J.C., Young, H.M. (2010) The first intestinal motility patterns in fetal mice are not mediated by neurons or interstitial cells of Cajal. *J Physiol.* 588, 1153-69.
74. Wang XY, Huizinga JD, Diamond J, Liu LW. (2009) Loss of intramuscular and submuscular interstitial cells of Cajal and associated enteric nerves is related to decreased gastric emptying in streptozotocin-induced diabetes. *Neurogastroenterol Motil.* 21, 1095-e92.
75. He CL, Soffer EE, Ferris CD, Walsh RM, Szurszewski JH, Farrugia G. Loss of interstitial cells of cajal and inhibitory innervation in insulin-dependent diabetes. Gastroenterology. 2001 Aug;121(2):427-34.
76. Nagl, F., Schönhofer, K., Seidler, B., Mages, J., Allescher, H.D., Schmid, R.M., Schneider, G., Saur, D. (2009) Retinoic acid-induced nNOS expression depends on a novel PI3K/Akt/DAX1 pathway in human TGW-nu-I neuroblastoma cells. *Am. J. Physiol. Cell. Physiol.* 297, C1146-56 (2009).
77. Gangula, P.R., Mukhopadhyay, S., Ravella, K., Cai, S., Channon, K.M., Garfield, R.E., Pasricha, P.J. (2010) Tetrahydrobiopterin (BH4), a cofactor for nNOS, restores gastric emptying and nNOS expression in female diabetic rats. *Am. J. Physiol. Gastrointest. Liver. Physiol.* 298, G692-9.
78. Welsh C, Enomoto M, Pan J, Shifrin Y, Belik J. (2013) Tetrahydrobiopterin deficiency induces gastroparesis in newborn mice. *Am J Physiol Gastrointest Liver Physiol.* 305, G47-57.
79. Chaudhury, A., He, X. D., Goyal, R. K. (2011) Myosin Va plays a key role in nitrergic neurotransmission by transporting nNOSα to enteric varicosity membrane. *Am. J. Physiol. Gastrointest. Liver. Physiol.* 301, G498-507.
80. Chaudhury, A., He, X. D., Goyal, R. K. (2012) Role of myosin Va in purinergic vesicular neurotransmission in the gut. *Am. J. Physiol. Gastrointest. Liver. Physiol.* 302, G598-607.
81. Chaudhury, A., Cristofaro, V., Goyal, R.K., Sullivan, M.P. (2013) Nitrergic smooth muscle relaxations are impaired in myosin Va deficiency. *PlosOne*, Submitted (peer review).

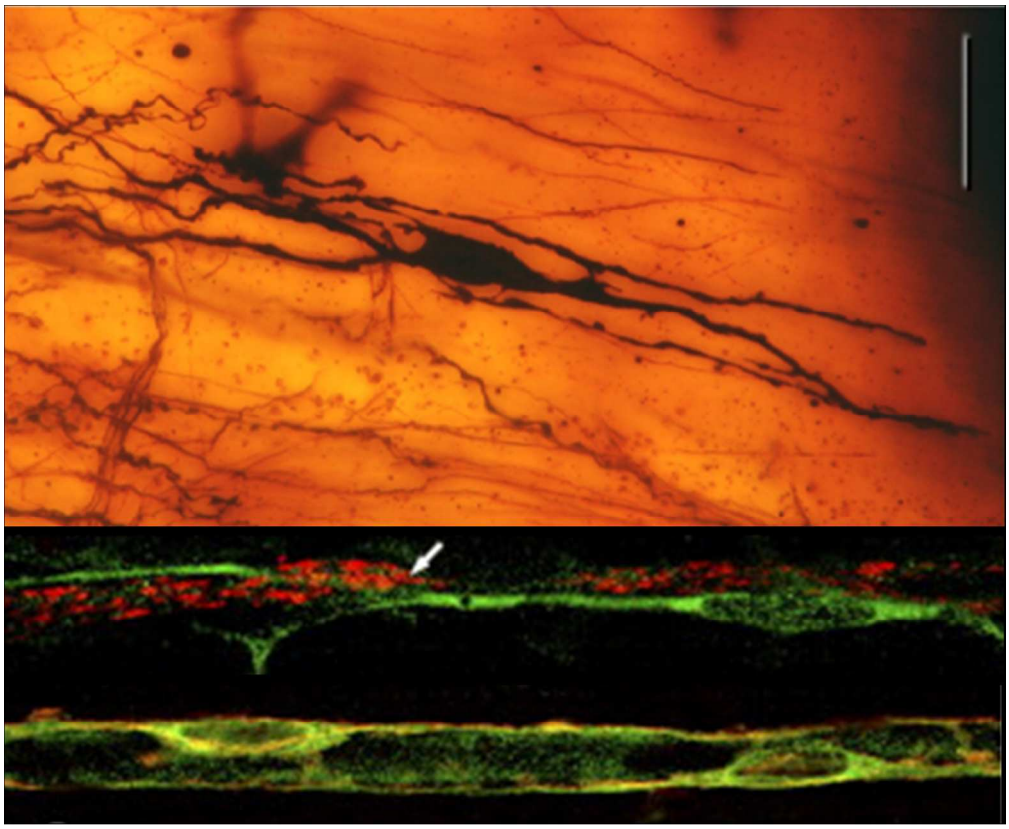
82. Bautista-Cruz, F., Paterson, W.G. (2011) Evidence for altered circular smooth muscle cell function in lower esophageal sphincter of W/Wv mutant mice. *Am J Physiol Gastrointest Liver Physiol.* 301, G1059-65.
83. Szurszewski, J.H. (1997) American Gastroenterological Association. The centennial year: the development of important ideas during the last 100 years. The enigmatic electrical slow wave: a mirror on 100 years of research in gut motility. *Gastroenterology.*113, 1785-7.
84. Szurszewski, J.H. (1998) A 100-year perspective on gastrointestinal motility. *Am J Physiol.* 274, G447-53.
85. Sarna, S.K., Daniel, E.E., Kingma, Y.J. (1971) Simulation of slow-wave electrical activity of small intestine. *Am J Physiol.* 221,166-75.
86. Sarna, S.K. (1991) MMC migration: neural or muscular? *Am J Physiol.* 260, G665-7.
87. Gangula, P.R., Maner, W.L., Micci, M.A., Garfield, R.E., Pasricha, P.J. (2007) Diabetes induces sex-dependent changes in neuronal nitric oxide synthase dimerization and function in the rat gastric antrum. *Am J Physiol Gastrointest Liver Physiol.* 292, G725-33.
88. Domènech, A., Pasquinelli, G., De Giorgio, R., Gori, A., Bosch, F., Pumarola, M., Jiménez, M. (2011) Morphofunctional changes underlying intestinal dysmotility in diabetic RIP-I/hIFNβ transgenic mice. *Int J Exp Pathol.* 92, 400-12.
89. Garcia-Lopez, P., Garcia-Marin, V., Martínez-Murillo, R., Freire, M. (2009) Updating old ideas and recent advances regarding the Interstitial Cells of Cajal. *Brain Res Rev.* 61, 154-69.

Figure legends

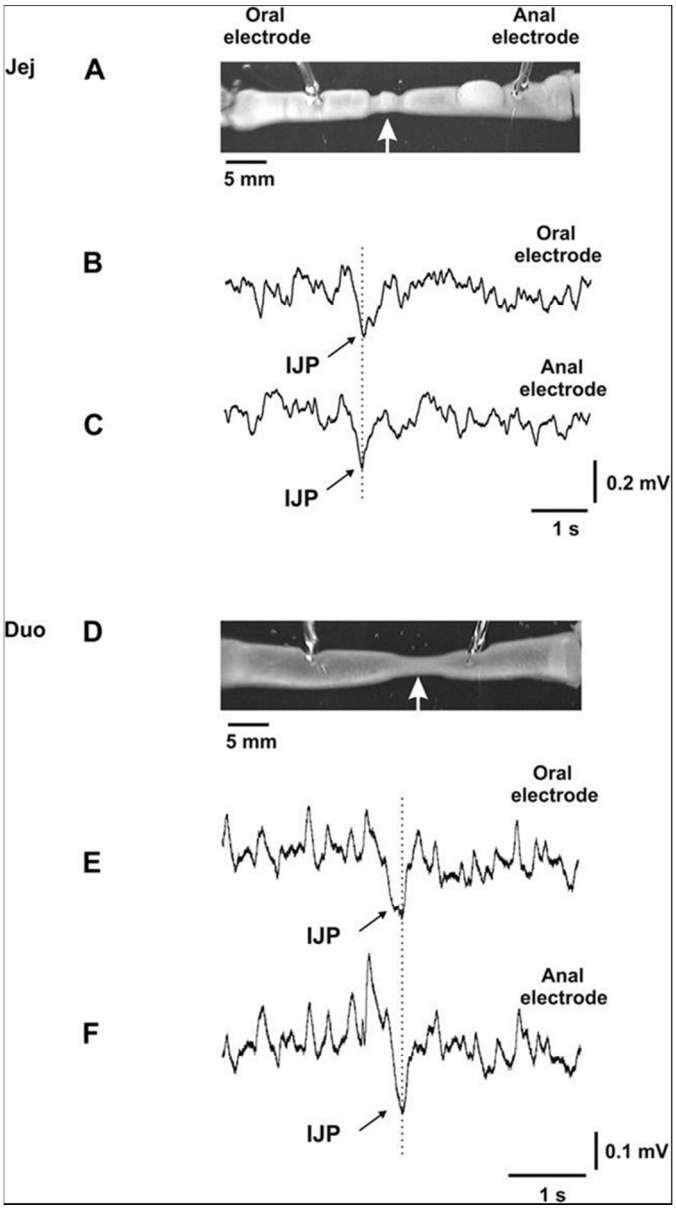
Figure 1 Upper panel is an original preparation of Ramon y Cajal. This Golgi stained section shows an interstitial cell, and extensive arborization of its processes, along with punctate appearing nerve terminals stochastically distributed across the dimensions of the gastrointestinal tissues [with permission from (Garcia-Lopez et al 2010)[89]]. Middle and lower panels show microscopic scale *en face* appearance of ICCs and nerve terminals arising from tertiary plexi in 2D sections of gut showing proximate location of these structures [with permission from Iino et al (2008)[16]]. The middle and lower panels are whole mounts obtained from guinea-pig colon. In the middle panel, nNOS positive nerve terminals are stained in red, while the ICCs are stained by sGC in green. Note the bipolar shaped ICC in the lower panel stained for c-kit and soluble guanylyl cyclase (sGC).

Figure 2 Inhibitory junction potentials (IJPs) are *sine qua non* of inhibitory neuromuscular neurotransmission. Small intestinal IJPs were recorded simultaneously both oral and anal to isolated segmentation contractions, along with simultaneous video recordings of muscular relaxations at the sites of IJP recordings [with permission from (Gwynne and Bornstein 2007)[38]]. Note the recorded IJPs at the sites of the relaxed intestinal segments [with permission from (Gwynne and Bornstein 2007)[38]]. Klein et al (2013)[25] provides qualitative evidence for the first time that despite specific genomic depletion of ICCs (tamoxifen treated *c-Kit*$^{CreERT2/+}$;*LSL-R26*$^{DTA/+}$ mice), both fast and slow IJPs were intact, as well as normal post-electrical field stimulation (EFS) mechanical relaxations were recorded, thus providing unambiguous evidence for the lack of role of ICCs in genesis of IJPs. Recent evidence show that exquisite molecular precision brought about by cellular motor proteins like myosin Va facilitates the co-transmission process involving the tandem release of ATP and NO that produce the fast and slow IJPs, respectively [with permission from {Chaudhury et al 2011, Chaudhury et al 2012)[79,80]].

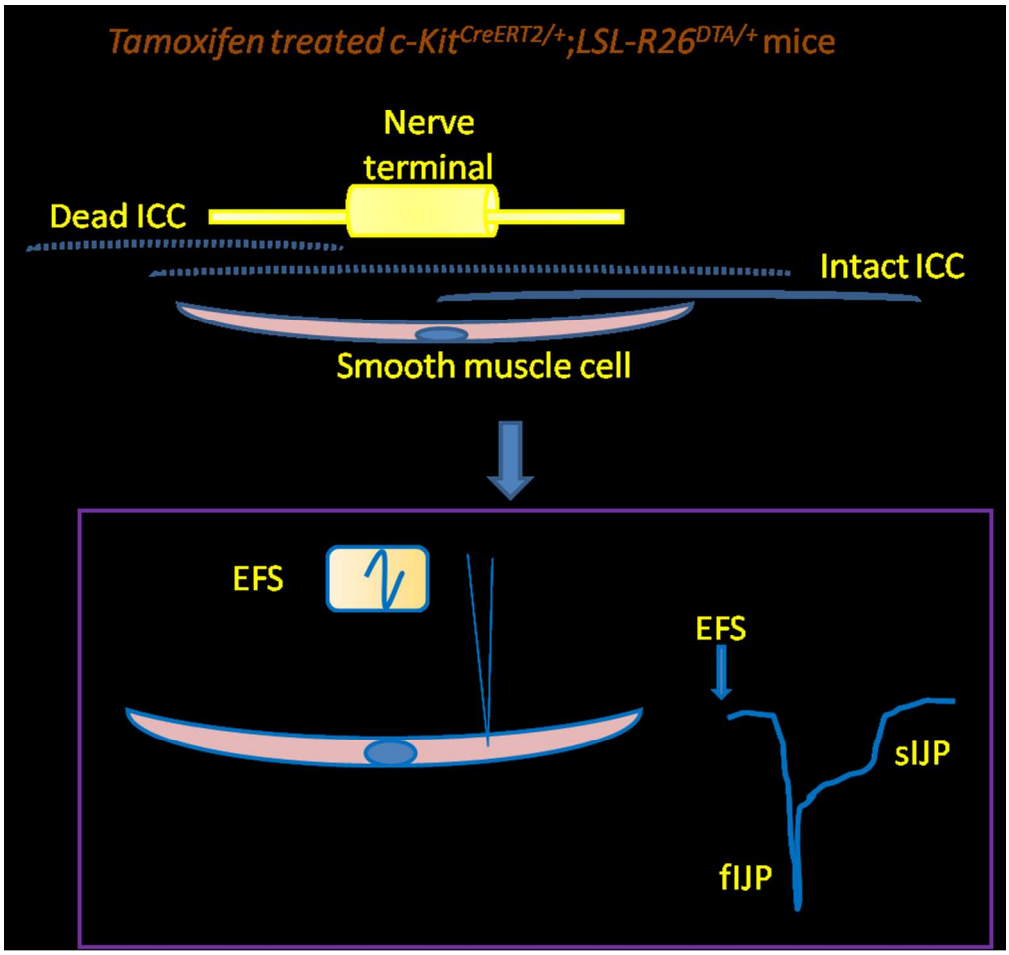
Figure 3 Cartoon depicting key neurophysiological findings recorded from gastrointestinal muscular strips after genomic depletion of ICCs [with permission from (Klein et al. 2013) [25]]. Even after partial depletion (50-60%) of ICCs, fast(f) and slow(s) IJPs could be recorded. This is unambiguous evidence of intact inhibitory neurotransmission in the absence of ICCs, strongly suggesting the lack of role of ICCs in transducing inhibitory neurotransmission signals to the smooth muscles. ICC, interstitial cell of Cajal; EFS, electrical field stimulation; IJP, inhibitory junction potential.



212x173mm (150 x 150 DPI)



106x190mm (150 x 150 DPI)



169x160mm (150 x 150 DPI)