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and

FONDAZIONE INTERNAZIONALE MENARINI

ABSTRACT BOOK

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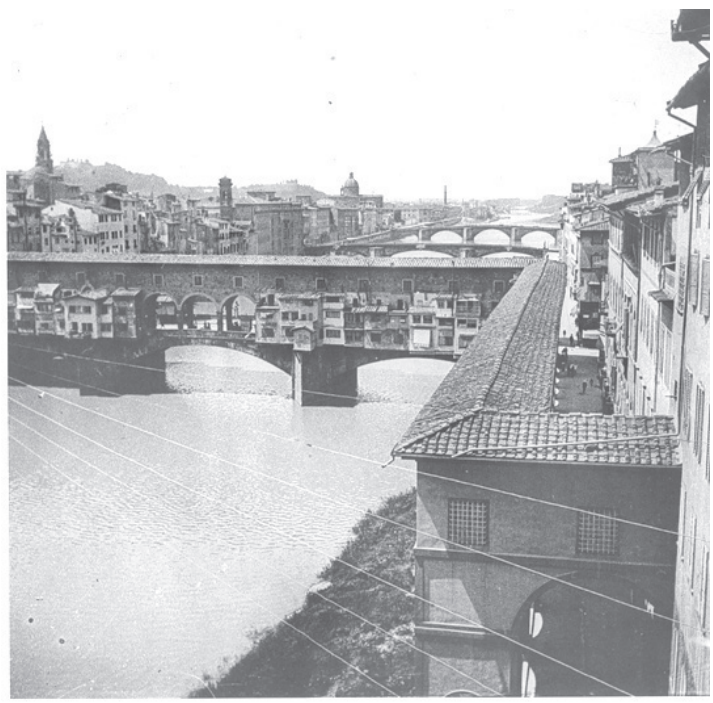
From: *Viajes fotograficos de Santiago Ramon y Cajal, 1903*



23. Vista del rio Arno a su paso por el Ponte Vecchio, Florencia.

2nd of September 2012

Registration and Welcome Reception, Hotel Albani

3rd of September 2012

Welcome Addresses:
Authorities
Maria-Simonetta Faussone-Pellegrini (Florence, Italy)
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Special Lecture with two voices:
Santiago Ramon y Cajal Junquera ((Zaragoza, Espana)
Concepciòn Junquera (Zaragoza, Espana)

Session I - Interstitial cells of Cajal from morphology to function and dysfunction

Session II – ICC in organ function

Session III – ICC development and recovery

Session IV - Communication between ICC and the enteric nervous system

4th of September 2012

Session V - Calcium signalling in ICC

Session VI - ICC ion channels and pacemaking

5th of September 2012

Session VII - ICC companions?

Session VIII - ICC outside gut

Poster Session

Concluding remarks:
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ABSTRACTS

Art and Science in Santiago Ramon y Cajal´s life

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Santiago Ramon y Cajal (1852 Petilla de Aragon- 1934 Madrid) is considered the persevering, sacrificed and visionary neuroscientist who funded the basis of modern neuroscience. His mayor contribution to the knowledge of the nervous system structure was achieved in heroic conditions working at his self-funded modest laboratory. In order to study in depth his life, nothing better than reading his autobiography 'Recuerdos de mi vida'.
S. Ramon y Cajal was born in 1952 in Petilla de Aragon, a remote small village in northern Spain. His father, Justo Ramon, practiced medicine in the area. He was a person with a strong character, raised in very austere and positive habits. His son, Santiago, brought up in this rural environment, was interested in Nature early. He went around all the surroundings and coves, collected bird eggs, observed insects life. He was particularly impressed with the sun eclipse that occurred in 1860…

During his adolescence, he felt such a marked fondness for drawing and pictures that his parents thought his intellectual future would be connected with the arts. His early vocation for the arts together with his poor marks at school and the typical never-ending pranks of his imaginative and restless mind forced his father to have him learn a few trades, first as a shoemaker and later on as a barber. This kind of punishment led him to promise he would improve in his studies in exchange of attending drawing classes. Young Cajal began his studies in Zaragoza Medical School and practiced his pictorial skills at the Anatomy dissection ward in 1868, this time with his father´s approval. For the first time, Cajal linked arts with science, vocations he would develope some years later initially specialising in macroscopic Anatomy and subsequently in Histology, two art-related medicine branches. Undoubtedly, his perfect command of drawing helped Cajal in his microscopic observations of the nervous cells. He performed excellent pictures of interneuronal connections that helped him to explain his research and discoveries. He followed Darwin´s and Virchow´s Theory of Evolution and Cell Theory, respectively, both of which were very useful for his later studies on the independence of nervous cells.

Once he graduated from Medical School, Cajal joined the health military service and was sent to Cuba where the first Cuban War of Independence was taking place. Assigned to hospitals placed in dangerous and unhealthy areas, he soon caught malaria and disentery, which made him apply for discharge from the Army. After he went back to Spain in 1875 and recovered, he resumed his anatomic studies, prepared to become a professor and got married with Silveria Fañanas. During these years, he started his first research studies and obtained the Anatomy chair at Valencia University (1884-1887). Then, he started his histological studies and soon realised that we would never be able to examine the nervous tissue using the staining methods that were broadly available at that time. Being familiar with the argentum nitricum method developed by Camillo Golgi, Cajal began to use it to stain embrionary tissues in order to avoid the complexity of the adult nervous tissue. Once he was named Professor of Histology by the University of Barcelona (1887-1892) he worked restlessly with an improved version of the Golgi method and proved the free ending of axons which was the basis of his Neuronal Theory (1888). He advanced that the neurons body and its dendrites made contact with axon endings from other cells and the nervous impulse did not pass by continuity but through a gap between cells named ´synapse´.

When Cajal was forty, he moved to the University of Madrid as Chair of Histology and Pathology. Cajal worked actively in this new period (1892-1934). He studied the embrionary development of the nervous system and the microscopic anatomy of the nervous centers and their interconnections. He published his great work ´Textura del sistema nervioso del hombre y de los vertebrados´. In 1903, he presented the modified argentum nitricum method that allowed him to study the degeneration and regeneration of the nervous system. In 1905 and 1906 he achieved international recognition by receiving the highest honours and awards, such as the Helmholtz medal and the Nobel prize that he shared with Golgi.

Cajal always had many interests aside from his work in the laboratory. He was the precursor of colour photography in Spain, the inventor of the steroscopic microphotography and a tireless traveler who used to carry around his heavy photography equipment. In addition, he was very fond of Astronomy and an expert chess player. We should also mention his activity as a writer with quite popular books such as *`Recuerdos de mi vida´, 'Charlas de café´, ´El mundo visto a los 80 años´, ´Cuentos de vacaciones´ or ´Reglas y Consejos sobre la investigacion cientifica.´* This last work was aimed at young scientists and politicians responsible for the promotion of research and has been translated into many languages.

Ramon y Cajal´s existence was based on a series of values he practised from the beginning of his life as a scientist: open and independent mind, intellectual curiosity and perserverance. His phylosophical thoughts were coherent with the historical and social world he lived in his early childhood and youth. His intellectual development was very influenced by Berkeley´s idealism, among other thinkers, that he adopted as his own philosophy, a positive naturalism *´Any man can be, if he is determined, his own brain sculptor´.*

Interstitial Cells of Cajal: Original histological slides and drawings seen today

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Among the extensive Cajal's scientific work about nervous system, perhaps the least known part is his investigations on Autonomic Nervous System, which is compiled in the last chapter of his book `Texture of the Nervous System of Man and the Vertebrates' (1899-1904). The most interesting observations taken about the innervations of pancreas and digestive tract are presented in this chapter. Cajal not only first established in the intestine the presence of two new nervous plexuses (periglandular and intravillous plexi), but also described a peculiar type of cells in all of them. Dogiel, a Russian histologist who was contemporary with Cajal, was also able to recognize these new cells and appointed these cells with the name of Interstitial Cells of Cajal (*Cajal'sche Zellen*), which are universally known (ICC).
This last chapter summarizes those findings and ideas shown in 5 monographs which were published by Cajal in consecutive years (1889, 1891, 1892a-b, 1893). We have thoroughly analyzed these publications in order to faithfully transmit Cajal's descriptions and thoughts about ICCs. Moreover, we have studied and photographed original slides and drawings which are kept in Cajal Institute (Madrid, Spain). We also analyzed some drawings made by his disciple La Villa, which we considered actually interesting. We will present these monographs in this communication, emphasizing some aspects with actual significance.
The first description of these cells was developed by Cajal in 1889 in his article `New applications of Golgi procedure'. He applied the method standardized by the notable Italian researcher with the aim of analyzing the olfactory nerve endings in nasal mucosa, hepatic and submandibular glands and muscle. He also described nerve cells located in intestinal villi in young rats and guinea pigs: *'Silver chromate highlights nerve cells as dark coffee on light background… The lowest nervous corpuscles are elongated and usually fusiform; those located close to the apex of the villi are the most voluminous and rounded; but all of them, whichever their shape, present large*

diverging prolongations with anastomosis, constituting a network of irregular meshes which spanning blood vessels and connective components. (…) No fibre compatible with characteristic axonal process is seen from them'.

The relationship between ICCs and blood vessels which Cajal already established in his first work seems to be frequently forgotten by some researchers. Despite these cells were stained in a similar way than neurons, he observed how ICC prolongations were morphologically indistinct, not being able to identify the axonal processes among them.

Two years later he found again these same cells in avian (sparrow) and mammalian (hedgehog) pancreas. He modified Golgi black staining, providing the double impregnation. '*They show themselves as triangular or stellate and perfectly independent*'… '*They are numerous and spread all over the pancreas thickness lay between the acini, in the convexity of which fit the cell processes… Do such elements have a genuine axonal process?'* The doubt arises again. Furthermore, Cajal wrote '*We have believed to notice anastomosis among expansions corresponding to neighboring cells in some cases… as previously we have observed in the plexus at intestinal villi*' (1889).

Although Cajal initially considered ICCs as nervous cells because of their tinctorial features, he always pointed out that they were primitive neurons, similar to those described in coelenterates, as he was unable to recognize an axon. In fact, he avoided denominating them neurons, by using different names: sympathetic interstitial cells, sympathetic cells, interstitial cells and interstitial corpuscles. He only called them sympathetic interstitial neurons in one occasion.

These cells remained in his mind throughout his whole life. When he finally classified all those nervous cells which he had visualized, in the first chapter of `Texture of the Nervous System of Man and the Vertebrates', ICCs are included in a separate group, basing on the fact that they just present a single type of very long prolongations (*apendices somatófugos larguisimos),* that he names *interstitial corpuscles of glands and the great intestinal sympathetic.* These corpuscles actually constituted the exception for the neuronal theory in a moment where the controversy between reticularist and neuronal doctrine existed because interstitial cells showed a network with anastomosis among their prolongations.

Therefore, with an absolute honesty and scientific rigor, Cajal wrote in his last published article '*Neuronismo o Reticularismo*?' (1933), considered as his scientific testament: '*We are boasting about our mental flexibility that is not ashamed of corrections. Neuronal discontinuity, really evident in innumerable examples, could present some exceptions. We have referred some of them in fact. For instance,*

those probably existing in glands, vessels and gut (our interstitial neurons)'. Many years should be necessary in order to discover gap junctions.
When Noble Prize is shared by both Golgi and Cajal in 1906, Cajal described this fact: '*Cruel irony of the luck, to pair like Siamese twins united on their backs, scientific adversaries of such antithetical character'*.
The techniques developed by Golgi, and neuronal and reticular doctrines merge on both geniuses backs. ICCs can be considered as the symbol of this merger.

The start of ICC research in modern times

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Nowadays the pacemaker function of the Interstitial cells of Cajal (ICC) between the muscle layers in the gastrointestinal tract is acknowledged as well as their origin as modified muscle cells. The concept of ICC as regulatory cells was first suggested by S.R. Cajal (1911), who considered them to be terminal neurons, later A. Keith (1914, 1915, and 1916) advocated for them to be pacemaker cells, and Feyrter (1951) that they were nonneuronal, intercalated cells. However, during the beginning of the twentieth century up till the late seventies, these cells were only studied morphologically and the concept of their origin varied, so they were considered to belong to several cell types from nerve cells, Schwann cells, and ordinary connective tissue cells or to cells involved in neural transmission. Mostly the studies were based on light microscopically observations, but also electron microscopic observations took place from the sixties; however their functional significance was uncertain. In the late seventies the revival of the idea that ICC could be pacemaker cells came in to being again, partly in Italy where Maria Simonetta Fausonne-Pellegrini made electron microscopically studies on the human gastro esophageal junction and suggested that pace maker cells were present here, and partly in Denmark where Lars Thuneberg based on morphological and to some extent functional studies on mouse muscularis externa came to the conclusion that they were pacemaker cells and named his doctoral thesis: Interstitial Cells of Cajal: Intestinal Pacemaker cells. In 1981 he attended the 8th Symposium on Gastrointestinal Motility in Königstein and presented his ideas. The following years he attended all motility meetings advocating for ICC and by 1987 their role had nearly become established, as at the Motility Symposium in Oxford where he and Simmonetta were asked to make the introductory talks.
My talk will mainly focus on Lars and his work in the late seventies and beginning of the eighties.

ICC in tissue and stromal tumors

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The discovery that the receptor tyrosine kinase Kit (c-kit) is required for the development and function of the interstitial cells of Cajal (ICC)[1], the identification of gain-of-function mutations of Kit in human gastrointestinal stromal tumors (GIST)[2] and the introduction of compounds inhibiting Kit activation [3]represent milestones in the field of ICC and gastrointestinal stromal tumors. They have opened an entire area of research on their embryological origin, signaling pathways, immunomarkers and putative therapeutic targets.
Several ICC immunomarkers were found on GIST and, reciprocally, a marker discovered on GIST (DOG1) was also found to be expressed in normal ICC[4].
We have been interested in gene expression profiles and signal transduction pathways of KIT-ir ICC in wild-type (WT) mice and in transgenic mice harboring the oncogenic KitK641E mutation[5] previously identified in a familial form of human GIST[6].
Gene expression profiles of antrum of KitK641E homozygous mice and their WT littermates were compared using cDNA microarrays. Up-regulated genes were further validated using quantitative PCR and immunofluorescence (-ir).
Most up-regulated genes belong to the gene expression profile of human GIST but also to the profile of normal Kit-ir ICC in the mouse small intestine[7]. Conversely, several candidates, e.g. Spry4, Tpbg/5T4 and Ntsr1, were not detectable in Kit-ir ICC of WT mice, but were present in the Kit-ir hyperplasia of KitK641E mice[8;9].
Alterations of signal transduction pathways have also been identified in the Kit-ir ICC of KitK641E mice[10].
Recently, CD105/Endoglin (Eng) was identified not only in endothelium but also in normal Kit-ir ICC and in human GIST[11].
Genes identified in the KitK641E mouse model may contribute to the histopathological profiling of human GIST and are promising candidates for novel targeted therapeutic approaches in GIST with oncogenic KIT mutations.

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Updating on the Interstitial Cells of Cajal located at the deep muscular plexus (ICC-DMP) in humans and other mammals

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In 1893 Ramon Santiago y Cajal, for the first time, described in the most internal part of the circular muscle layer of the guinea pig ileum 'primitive neurons' intermingled with several nerve fibers that he named 'plexus muscularis profundus' (1) and now known as deep muscular plexus or DMP. Those cells, that impregnated as the neurons, are now known as interstitial cells of Cajal (ICC) and those at the DMP, ICC-DMP.

The next researches have demonstrated that the great morphologist missed the nature of these cells which, indeed, are connective and not neuronal in origin, but perfectly described their shape, location and cellular relationships: the ICC-DMP are intercalated between the nerve endings which make many specialized contacts on them and the smooth muscle cells on which the ICC form numerous gap junctions and mechanical junctions called pegs and sockets (2). To date, however, the precise role of the ICC-DMP in the small intestine, the only gut region sharing this ICC population and the related non-ganglionated nerve plexus, has not been definitively clarified.

The ICC are commonly considered the gut pace-making cells (3). Physiologically, it has been clearly demonstrated that the ICC generate the slow-waves driving the gut peristalsis. In the small intestine this function depends almost completely on the ICC located at the myenteric plexus (ICC-MP) and in animal models devoid of this ICC population the peristaltic activity is greatly compromised in spite of the presence of healthy ICC-DMP (4).

In the past decade, several papers have been focused on the ICC-DMP and the synthesis of the information is the following: they cannot be considered pace-maker cells; the huge number of nerve endings contacting the ICC-DMP share almost all the transmitters described in the gut and these ICC express each of the specific receptor for the related ligand; although a vicinity between nerve endings and ICC is common, the ICC-DMP present only contacts assimilated to synapse; finally, the network made up by ICC-DMP

does not contain intercalated fibroblast-like cells which is instead common for the ICC-MP network.

Ambiguous is also the role of the c-Kit receptor in the ICC-DMP. This is a specific marker commonly used to identify the ICC. It corresponds to a tyrosine-kinase membrane receptor whose ligand is represented by a nerve growth factor called steel factor. This receptor seems to regulate the ICC differentiation and to maintain the differentiated state. Indeed, gene mutations able to affect the c-Kit receptor expression or the steel factor production, deeply compromise the presence and function of most of the ICC populations but not those of the ICC-DMP (5). The administration in adult animals of antibodies against the c-Kit receptor did cause ICC-DMP damage associated with ineffective neurotransmission. However, in this model, all the ICC populations were definitively destroyed (3). To date, there is no mean to selectively destroy the ICC-DMP in the attempt to finally understand their role(s).

During ontogeny ICC-DMP differentiate after birth either in humans or mice (6). Interestingly, these cells differentiate not only in mutants for the c-Kit/Steel factor, but also in case of enteric nerve system failure (7). Studies in rats have shown that at birth the ICC-DMP, while are still c-Kit negative, start to express the NK1 receptor and one week later the levels of the NK1r-immunoreactivity (NK1-IR) are comparable to those of the adult animals. The appearance and the increase in the NK1-IR always precede that of the SP-IR in the nerve fibers of the DMP. These findings suggest that the ICC-DMP are committed to synthesize functioning molecules independently on the presence of the ligand. Moreover, it could be also hypothesized that the ICC-DMP actively interact with the nerve endings conditioning their ability to express an appropriate ligand. In favor of this possibility is the observation that in c-Kit mutant mice, the ICC-DMP did not express NK1r and the nerve endings containing SP are significantly increased (8).

Several papers have clearly demonstrated that the ICC-DMP are intermediate in the enteric neurotransmission and physiological experiments have brought to the conclusion that they behave as the gastric intramuscular ICC (ICC-IM) (9,10). However, the ICC-DMP although similar to ICC-IM, have to be considered unique. Indeed, besides all the peculiarities quoted above, they are intercalated between the inner and the outer portion of the circular muscle layer (CML). In particular, the cells located in the inner portion (ICML), organized in a variable number of row depending on the specie, are always smaller than those of the outer portion (OCML) and do not form gap junction neither between themselves nor with the ICC-DMP. Because of the neighbor elements, these ICC can be considered as part of a complex system endowed in the longest region of the gut, the region responsible for food absorption. Taking into account these data and looking at some animal models or human pathologies, it has been possible to converge towards a unique role for this complex system that is that to be a stretch receptor.

This role was firstly considered in the paper by Faussone-Pellegrini et al, (11), studying, under the TEM, the fate of the ICC-DMP in some cases of ileal-bladder and correlating the loss of the ICC-DMP with the loss of the distention reflex. Few years later, Thuneberg & Peter (12) showed the appearance and the increase among the ICC-DMP and the neighbor SMC, comprised those of the ICML, of mechanical junctions, the peg and sockets ones, either in suckling or adult mice carrying or not the c-Kit mutation. This cellular response corresponded to segmental contractions due to the food presence. A third model showing the role as stretch sensor of this cell complex was described by Wang et al. (13) in a model of gut infection. Finally, we published recently a paper showing the extreme richness of nerve endings carrying the receptor for the glucagon-like peptide2 (GLP2) in the DMP and contacting the local ICC. This hormone is strictly involved in regulating local stimuli depending on the presence of food (14).

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High definition gastric electrical activity analysis in the human

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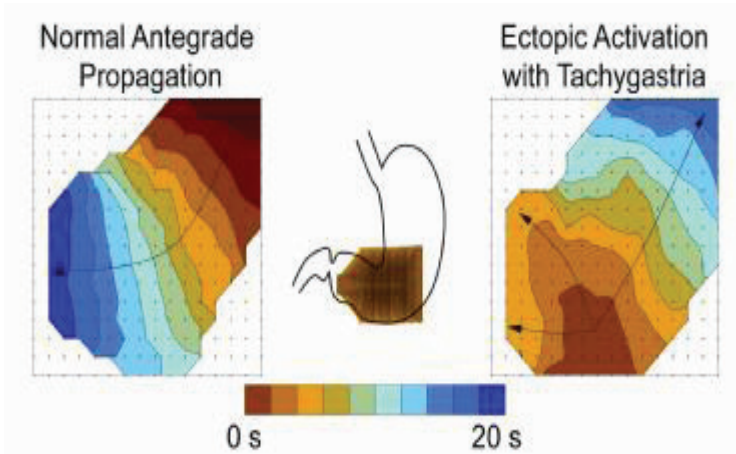
Extracellular recordings enable investigations into the integrated electrophysiological functions of intact tissues. In high-resolution (HR; multi-electrode) mapping, dense arrays of many electrodes are used to track electrical activation patterns in fine spatiotemporal detail.

This talk will present recent progress on the HR mapping of human gastric slow wave activity. The development of human serosal mapping has been facilitated by the development of flexible printed circuit board arrays suitable for intra-operative use. A comprehensive data analysis pipeline and software packages have been developed for identifying slow wave events, grouping them into wavefronts, and for generating activation maps, velocity and amplitude profiles, and animatirons, including for both off-line and real-time recordings.

To date, translational studies have applied these techniques to quantify human gastric slow wave patterns in health and gastroparesis. Normal human mapping has enabled a refined understanding of gastric conduction, including localization of the normal gastric pacemaker area, quantification of the multiple propagating wavefronts, and redefining regional changes in activation. A high-amplitude, high-velocity activity has been found in association with the normal pacemaker region, which recent evidence suggests is a consequence of the circumferential propagation occurring locally in this region.

HR mapping in diabetic and idiopathic gastroparesis was accompanied by full-thickness biopsy studies, demonstrating reduced ICC counts compared to matched controls (2.3 vs 5.4 bodies/field; $P<0.01$). Several novel patterns of abnormal human slow wave activation have been observed in gastroparesis, and were classified as 'abnormal initiation' (10/12; stable ectopic pacemakers or diffuse focal events; median 3.3 c/min, range 2.1–5.7), or 'abnormal conduction' (7/10; reduced velocities or conduction blocks; median 2.9 c/min; range 2.1–3.6). Circumferential conduction emerges during a range of dysrhythmias, and is associated with higher velocity (7.3 vs

2.9 mm s^{-1}; P=0.002) and increased extracellular amplitudes beyond a low gastroparesis base value (415 vs 170 μV; P=0.002).



In conclusion, this work presents a new technical and physiological foundation for analyzing abnormalities of human gastric slow wave conduction. The results provide an improved understanding of normal gastric activation, as well as novel pathophysiological insights that we hope will ultimately contribute to diagnostic advances for gastric motility disorders.

Figure. Human HR gastric mapping at the corpus-antrum border. Each black dot represents an electrode, and each color band corresponds to 2 s of propagation time. *Left*: The normal gastric activation profile, showing antegrade propagation (3 c/min), with wavefronts oriented in the transverse gastric axis. *Right:* An example of an ectopic initiation event in gastroparesis, arising from near the distal greater curvature. Conduction in the circumferential axis is shown to be rapid, frequency was elevated (4 c/min), and propagation is retrograde.

The propagation of electrical signals and networks of Interstitial Cells of Cajal

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OBJECTIVE: In the past decennia's, it has become clear that the propagation of the slow wave, in the stomach and the small intestine for example, is determined by the network of interstitial cells of Cajal (ICC-MY). What is not clear is what will happen to the slow wave (SW) propagation if the numbers of myenteric ICC's are reduced, as for example in diabetes. The aim of this study was to determine the impact of a decreased number of ICC-MY on the propagation pattern of the slow wave.

METHOD: Eleven rats were treated with streptozotocin (STZ) and housed in pairs with 11 age-matched controls. After 3 and 7 months, segments of duodenum, jejunum and ileum were isolated and divided into two parts. One part was processed for immediate freezing, cryosectioning and immunoprobing using anti-c-kit antibody to quantify ICC-MY. The second part was superfused in a tissue bath and SW propagation was recorded with 121-extracellular electrodes. In addition, a cellular automaton was developed to study the effects of randomly distributed inactive cells on overall propagation.

RESULTS: ICC-MY's were significantly reduced after 3-months of diabetes, but rebounded to control levels at 7-months diabetes. SW frequencies, velocities and extracellular amplitudes were unchanged at any stage of diabetes. The cellular automaton showed that SW velocity was not linearly related to the number of inactive cells. In addition, in 10 out of a total of 66 intestinal segments (15%), a circus movement of the slow wave was detected. These re-entries were seen in control (n=2), in 3-month (n=2) and especially in 7-month (n=6) diabetic rats. Local conduction velocities (3.03 ± 0.67 cm/sec) and beat-to-beat intervals (0.42 ± 0.15 sec) during the re-entries were measured leading to a wavelength of 1.3 ± 0.5 cm and a circuit diameter of 4.1 ± 1.5 mm.

CONCLUSION: ICC-MY depletion is not as severely affected as is often assumed and may in fact rebound after some time. In addition, at least in the STZ-model, initial reduction in ICC-MY is not enough to affect SW propagation. However, a functional impairment may occur in the form of circus movement arrhythmias, especially in the chronic diabetic rats. Calculations of the size of the circuit indicate that they are small enough to fit inside the intestinal wall. Extrapolation based on measured velocities and rates indicate that re-entrant arrhythmias are also possible in the distal small intestine of larger animals including humans. Further studies however are now required to determine why re-entrant arrhythmias occur more often in the diabetic state.

Colonic pacemakers and gaseous transmitters

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Background. Previous studies have demonstrated the presence of two pacemakers in the rat colon. In vitro circular oriented strips developed spontaneous *low frequency contractions of high amplitude* and a second pattern consisting on *high frequency contraction of low amplitude*. These two patterns of motility are associated with "cyclic depolarisations" of about 0.5-1/min and "slow waves" 8-10/min. Both electrical and mechanical activities are TTX insensitive demonstrating that non-neural input is required to develop both motility patterns at least in strips studied "in vitro". Dissection experiments combined with c-kit staining have demonstrated the presence of two ICC networks associated to the submuscular (ICC-SMP) and myenteric (ICC-MY) plexus which might be responsible for slow waves and cyclic depolarization respectively. Purinergic and nitrergic neurotransmitters are the main inhibitory mediators of gastrointestinal tract. Purinergic neurotransmission is mediated by P2Y1 receptors and nitrergic neurotransmission activates Guanylate cyclase causing cGMP elevation in post-junctional cells. Hydrogen sulphide is an endogenous gaseous signalling molecule with putative inhibitory functions in the gastrointestinal tract.
Aims. The aim of the work was to study the effect of inhibitory neurotransmitters on pacemaker function.
Results.
Characterization of Inhibitory neurotransmission using constant EFS. To be able to characterise the effect of inhibitory neurotransmission on pacemaker function we studied the effect of "constant" electrical field stimulation (EFS) on inhibitory junction potentials. Two parameters were studied: voltage and frequency. The amplitude of both purinergic and nitrergic junction potential was increase in a voltage dependent manner. Low frequencies of stimulation 0.1-1 Hz predominantly elicited purinergic (MRS2500 sensitive) junction potentials that partially ran down when the frequency increased. In contrast nitrergic junction potentials (L-NNA sensitive) increased with frequency. Junction potentials elicited at 5

Hz caused a hyperpolarization (60% nitrergic and 40% purinergic). Taking in account all these results a mathematical model was build to define inhibitory neurotransmission in the rat colon. Comparison with human colon was established.

Characterization of Inhibitory neurotransmission in pacemaker function. To be able to analyse the effect of both neurotransmitters on pacemaker function the frequency of 5Hz was used. At 5Hz a reduction (both in amplitude and frequency) of low frequency contractions was observed in a voltage dependent manner. The result was observed even when the tissue was incubated with ODQ (to isolate the purinergic component) or with MRS2500 (to isolate the nitrergic component). Blockade of the residual neurotransmission i.e. a- MRS2500+ODQ (in control); b- MRS2500 in tissue previously incubated with ODQ and c- ODQ in tissue previously incubated with MRS2500 caused a "pharmacological" off-contraction and completely restored the two motility patterns even during constant EFS. High frequency contractions were insensitive to 5Hz EFS. These results correlate with electrophysiological recordings were slow wave activity but not cyclic depolarizations are "insensitive" to hyperpolarizations induced by EFS (5Hz). Exogenous addition of NaNP and H2S caused smooth muscle hyperpolarization being cyclic depolarizations and low frequency contraction more sensitive than slow waves and high frequency contractions. NaHs a H2S donor mimic NaHS effects.

Conclusion: Purinergic and nitrergic neurotransmission modulate pacemaker function. Cyclic depolarisations and low frequency contractions are modulated by inhibitory enteric neurons. Slow waves and high frequency contractions are comparatively les sensitive to inhibitory neurotransmitters. Although we could not demonstrate H2S neural release, exogenously applied gasotransmitters caused inhibition of low frequency contractions due to smooth muscle hyperpolarization.

S-III

ICC stem cells: Interaction between nutrient-sensing and morphogenetic pathways to control self-renewal and senescence

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Aging-associated tissue dysfunction has been linked to lifelong stimulation of nutrient-sensing pathways mediating the effects of circulating insulin and IGF1. Key downstream mediators of insulin/IGF1 signaling, mTOR and ribosomal S6 kinase, also mediate stem cell exhaustion induced by persistent Wnt signaling. Consistent with this model, chronic caloric restriction and inhibition of mTOR/S6 kinase activity promote longevity and health span. We have studied the mechanisms underlying the maintenance and differentiation from local stem cells of interstitial cells of Cajal (ICC), electrical pacemaker and neuromodulator cells of the gut, in premature and natural aging, chronic caloric restriction, as well as in type 1 and type 2 diabetes. In both rodent models and human tissues, ICC were depleted under conditions involving reduced insulin/IGF1 signaling such as type 1 diabetes, aging and caloric deficit. ICC were also decreased by chronic low-dose rapamycin treatment. In contrast, ICC were hyperplastic in uncomplicated, hyperinsulinemic type 2 diabetes. Results in humans indicate that loss of ICC correlates with gastric neuromuscular dysfunction manifesting in early satiation and reduced food intake in primary motility disorders such as gastroparesis, aging and protein-energy malnutritions. Together, these findings suggest the existence of a self-reinforcing loop formed by reduced nutrient-sensing mechanisms, ICC depletion and reduced food intake and raise the intriguing possibility that aging-associated gastric dysfunction may serve as an occult pro-longevity mechanism. In this talk I will discuss the mechanisms whereby murine ICC stem cells can undergo senescence in the absence of chronic activation of nutrient-sensing pathways and limit caloric intake and aging.

ICC changes in diabetic gastroparesis and other motility disorders

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An association between depletion of networks of interstitial cells of Cajal (ICC) and gastrointestinal motility disorders is now well established. This has been demonstrated in patients with slow transit constipation, diabetic gastroparesis and intestinal pseudo-obstruction and is reproduced in animal models of these diseases. Specifically the development of delayed gastric emptying in mice with diabetes is directly correlated with the depletion of ICC networks. We have demonstrated that failure to sustain expression of heme oxygenase-1 (HO1) in macrophages of the muscularis propria is associated with ICC loss and development of gastroparesis in these animals. Restoration of HO1 by treatment with hemin or administration of carbon monoxide, a product of HO1 activity, restores ICC networks and reverses the delay in gastric emptying. These observations have led to the investigation of drugs for safely inducing HO1 with the objective of using the compounds to treat patients with the disease. In mice we have examined the physiological and morphological changes that occur in the stomach of animals with gastroparesis and those treated to reverse the delay in emptying. The observations confirm disruption of ICC networks and the presence of abnormal electrical slow wave activity, as previously reported. However spatial mapping and correlation of sites of electrical recording with immunohistochemical results indicates that, in mice with delayed gastric emptying, the most significant changes in slow wave are in the distal antrum, whereas damage to ICC networks is patchy but distributed across all of the body and antrum of the affected mice.
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Gut organ development - Investigation using in vitro system

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Background: The GI tract is composed of three distinct layers, i.e., epithelium, connective tissue layer and musculature including enteric neurons. The development of the GI tract in the embryo requires consecutive interactions between these three layers. Recently, although differentiation of each tissue layers has been clarified much using a culture system in vitro, the development of the GI tract as a three-dimensional organ remains to be elucidated. I described the development of the GI tract using mouse ES cells in vitro that mimic the developmental process in vivo. I also established the isolation, culture and characterization of mesenchymal stem cells (MRCs) derived from mouse ES cells.
Materials and Methods: Embryoid bodies (EBs) corresponding to mouse early embryos were formed by hanging drops of mouse ES cells for 6 days. They were embedded into the AteloCell (type one collagen gel) and incubated in a medium including activin, followed by a medium with retinoic acid. They were then treated with BMP2 and PDGF-A for a few days. Formation of gut-like structures in the collagen gel was recorded and processed for immunofluorescent staining. The expression of mRNAs was analyzed by PCR. Mesenchymal stem cells (MSCs) were isolated by a Magnetic Cell Sorter using CD105 expression. They were then cultured with BMP2, PDGF-A and/or Steel factor.
Results and conclusion: Culture of EBs in AteloCell formed more compact and three- dimensional appearing of gut-like structures. Activin induced definitive endoderm in EBs that differentiated into GI epithelium. Retinoic acid prolonged the gut tubes. BMP2 increased the smooth muscles surrounding gut and also sporadic differentiating smooth muscles. MSCs expressed PDGFr-α, and PDGF-A induced the GI muscles. These results strongly suggest that these two in vitro systems are unique and suitable experimental tools for investigation of the signals involved in the development of the GI tract and the differentiation of the smooth muscle cells in the musculature.

Neurogenic and myogenic factors of intestinal motility

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Intestinal motor functions are involved in storage, mixing and propulsion of contents along the digestive tract. The cellular mechanisms underlying intestinal motility have been investigated in great detail. These include systematic studies of the enteric neural circuits in different species underlying neural motor functions and the pacemaker systems underlying myogenic motor activity. However the relative role and interaction of neurogenic and myogenic mechanisms during physiological activation of intestinal motor patterns is still at its infancy. We have developed methods of recording and analyzing motor patterns in isolated segments of small and large intestine of rabbits, guinea-pigs and rats that enable to describe the biomechanical state of the intestinal wall and describe the state of tone of the smooth muscle during myogenic and neurogenic motor activity.
The **objective** was to distinguish active states of isometric, isotonic and auxotonic contractions and relaxations of the intestinal muscle distinguishing myogenic and neurogenic underlying mechanisms.
Methods. We used segments of large intestine taken from rabbit, guinea-pig and rats killed according to the AWC of Flinders University. The segments were set up in organ baths with Krebs solution bubbled with carbogen and kept at 36°C. The motor patterns of the intestinal segments were recorded with a combination of video recording and a high definition intraluminal manometry. We constructed spatio-temporal maps detailing changes in diameters (Dmaps) from video recordings as developed originally at Flinders (Hennig et al 1999) and changes in intraluminal pressure (PMaps) by a fibre-optic manometry catheter (Arkwright et al 2010). The two spatio-temporal maps were combined and compared as was reported in the first publication (Dinning et al 2010). We developed a more comprehensive analysis package based on MatLab, which enables to construct spatio-temporal maps with the combined information (D-PMaps). Dynamic parameters extracted from these spatio-temporal

maps enable the state of the intestinal muscle to be determined according to isotonic, auxotonic, and isometric active and passive contractions and relaxations of the circular muscle. Spontaneous and distension induced motility was studied in preparation in which oral inflow of fluid was controlled and the outflow measured, or with maintained distension with outflow blocked. Pharmacological agents including tetrodotoxin, the nicotinic antagonists hexamethonium and mecamylamine and the cholinergic receptor agonist carbachol, were used to distinguish neurogenic from myogenic nature of the motility patterns observed.

Results. In all species distension of the segments of colon was accompanied by propagating contractions which were blocked by TTX and which could not be restored by adding increasing muscle and neural excitability by carbachol. These neurally mediated propagated contractions in the rabbit colon consisted in isotonic followed by isometric contractions of the circular muscle that resulted in a lumen occlusive contraction that propagated aborally to empty the segment or to bulge the intestine if prevented to empty. The muscle ahead of the advancing contraction was initially actively relaxed isotonically and then passively distended isometrically. In none of the species propagating contractions of these characteristic were observed after the cholinergic agonist carbachol. In rat colon some irregular and poorly propulsive myogenic contractions were observed to propagate slowly in both directions. In rabbit and guinea-pig the only motor activity observed after blocking neural activity even after application of carbachol, was a myogenic rhythmic activity, probably due to the ICC pacemaker cells driving the intestinal muscle. These contractions and relaxations in the rabbit colon were investigated with biomechanical measures, and showed both isotonic and isometric properties. Myogenic motor activity did not contribute significantly to the propulsion of contents and consisted in contractions propagating in both directions in an irregular chaotic manner. Neurogenic and myogenic mechanisms interacted to generate the complex but predictably pattern of motor behaviour.

Discussion. Our investigations indicate that is possible to apply simple principles of biomechanics to localize where and when active contractions and relaxations occur during physiological movements of the intestine and to establish were and when passive changes occur. In parallel we could confirm that myogenic activity alone is not involved in significant propulsive behavior in the colon. The degree and patterns of interaction between myogenic and neurogenic mechanisms can be revealed readily in the spatio-temporal maps and the combination of PMaps and Dmaps gives new ways to analyze quantitatively the mechanical consequences of the myogenic and

neurogenic processes in normal and in experimental disease conditions.

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Cooperation between ICC and nerves

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Remarkably, the large intestine is always under the influence of ongoing neural activity both between and during colonic migrating motor complexes (CMMCs), (1-4). Between CMMCs the colon is under tonic inhibition: spontaneous inhibitory junction potentials (IJPs) in the circular muscle (CM) and suppressed activity in ICC-MY and ICC-SM, resulting from ongoing activity in descending inhibitory (serotonergic) nerve pathways (4). CMMCs are neurally mediated, cyclical contractile and electrical events, which typically propagate along the colon every 3 min in the mouse, and are of sufficient strength that they can propel fecal contents. CMMCs occur spontaneously or can be evoked by stimulating the mucosa locally, or by brushing it at either end of the colon (1-5). Intracellular recordings from the circular muscle demonstrate that the CMMC consists of a brief hyperpolarization of the circular muscle followed by a prolonged (20-40s) slow depolarization (~14.0 mV) upon which is superimposed fast oscillations (frequency ~1.6Hz) that give rise to action potentials that produce muscle contraction. During the CMMC, both the longitudinal (LM) and circular (CM) muscle layers are activated at the same time suggesting their synchronized activities are coupled by nerves and/or by ICC-MY that appear to form a thin network spanning the two muscle layers (1,3).

The mechanisms underlying the generation of the CMMC were controversial because it had been proposed that turning off tonic inhibition (disinhibition) or excitatory nerve activity generates the slow depolarization of the circular muscle underlying the CMMC. Our recent studies suggest that the CMMC cannot be considered as just a neurally generated muscle event but must be regarded as a synergistic interaction between neural and ICC-MY (myenteric ICC) and ICC-SM (submucosal ICC) pacemaker networks and a network of ICC-IM (Intramuscular ICC). Between CMMCs, most myenteric ICC-MY, which appeared to form a thin layer between the LM and CM layers, were often quiescent, as were also ICC-IM; their lack of activity was correlated with ongoing Ca^{2+} transients in varicosities, which are

neurotransmitter release sites, on the axons of nNOS motor neurons that were on or surrounded ICC-MY. Following TTX (1 μM), or blockade of inhibitory neurotransmission with N(ω)-nitro-L-arginine (L-NA, a NO synthesis inhibitor, 10 μM) and MRS 2500 (a P2Y1 antagonist, 1 μM), ongoing spark/puff like activity and rhythmic intracellular calcium waves (38 cycles per min) were observed, yet this activity was uncoupled, even between ICC-MY in close apposition. This suggested that ICC-MY don't form an integrated network through which slow wave activity can propagate, as do ICC-MY in the small intestine. SNP (an NO donor, 10 μM) abolished all activity in ICC-MY.

During a spontaneous or evoked CMMCs there was an increase in the frequency (63 cycles per min) and amplitude of Ca^{2+} transients in ICC-MY and muscle, which often had synchronized activity. Ca^{2+} transients in ICC-MY, which consisted of fast oscillations superimposed on a slow rise in Ca^{2+}, appeared to be initiated slightly before similar activity in the muscle, suggesting that the electrical activity underlying the Ca^{2+} transients in ICC-MY spreads into the LM and CM. At the same time, activity in varicosites along excitatory and inhibitory motor nerve fibers increased and decreased respectively, leading to an overall excitation of ICC-MY. Atropine (1 μM) reduced the evoked responses in ICC-MY, and subsequent addition of an NK1 antagonist completely blocked the responses to stimulation.

Conclusions: ICC-MY, which are normally under tonic inhibition, can function as intestinal pacemakers since they likely generate fast electrical oscillations called myenteric potential oscillations (MPOs) that conduct into the neighboring LM and CM cells. Although pacemaker activity doesn't propagate through the colonic ICC-MY network as it does in other regions of the GI tract where there is strong gap junctional coupling between ICC-MY and between ICC-MY and the muscle. However, the activity in ICC-MY can be synchronized by activation of excitatory motor nerves to cause contraction of the muscle layers at the same time. Therefore, neurally generated motor patterns in the large bowel are dependent on a complex integration between myenteric neuronal and ICC networks.

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Enteric nervous system input into small intestine ICC-MP and ICC-DMP calcium transients

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Objective: The aim of this study was to investigate potential communication between enteric nervous system and ICC-MP/ICC-DMP network of the small intestine.
Methods: To determine the functionally communication from enteric neurons to ICC-MP network, ICC-MP network was loaded with the calcium dye Fluo-4, Neuron on the myenteric plexus was impaled with sharp electrodes and verified by electrophysiology and morphology, a series of stimulus was provided on the neuron to study possible effects on a nearby network of ICC-MP; To determine the functionally communication from enteric neurons to ICC-DMP network, ICC-DMP network was loaded with the calcium dye Fluo-4neurons, substance P (25 μ*M*) was applied to study possible effect on a network of ICC-DMP.
Results: Like stimulation of excitatory motor neurons, stimulation of AH sensory neurons, evoking action potentials in these neurons, caused a marked increase of ICC intracellular calcium concentrations with increasing of calcium oscillations in ICC-MP, ICC-MP demonstrated significantly increased calcium basal point after stimulation (N = 5, P = 0.0232). ICC-MP received excitatory input that release acetylcholine acting on muscarinic receptors; Most ICC-DMP was often quiescent, showing slow frequency in calcium oscillation. Following substance P application, ICC-DMP produced significantly faster frequency spikes in calcium transients (N = 7, P = 0.0074). ICC-DMP received excitatory input that release tachykinins acting on NK1 receptors.
Conclusions: Enteric nervous system has the potential to modify gut pacemaking through action on calcium transients, both on ICC-MP and ICC-DMP. These imply the ICC as the potential mediaries in transmission from enteric neurons to the muscle.

Involvement of Na^+-leak Channel in SubstanceP-induced Depolarization of Pacemaking Activity in Interstitial Cells of Cajal

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Interstitial cells of Cajal (ICCs) are the pacemaking cells in the gastrointestinal muscles that generate the rhythmic oscillations in membrane potential known as slow waves. ICCs also mediate or transduce inputs from the enteric nervous system. Substance P (SubP) is a member of the family of mammalian tachykinin peptides that are predominantly released by enteric neurons. This study assessed the relationship of Na^+- leak channel (NALCN) in the SubP-induced depolarization in pacemaking activity in the gastrointestinal tract. The patch-clamp technique for whole-cell recording was used in cultured cluster and single ICCs. Electrophysiological and pharmacological properties of SubP in ICC pacemaking activity were similar to those of NALCN. Reverse-transcription polymerase chain reaction, Western blotting, and immunohistochemistry all showed abundant and localized expression of NALCN messenger RNA and protein in mouse small intestine. NALCN is involved in the SubP-induced depolarization of intestinal pacemaking activity. The protein is a potential target for pharmacological treatment of motor disorders of the gut.

Ca^{2+} imaging in the gut

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The ability to visualize activity in many cells within the gut wall using Ca^{2+} imaging has confirmed many of the interrelationships within and between networks of cells and revealed complex patterns of network activity. Using Ca^{2+} imaging, the wavefront of a slow wave in the ICC-MY network can be visualized, consisting of the area that is undergoing an upstroke in Ca^{2+} -induced fluorescence. Analysis in a number of regions of the gut shows that the slow wave wavefront in the ICC-MY network spreads isotropically (radially out from the site of initiation), and that not every cell is activated with each slow wave. Although slow waves are often initiated at the same regions within an organ, each ICC-MY is capable of generating slow waves, as demonstrated after uncoupling the ICC-MY network with the gap junction blocker, beta-Glycyrrhetinic acid. Intracellular Ca^{2+} waves are common in ICC-IM and ICC-MY network in some regions of the gut (e.g. colon). Normally, these intracellular Ca^{2+} waves are unsynchronized between adjacent cells, indicating no coupling of this activity, however, in certain conditions (e.g. nerve stimulation), the frequency of intracellular Ca^{2+} waves and their overall synchronization can be modulated. The main purpose of slow waves in ICC networks is to activate smooth muscle (SM) to produce contraction. Using Ca^{2+} imaging this process can be visualized and quantified. By observing the pattern of Ca^{2+} transients in SM, it is possible to infer information about the underlying slow wave. In the small intestine, the longitudinal muscle is active in bands (200-400 μm wide) that propagate in the oral or anal direction, but is quiescent outside of these bands. This suggests that the underlying slow wave wavefront/plateau depolarizes an area allowing muscle activity to occur, but outside of this region, muscle activity is suppressed. Without ICC to organize SM activity into bands, activity in SM is chaotic, as demonstrated in W/Wv mice.
Ca^{2+} imaging has been, and will continue to be an important tool to examine the activity of ICC and relationships with other cell types in the gut at the microscopic and macroscopic level.

Mechanisms of pacemaker activity in ICC of the urethra

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Interstitial cells of Cajal (ICC) in the urethra have been proposed as specialized pacemaker cells that are involved in the generation of urethral tone and therefore the maintenance of urinary continence. Studies on freshly dispersed ICC from the urethra of rabbits have demonstrated that pacemaker activity in urethra ICC is characterized by spontaneous transient depolarizations (STDs) under current clamp and spontaneous transient inward currents (STICs) under voltage clamp. These events result from activation of Ca^{2+}-activated Cl^- channels by spontaneous intracellular Ca^{2+} waves. Studies have shown that this activity arises through a complex interplay between Ca^{2+} release from intracellular stores and Ca^{2+} influx across the plasma membrane. In this presentation I will discuss recent work on the cellular basis of this activity, with particular emphasis on the molecular identity of the Ca^{2+}-activated Cl^- current and on the nature of the underlying Ca^{2+} signals responsible for activation of STICs in urethral ICC.

Does Ca^{2+} signaling drive pyeloureteric peristalsis? Involvement of atypical smooth muscle cells and interstitial cells

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Over the last 40 years, increasing evidence suggests that specialized smooth muscle cells (SMCs) called 'atypical' SMCs, clustered in regions within the papilla kidney border, are in fact the pacemaker cells driving pyeloureteric peristalsis. These atypical SMCs are thought to fire spontaneous transient depolarisations (STDs), which sum to trigger action potential discharge, calcium entry and muscle wall contraction. These contractions propagate along the renal pelvis into the ureter propelling urine and waste products towards the bladder for storage until micturition.

Single atypical SMCs of the mouse renal pelvis, voltage clamped (at 37°C) using the nystatin perforated-patch technique, were distinguished by their high resting input resistance and the absence of a 4-aminopyridine-sensitive voltage activated K^+ current (I_K) and spontaneous transient outward currents (STOCs) arising from the opening of large conductance Ca^{2+}-activated K^+ (BK) channels; both currents being readily recorded in typical SMCs of the renal pelvis (Lang *et al*., 2007c) and ureter (Lang, 1989).

Instead, single atypical SMCs displayed spontaneous inward currents (STICs) that arose from the opening of inward current channels that were either selective for Cl^- and blocked by niflumic acid, or cation-selective and blocked by La^{3+}. Intact tissue imaging suggest that these STICs are activated by transient increases in internal Ca^{2+} as Ca^{2+} is released from IP_3-coupled Ca^{2+} stores, in a manner amplified by ryanodine-receptor channels (Lang *et al*., 2007b; Iqbal *et al*., 2012) and by Ca^{2+} uptake/ release by adjacent mitochondria (Hashitani *et al*., 2009). We propose that these inward currents contribute to the generation of the membrane potential in atypical SMCs, as well as their STD discharge.

In the mouse renal pelvis, it is now apparent that interstitial cells (ICs) selectively expressing K_V7/ KCNQ ('M' channel) currents and located in sub-urothelium adjacent to the smooth muscle wall are also fundamentally involved in pyeloureteric peristalsis. Exposure to

K_V7 channel blockers (Xe991, linopirdine) and activators (flupirtine, meclofenamic acid MCA) increased and decreased, respectively, the frequency of the propagating pelvic contractions. The time course of the spontaneous action potentials recorded in the typical SMC wall with intracellular microelectrodes was also not affected by Xe991 suggesting that typical SMCs do not express these channels (Iqbal *et al.*, 2012).

Double labelling with $K_V7.5$ (KCNQ5) channel subunit antibodies established that $KCNQ5^+$ ICs were located in the sub-urothelial region and that they increased in number with distance from the papilla kidney junction. These ICs were also negative for antibodies raised against Kit (AK2) or α-smooth muscle actin (α-SMA)(Iqbal *et al.*, 2012).

Perforated-patch voltage clamp experiments (at 37°C) confirmed that freshly isolated ICs of the mouse renal pelvis were distinguished by the presence of a transient K_V7/ KCNQ/ M current, not present in either typical or atypical SMCs (Iqbal *et al.*, 2012). These ICs also displayed a small 4-AP-sensitive I_K as well as large TEA-sensitive STOCs and niflumic acid-sensitive STICs, readily observed upon membrane depolarization. The K_V7 current was blocked by Xe991 and reduced by MCA.

In intact preparations of the mouse renal pelvis, low frequency long-duration Ca^{2+} transients are recorded in ICs within the sub-urothelial space in the absence or presence of nifedipine. Intracellular microelectrode recordings revealed that ICs also fired action potentials at similar low frequencies and long duration and that they don't propagate (Lang *et al.*, 2007a).

We propose that modulation of K_V7/ KCNQ/ M current flow with channel blockers (XE991 or linopirdine) or activator (flupirtine) alters the spontaneous electrical and Ca^{2+} signalling in $KCNQ5^+$ ICs. This modulation directly influences the excitability of neighbouring typical SMCs in the pelvic wall, resulting in a change in the frequency of action potential discharge in the typical SMC layer.

The action of MCA acid is less clear. Analogous to Xe991, the partial blockade of K_V7 observed in MCA at the single IC level might be expected to increase contraction frequency. However, the net effect of MCA on renal pelvis contractility was inhibitory. Two possible mechanisms of MCA action can be proposed. First, MCA is a peripherally acting nonsteroidal anti-inflammatory drug (NSAID). The inhibitory effects of MCA on renal pelvis contractility were readily mimicked by the nonspecific cyclooxygenase (COX) inhibitor, indomethacin and reversed upon exposure to prostaglandin E_2 or the prostaglandin $F_{2\alpha}$ analog, dinoprost. In addition, COX2-selective NSAIDs have been shown to directly modulate K_V7 channel activity

independent of their COX inhibitory actions. For example, diclofenamic acid increases current flow through expressed KCNQ4 homomeric channels, but decreases current flow through native or expressed KCNQ5 homomeric and heteromeric KCNQ4/5 channels (Brueggemann *et al.,* 2011). While celecoxib, but not rofecoxib, enhances KCNQ5 current flow but suppresses L-type Ca^{2+} channel currents producing a net inhibitory effect in vascular muscle (Brueggemann *et al*., 2009).

In conclusion, $KCNQ5^{+}$ ICs within the sub-urothelial region of the renal pelvis represent the first example of an IC population having a direct modulating effect on pyeloureteric peristalsis. The increasing number of $KCNQ5^{+}$ with distance from the papilla suggests that their influence on neighbouring smooth muscle cells also increases with distance, particularly in the absence of a proximal atypical SMC pacemaker drive.

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Lypopolysaccharide effects on ICC pacemaker activity

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Background and Purpose: Lipopolysaccharide (LPS) induces intestinal dysmotility by alteration of smooth muscle and enteric neuronal activities. However, there is no report on the modulatory effects of LPS on interstitial cells of Cajal (ICCs). We investigated the effect of LPS and its signal transduction in ICCs. ***Methods:*** We performed whole-cell patch clamp and RT-PCR in cultured ICCs from mouse small intestine. ***Results:*** LPS suppressed the generation of pacemaker currents of ICCs. The mRNA transcripts for Toll-like receptor (TLR) 4 were expressed in ICCs. However, the inhibitory action of LPS on pacemaker currents from $TLR4^{+/+}$ mice was not present in $TLR4^{-/-}$ mice. The inhibitory effects of LPS on ICCs were blocked by glibenclamide (an inhibitor of ATP-sensitive K^+ channels), NS-398 (a COX-2 inhibitor), AH6808 (a prostaglandin E_2-EP_2 receptor antagonist), ODQ (an inhibitor of guanylate cyclase), and L-NAME (an inhibitor of nitric oxide synthase). Furthermore, genistein, and herbimycin A (tyrosine kinase inhibitors) blocked the LPS-induced inhibitory action on pacemaker activity in ICCs. ***Conclusions:*** LPS can activate ICCs to release nitric oxide and prostaglandin E_2 through TLR4 activation. The released nitric oxide and prostaglandin E_2 inhibit pacemaker currents by activating ATP-sensitive K^+ channels. The LPS actions are mediated by tyrosine kinase signaling pathways.

How Many Chloride Channels Do You Need?

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Calcium-activated chloride channels (CACCs) have been proposed to play a role in the generation of pacemaker potentials by ICC. We set out to identify calcium-activated chloride channels in ICC by experimentally manipulating intracellular calcium. Currents were recorded from voltage-clamped cell-attached patches. Cyclopiazonic acid (CPA) causes an increase in intracellular calcium by inhibiting calcium uptake into intracellular stores. CPA first caused the activation of an inwardly rectifying chloride channel which had on occasion been observed under control conditions. After some minutes a large outward current developed which inactivated sigmoidally. The time of inactivation varied stochastically, from depolarizing pulse to pulse, but generally increased over time. When patches were excised an outwardly rectifying maxi-anion channel appeared. On rare occasions this channel would change voltage-sensitivity so that it appeared like the CPA-induced, inwardly rectifying current. Do these chloride currents each result from a separate channel or are they the same channel in different guises? A similar situation, of time-dependent activation of multiple chloride currents by calcium, has been observed in the Xenopus oocyte, the classic "model" for CACCs. Therefore it is perhaps not surprising that we find ourselves in the same situation with ICC. With Xenopus oocytes the jury is still out on how many chloride channels are needed to explain the data. We make some speculations on what might be the case with ICC.

A Different Role for Ano1 in ICC

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A recent addition to the broad and diverse Cl^- channel family is Ano1 (TMEM16A). Ano1 is a Ca^{2+} activated Cl^- channel expressed in several epithelia. Ano1 is also expressed on interstitial cells of Cajal (ICC). All classes of ICC in both human and mouse express Ano1. Ano1 are required for normal gastrointestinal physiology as $Ano1^{-/-}$ mice have reduced smooth muscle contraction in the stomach and block of Ano1 or knockout of Ano1 results in loss of the slow wave. However, given that Ano1 is expressed on all classes of ICC, even those which are not responsible for the pacemaker function, raises the possibility that Ano1 has additional functions in these cells.
This talk will focus on a potential role for Ano1 in proliferation. Ano1 was first discovered in Gastrointestinal stromal tumors (GISTs) and given the initial name of DOG1 (discovered on GISTs). Ano1 is described as being ubiquitously expressed on all GISTs, although a low percentage of Ano1 negative GISTs have been reported. The expression of Ano1 on all classes of ICC, including those that do not generate slow waves, the expression of Ano1 in GISTs and the known role Cl^- plays in regulating cell cycle suggested that Ano1 could be involved in the proliferation of ICC. Data supporting this role include that mice lacking Ano1 have fewer proliferating ICC in whole mount preparations and in culture. Cl^- channel blockers, both the non-specific Cl^- channel blockers as well as the more recently described more specific Cl^- channel blockers, decrease proliferation in cells expressing Ano1, including primary cultures of ICC and in the pancreatic cancer-derived cell line, CFPAC-1. Cl^- channel blockers have a reduced effect on $Ano1^{-/-}$ cultures confirm that the blockers are acting on Ano1. Ki67 immunoreactivity, EdU incorporation and cell cycle analysis of cells grown in low Cl^- media show fewer proliferating cells than in cultures grown in regular medium. Mice lacking Ano1 have less phosphorylated retinoblastoma protein compared to controls. These data suggest that, together with Ano1 role in physiology, Ano1 also regulates proliferation at the G1/S transition of the cell cycle.

Pacemaker potentials recorded from ICC-MY in the rabbit small intestine

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The nature of pacemaker potentials recorded *in situ* from myenteric interstitial cells of Cajal (ICC-MY) in the rabbit small intestine were investigated using intracellular recording techniques in the presence of nifedipine. Rabbit pacemaker potentials consisted of upstroke and plateau components. Ni^{2+} and nominally Ca^{2+}-free solution partially inhibited the upstroke component of pacemaker potentials. Replacement of Ca^{2+} with Sr^{2+} enhanced the upstroke component and decreased the plateau component of rabbit pacemaker potentials. The plateau component of rabbit pacemaker potentials was inhibited by low$[Cl^-]_o$ solution, 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS), a blocker of Cl^- channels, cyclopiazonic acid (CPA), an inhibitor of the internal Ca^{2+} pump or bumetanide, an inhibitor of Na^+-K^+-$2Cl^-$ cotransporter (NKCC1). NKCC1-like immunoreactivity was mainly observed in ICC-MY in the rabbit small intestine. Depolarized rabbit pacemaker potentials by high-K^+ solution was abolished by DIDS, CPA or bumetanide. These results suggest that the upstroke component of rabbit pacemaker potentials is partly mediated by voltage-dependent Ca^{2+} influx, whereas the plateau component is generated by Ca^{2+}-activated Cl^- efflux. NKCC1 is likely to be responsible for Cl^- accumulation into ICC-MY.

Fibroblast-like cells

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Purinergic and nitrergic neural pathways both contribute to inhibitory neuromuscular transmission (NMT) in the gastrointestinal tract. However, the contribution of these pathways to NMT in the internal anal sphincter (IAS) and the role of interstitial cells are still controversial. Intramuscular interstitial cells of Cajal (ICC-IM) have long been recognized as participants in nitrergic NMT while more recent studies suggest an additional role for a second class of interstitial cells referred to as "Fibroblast-like cells" (FLC). Since FLC selectively express the receptor tyrosine kinase PDGFRα we have referred to them as PDGFRα$^+$ cells. Studies in our laboratory have focused upon examining the role of ICC-IM and PDGFRα$^+$-IM in purinergic and nitrergic NMT in the mouse and monkey IAS. Changes in electrical and contractile activity during nerve stimulation were measured in the mouse and Cynomolgus monkey IAS. Dual labeling immunohistochemical techniques and confocal microscopy were also used to examine the distribution of two soluble guanylate cyclase isoforms α and β (GC), cGMP-dependent protein kinase I (PKGI), and small conductance Ca^{2+} activated K^+ channels (SK3) in ICC-IM and PDGFRα$^+$-IM. NMT in the mouse IAS consisted of purinergic and nitrergic components. In the IAS of the ICC-deficient *W/W^v* mouse, purinergic NMT was intact while nitrergic hyperpolarization was reduced. Nitrergic but not purinergic NMT was present in the monkey IAS. In mouse, GC was detected in all PDGFRα$^+$-IM and in some ICC-IM whereas in monkey only a subpopulation of PDGFRα$^+$-IM and ICC-IM expressed GC. PKGI immunoreactivity was detected in nerves but not in interstitial cells of either species. SK3 immunoreactivity was present in PDGFRα$^+$-IM of mouse but not monkey while it was absent from ICC-IM in both species. In *W/W^v* mice, ICC-IM were absent but PDGFRα$^+$-IM still expressed GC and SK3. In conclusion, the role of PDGFRα$^+$-IM in the IAS appears to differ between species. The persistence of both purinergic NMT and SK3-expressing PDGFRα$^+$-IM in the *W/W^v* mouse is compatible with a role for these cells in purinergic NMT in the mouse IAS. The high expression levels of GC in mouse PDGFRα$^+$-IM suggests that these

cells may also participate in nitrergic NMT. In contrast, both purinergic NMT and SK3-expressing PDGFRα^+-IM were absent in monkey and GC levels were considerably lower. Thus, the role of both ICC-IM and PDGFRα^+-IM in NMT in the monkey IAS is less clear. Grant support DK 078736.

Pericytes - Genuine pacemaker cells in suburothelial venules of the bladder

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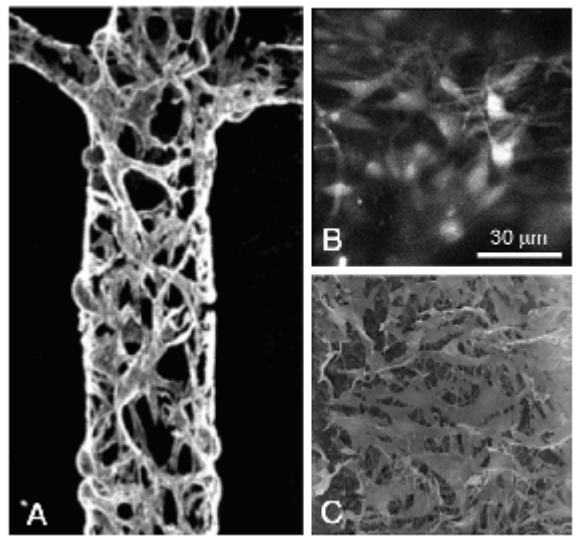
Suburothelial venules of the rat bladder exhibit spontaneous phasic constrictions that may be beneficial for preventing venular stagnation during bladder filling (Hashitani *et al*., 2011). Since both venular smooth muscle cells and pericytes (perivascular cells) are capable of generating Ca^{2+} transients in the presence of nicardipine, it is difficult to determine which cell population plays a fundamental role in driving this system. Therefore we have explored the functional and morphological characteristics of pericytes in the mouse bladder.
Changes in the diameter of suburothelial venules were measured using video microscopy and analyzed using Diamtrak edge-tracking software, while intracellular Ca^{2+} dynamics were visualized using fluo-4 fluorescence Ca^{2+} imaging. Electron microscopy and fluorescence immunohistochemistry investigated the morphological features of pericytes.
A network of stellate-shaped pericytes surrounded suburothelial venules and exhibited spontaneous Ca^{2+} transients (Fig B), which were accompanied by phasic venular constrictions. These stellate-shaped pericytes interdigitatied via their processes and were immunoreactive for α-smooth muscle actin (Fig A). Scanning electron microscopy revealed that this network of stellate-shaped pericytes covered the venules (Fig C), while transmission electron microscopy demonstrated that the venular wall consisted of endothelium and adjacent pericytes, but lacked an intermediate smooth muscle layer. Nicardipine (1 μM) disrupted the synchrony of spontaneous Ca^{2+} transients in pericytes and greatly diminished associated constrictions. Residual asynchronous Ca^{2+} transients were suppressed by CPA (10 μM), 2-aminoethoxydiphenyl borate (10 μM), U-73122 (1 μM), oligomycin (1 μM) and SKF96365 (10 μM), but not affected by ryanodine (100 μM) or YM-244769 (1 μM), a blocker for NCX3, suggesting that pericyte Ca2+ transients rely on InsP3 receptor-

mediated Ca^{2+} release from the endoplasmic reticulum as well as Ca^{2+} influx through store-operated Ca^{2+} entry channels.
Pericytes in the mouse bladder are capable of generating spontaneous Ca^{2+} transients and contractions, thus acting as the basic machinery driving spontaneous constrictions of suburothelial venules.



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Telocytes

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Telocytes represent a novel cellular type discovered in the interstitial space and defined in 2010. The peculiar ultrastructural features of telocytes include a small cellular body and very thin, long prolongations (telopodes). The moniliform aspect of telopodes is given by an alternation of thin segments (podomers, with a caliber below the resolving power of light microscopy) and dilated portions (podoms, accommodating mitochondria, endoplasmic reticulum, and caveolae). Telocytes were found in vertebrates (fishes, reptiles, birds), including humans and other mammals. Telocytes have a specific immunocytochemical "portrait" as well as a specific microRNA signature. Telocytes interact with neighboring cells either by direct contact (creating a 3D network) or indirectly by shed extracellular vesicles or "microcrine" secretion. Stem cells and telocytes are cooperating as a tandem, mostly in stem cell niches of various organs (*e.g.* heart, lungs, skeletal muscle, skin, subventricular zone of brain, meninges). The telocyte could be a "rising star" for regenerative medicine.

Lymphatic pacemaking – a symbiosis of mechanisms

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Lymphatic vessels, which are divided into chambers by inter-connecting valves, propel fluid by a rhythmic constriction-dilation cycle, a process known as lymphatic pumping. A pacemaking mechanism, which is generated in the lymphatic smooth muscle (SM), underlies lymphatic pumping. Ca^{2+} release from IP_3-operated intracellular Ca^{2+} stores generates an inward Ca^{2+}-activated Cl^- current that depolarizes the SM membrane leading to triggering of an L-type Ca^{2+} channel-mediated action potential and resultant contraction. Pacemaker currents such as the hyperpolarization-activated HCN current (I_f), and the low threshold voltage-activated current such as the T-type Ca^{2+} channel current ($I_{T\text{-}Ca}$) have also been reported in the lymphatic smooth muscle. This cardiac-like ionic pacemaking mechanism interacting with the Ca^{2+}-store driven pacemaker drives lymphatic rhythmicity. Mechanical stretch of the lymphatic wall further modulates the Ca^{2+} store-ionic current pacemaking mechanism.

Interestingly, highly synchronous action potentials have been observed in the lymphatic smooth muscle which result in near synchronous or well organized propagating waves of contractions. This synchronicity has been suggested to arise due to a gap-junction dependent electro-chemical coupling between the pacemaking mechanisms of the smooth muscle. In some smooth muscle, such as the gastric smooth muscle, specialized cells known as the interstitial cells of Cajal (ICC) have been shown to underlie pacemaking. While ICCs have been reported in some lymphatic preparations, their role in lymphatic pacemaking remains unclear. Interestingly, a novel cell type known as the telocyte, which shares similarities to ICCs, has been reported in various organs. Here we report novel identification of telocytes in lymphatic vessels and provide insight into their role in lymphatic pacemaking and pumping.

Confocal imaging of immune-labelled lymphatics showed that α-actin positive lymphatic smooth muscle cells are oriented mainly in a

circular direction. Importantly, some α-actin negative immuno-labeled cells appeared to transverse the entire length of the lymphatic chamber and while at low density (1-2 per chamber) ran in close proximity to smooth muscle cells.
Numerical simulations were made to test the hypothesis that telocytes influence lymphatic pacemaking and synchrony if they were interconnected to the smooth muscle. The results of our simulations show that in the presence of telocytes a significantly more effective pacemaker mechanism emerges triggering action potentials of increased synchronicity across the lymphatic chamber.
In conclusion, the result of our studies indicate that lymphatic pacemaking arises through a symbiotic interaction of Ca^{2+} store and ionic pacemaker mechanisms, modulated by mechanical stretch, and possibly facilitated by telocytes and/or ICCs. Telocytes may provide a network for long range signaling in the lymphatic system.

Role of ICC in egg transport by the oviduct in health and disease

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Spontaneous contractions of the myosalpinx are critical for oocyte transport along the oviduct. Slow waves that underlie myosalpinx contractions, are generated by a specialized network of pacemaker cells called oviduct interstitial cells of Cajal (ICC-OVI). ICC-OVI, labeled with Kit antibodies, form a dense network associated with the smooth muscle cells along the entire length of the oviduct. Selective removal of ICC-OVI with KIT-neutralizing antibody resulted in loss of electrical rhythmicity and loss of propulsive contractions that drive egg movement in the oviduct. Molecular studies revealed most isoforms of L- and T-type calcium channels (Cav1.2,1.3,1.4,3.1,3.2,3.3) are expressed in the oviduct myosalpinx. Reduction of extracellular Ca^{2+} concentration results in the abolition of slow waves and myosalpinx contractions without significantly affecting resting membrane potential (RMP). Spontaneous Ca^{2+} waves spread through ICC-OVI networks at a similar frequency to slow waves and was inhibited by reduced Ca^{2+}. Nifedipine depolarized RMP and inhibited slow waves; however, slow waves returned when the membrane was repolarized with reduced extracellular K^+ concentration. Studies on the role of ryanodine and IP3 receptor-sensitive stores in ICC-OVI suggest that although both stores are involved in regulating slow wave frequency, neither are exclusively essential. The sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) pump inhibitor CPA, inhibited pacemaker activity and Ca^{2+} waves suggesting that a functional SERCA pump is necessary for pacemaker activity. Pacemaker activity is also absent in *Tmem16a*/ANO1 KO mice and is inhibited by the two chloride channel antagonists niflumic acid and 9-AC, supporting a role of calcium activated chloride channels (CaCC) in the generation of pacemaker activity.

Chlamydia trachomatis is a common sexually transmitted bacterial infection that causes damage to the oviducts, resulting in ectopic pregnancy and tubal factor infertility, but the reasons for defective oviduct function are poorly understood. Mice infected with Chlamydia display dilation of oviducts, pyosalpinx, and loss of

spontaneous contractions. Morphological inspection showed disruption of ICC-OVI networks, and electrophysiological recordings showed loss of pacemaker activity without change in basal smooth muscle membrane potential. Chlamydia infection is associated with upregulation of *Nos2* (iNOS) and *Ptgs2* (COX II) in leukocytes. Loss of ICC-OVI and pacemaker activity in Chlamyida infected oviducts causes pseudo-obstruction and loss of propulsive contractions for oocytes. This, accompanied by retention of oviduct secretions, likely contributes to the development of tubal factor infertility.

In conclusion, pacemaker activity in oviducts generated by ICC-OVI is dependent on extracellular calcium and functional SERCA pumps. ANO1 is a CaCC responsible for the generation of oviduct slow waves. ICC-OVI are sensitive to host inflammatory responses to infection of the female reproductive tract and loss of slow waves likely contributes to the pathophysiology of oviducts and leads to tubal factor infertility or ectopic pregnancy.

An updated view of interstitial cells of Cajal in the urinary bladder

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The wall of the urinary bladder comprises a complex cellular structure whose complement of specific cell types and physiological cellular interactions have been shown in recent years to be more extensive than previously understood. Classically, the urothelium was considered as a passive barrier providing an important separation of urine and bladder smooth muscle and nerves. Yet, the urothelial cells are now known to participate in bladder function by sensing and responding to changes in the environment such as pH, temperature and stretch by releasing signalling molecules including acetylcholine and ATP (1). Much attention has been given to the role of afferent nerves and how they interact with urothelial cells, intersitial cells of Cajal (ICC) and smooth muscle (2). In the last decade, much work on the novel interstitial cells which occupy the sub-urothelial lamina propria layer (ICC-LP) and the detrusor layer (intramuscular ICC-IM and interbundle ICC-IB) has provided new avenues of research into normal and dysfunctional bladder physiology (3).

The dual functions of bladder filling and emptying require coordination between the activities of smooth muscle, nerves, interstitial cells and the urothelium. During filling, relaxation of detrusor smooth muscle is important to enable urine storage at low intravesical pressures. Yet, low-level spontaneous, myogenic activity occurs during filling in detrusor smooth muscle which is considered to maintain the bladder wall in the optimal shape for efficient emptying. This activity appears to occur in discrete areas of the bladder wall which remain uncoordinated during filling. When a conscious decision has been made to void, the smooth muscle must then contract in a highly coordinated fashion and this is under parasympathetic neuronal control. Recent insights on the role of the mucosal layer on spontaneous activity will be presented with particular reference to interstitial cells. It has been shown that the urothelium provides inhibitory signalling to detrusor smooth muscle through urothelium derived inhibitory factor (4). Conversely others have shown that the

urothelium and potentially other sub-urothelial components enhance detrusor contractility (5).

There is substantial evidence that the distribution of ICC is altered in disease states including spinal cord injury (SCI), denervated bladder, bladder outlet obstruction and diabetes. Work from our laboratory on animal models of neurogenic dysfunctional bladder including SCI (6) and denervated bladders indicates different physiological roles for ICC-LP and detrusor ICC. In chronic SCI, loss of ICC-LP and detrusor ICC was associated with a hypercompliant phenotype and long-duration spontaneous contractions. In denervated bladders, maintenance of ICC-LP but loss of detrusor ICC was associated with high frequency, increased amplitude spontaneous contractions, indicative of an overactive phenotype. These observations suggest that (a) ICC-LP may have a pacemaker-type function, modulating or driving smooth muscle spontaneous activity and (b) detrusor ICC may have an inhibitory function, limiting spontaneous contractions of the detrusor during filling. Physiological evidence in support of these hypotheses include the findings that ICC-LP exhibit spontaneous depolarizing calcium activated chloride currents (7), not found in detrusor ICC which display hyperpolarizing spontaneous transient outward BK currents (RMJ Cunningham and KD McCloskey, unpublished observations). The current state of the field will be discussed with reference to the potential of ICC as therapeutic targets.

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Highlights and controversies in interstitial cell research; where to go from here……?

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Investigation into the function of interstitial cells has developed dramatically during the past 2-3 decades. The first population of interstitial cells highlighted is the cells now commonly referred to as interstitial cells of Cajal (ICC). Another population of cells, now referred to as PDGFRα+ cells, has been added to this field of investigation. The first observations suggestive of important functions were morphological for both classes of interstitial cells, and techniques were eventually developed to isolate ICC and PDGFRα+ cells to study their roles in the physiology of smooth muscles.
Labeling techniques, exploiting the expression of c-Kit, led to the realization that ICC networks are vulnerable to damage in several pathophysiological conditions, and new hypotheses for motor pathologies have resulted.
ICC have been linked to several important functions in the gastrointestinal (GI) tract: i) generation of pacemaker activity responsible for electrical slow waves; ii) providing a network for active propagation of slow waves and serving as a means to organize organ-level motor behaviors; iii) mediation of post-junctional responses to motor neurotransmission; iv) mediation of responses to stretch; v) stochastic discharge of spontaneous transient inward currents (STICs) that contribute to setting of membrane potential and regulating the excitability of the smooth muscle/ICC/PDGFRα+ cell (SIP) syncytium. PDGFRα+ cells have a role in mediating purinergic responses to enteric inhibitory neurotransmission. Changes in membrane conductance of any of the SIP cells will affect the excitability and behavior of smooth muscle tissues. Thus, as components of the SIP syncytium, interstitial cells have important roles in organizing and regulating smooth muscle behavior. This knowledge has provided new depth to the term ‘myogenic’ in regulation of GI motility.
ICC and PDGFRα+ cells are also present in other smooth muscles, however, less is known about their functions outside the gut. All visceral, and some vascular muscles display populations of c-Kit+

and/or PDGFRα+ cells. ICC have a pacemaker role in the oviduct and also display pacemaker activity in the urinary tract. PDGFRα+ cells were recently reported in the urinary bladder and may participate in neurotransmission and setting of bladder excitability. The functions of PDGFRα+ cells in smooth muscles will be a topic of intense research during the next few years a number of controversies and obstacles remain before a consensus view of the functions of ICC and PDGFRα+ cells in health and disease can develop. Several issues come to mind: i) Progress is slowed by the relatively small group of investigators using physiological and genetic approaches to study interstitial cells. ii) New reagents and animal models are needed to allow manipulation of these cells in adult animals and isolation and characterization of these cells in animals during the development of motor disorders. iii) A large body of literature has been developed based on various cell culture models of ICC. This information may be highly misleading in terms of the natural phenotypes and behaviors of these cells. iv) The role for ICC in neurotransmission has been questioned because electrical field stimulation elicits contractile responses in muscles with reduced ICC populations. Better understanding of the neuro-effector junction and the cell-specific contributions of interstitial cells and smooth muscle cells to the motor responses of the SIP syncytium are needed. v) A clearer understanding of what happens to ICC and PDGFRα+ cells in disease (particularly inflammatory conditions) is needed. vi) A better concept of interstitial cell development and the factors responsible for repopulation of tissues after an event causing loss of interstitial cells might provide rationales for therapies to treat interstitial cell-related motor pathologies. These issues, and others, will be put into context with new information presented during the ICC meeting.
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POSTERS

Responses to enteric motor neurons in mutant mice with reduced intramuscular ICC in the murine gastric fundus

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Objectives: Interstitial cells of Cajal (ICC) play an important role in the motor activity of the gastrointestinal tract. The role of ICC as pacemakers is well established, however their role in neurotransmission is more controversial. There are many reports describing the close proximity of enteric nerve terminals to ICC. Functional studies evaluating the role of ICC in enteric motor transmission have been performed using mutant animals in which ICC are decreased or absent, but these studies have yielded variable conclusions on the importance of ICC in enteric motor responses. Therefore, the aims of this study were 2 fold: i) to clarify the role of ICC (IM) in motor neurotransmission in the stomach, and ii) to evaluate possible remodeling of enteric motor responses in W/W^V mutants that lack ICC.

Methods: Immunohistochemical analysis for c-Kit was performed on gastric fundus whole mounts from wild-type and W/W^V mice. Contractile activity and post junctional responses to electrical field stimulation of fundus muscle strips were recorded using standard organ bath techniques. Real-time quantitative PCR (qPCR) was used to evaluate muscarinic (M2, M3) and neurokinin (NK1, NK2) receptors expression in gastric fundus of wild-type and W/W^V mutant fundus.

Results: Immunohistochemistry using antibodies against c-Kit revealed that spindle-shaped Kit^+ ICC-IM persisted in the gastric fundus of W/W^V mutants, although the number of cells was greatly reduced compared to wild-type controls.

Therefore, we divided these tissues into two distinct groups: W/W^V Group 1: Where ICC-IM were not resolved (46% of tissues). W/W^V Group 2: where ICC-IM were observed (40%). 14% of the W/W^V animals examined had severe mucosal scaring and were discarded from the study. Neural responses to electrical field stimulation in

muscle strips from the gastric fundus demonstrated biphasic responses, consisting of excitatory and inhibitory components. The inhibitory component (nitrergic) was completely absent in W/W^V group 1 and reduced in W/W^V group 2 compared to wild-type controls. An enhanced excitatory component (cholinergic) was observed in both W/W^V groups compared to controls. We used qPCR to compare expression of genes that encode neurotransmitter receptors to investigate the enhanced excitatory component in the W/W^V fundus. We found no significant change in the molecular expression of neurokinin (NK1, NK2) or muscarinic M2 receptors, however muscarinic M3 receptor expression was significantly augmented in the W/W^V fundus in comparison to wild-type animals.

Conclusions: The present study confirms that nitrergic inhibitory neurotransmission is indeed mediated via ICC-IM in the gastric fundus and provides evidence that some ICC-IM persist in the gastric fundus of W/W^V mutants. These data may explain the discrepancy in the functional data reported from studies that have utilized mutant animal models to examine the role of ICC-IM in enteric motor responses in the stomach. *(Supported by NIH, DK40569).*

The responsiveness of smooth muscle cells in the guinea pig ileum is affected by elements of dissection

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Previously, we have demonstrated that immediately after setting up preparations of guinea pig ileum and colon, in vitro, both inhibitory and excitatory junction potentials are suppressed. Simultaneously, resting membrane potential (RMP) is hyperpolarised, input resistance is increased, dye coupling between smooth muscle cells is reduced and responses to exogenous ATP are suppressed. Junction potentials recover over 120 minutes while RMP depolarizes, input resistance decreases and dye coupling increases. Once responses had recovered and stabilized, pharmacological blockade of gap junctions suppressed responses similar to the initial period (Carbone et al., 2012).
Aim: to determine which components of the setting up procedure may have caused the loss of gap junction coupling. **Method:** Intracellular recordings and dye fills were made from circular muscle cells of guinea pig ileum using microelectrodes containing 5% carboxyfluorescein. Results are presented as mean± SEM. **Results:** Dissecting preparations in Calcium-free solution did not prevent the initial suppression of neuromuscular transmission. Stretching the preparation (as occurred during setup) by 130% and 150% of resting circumference did not suppress responses. However, re-cutting all 4 edges of a fully responsive preparation did cause partial uncoupling, reducing fast IJPs from -17.2± 0.7mV to -9.6±1.5mV (P<0.0001, n=12) with recovery over the subsequent 40 minutes. Responses to local application of exogenous ATP were reduced following cutting. RMP hyperpolarized from -50.6±0.7 to -53.5±1.2mV (P<0.05) and the number of dye filled profiles reduced significantly from 10.1±0.9 to 7.4±1.0 (P<0.05, n=12). In contrast, when preparations were exposed to minimal dissection (ie: by leaving the mucosa attached), initial suppression of fast IJPs was reduced in amplitude and recovery occurred within 40 minutes (P<0.005, n=4). Input resistance and RMP did not significantly change within 40 minutes in the minimally

dissected preparations. Dissecting preparations in 3μM indomethacin (n=3) or 10μM ketotifen (n=4) did not block initial suppression.
Conclusion: Damage caused by cutting the edges of the preparation, and possibly by removal of the mucosa and submucosa, is responsible for the initial disruption of gap-junction coupling and suppression of neuromuscular transmission in in vitro preparations of gut smooth muscle. This does not appear to be due to influx of calcium or release of mediators such as prostaglandins.

Reference:

Carbone, S. E., Wattchow, D. A., Spencer, N. J. & Brookes, S. J. 2012. A loss of responsiveness of circular smooth muscle cells from the guinea pig ileum is associated with changes in gap junction coupling. *AJP: Gastrointestinal and Liver Physiology*, Mar 29. [Epub ahead of print]

High slow wave frequencies reduce the frequency of antral peristaltic contractions in the isolated murine stomach

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INTRODUCTION: Electro-mechanical (E-M) coupling of slow waves and gastric smooth muscle contraction ideally occurs at a 1:1 ratio to ensure that regular, ongoing peristaltic contractions are generated that propagate without interference to the pylorus. Deviation from the 1:1 E-M coupling ratio may affect gastric emptying independent of the force of contractions by reducing the number or coherence of antral peristaltic contractions.
AIM: In this study we aimed to determine:
1) the prevalence of dysrhythmic contractile activity in isolated mouse stomachs, and
2) whether increasing the frequency of slow waves or excitability of smooth muscle can uncouple their activities.
METHODS: Ca^{2+} imaging and video imaging from intact and/or flat-sheet preparations of mouse stomach. Spatio-temporal analysis was used to quantify movement and Ca^{2+} signals.
RESULTS: Dysrhythmic activity was observed in 50% of wildtype isolated mice stomachs (n=133). Of these 6.8% displayed an alternating pattern of strong and weak contractions ("skipping phenomenon"), while the rest showed moderate to high degrees of irregular contractions. These patterns of activity were not affected after the addition of TTX (1μM), suggesting that the underlying cause of the dysrhythmias was myogenic in origin. Similarly, there was no effect of TTX (1μM) compared to controls with regard to the frequency or the 1:1 E-M coupling ratio when Ca^{2+} imaging was used to monitor the frequency and spread of Ca^{2+} wavefronts in ICC/LM simultaneously with contractions (control 10.7±0.46 s versus TTX 10.8 ± 0.68 s interval). After the addition of the muscarinic agonist carbachol (1μM) the frequency of Ca^{2+} wavefronts in ICC/LM increased (6.5 ± 1.1 s interval), while the frequency of contractions remained similar to controls (12.5 ± 3.0 s interval). To increase the excitability of smooth muscle, but not ICCs, we added the L-type Ca^{2+}

channel opener (BayK 8644: 20-40μM). This also increased the frequency of Ca^{2+} waves (control 11.0 ± 1.1 s versus BayK8644 7.2 ± 0.56 s; interval; n=5) and resulted in an uncoupling between Ca2+ activity and contractions (contraction interval BayK8644 11.6 ± 0.9 s, n=4).

DISCUSSION & CONCLUSIONS: These results suggest that higher slow wave (Ca^{2+} wavefront in ICC/LM) frequencies cannot active smooth muscle at a 1:1 E-M coupling ratio. This is likely a property of the refractoriness of the smooth muscle and reduces the overall number of forceful contractions per minute that may contribute to poor gastric emptying. Prokinetic drugs that increase slow wave frequency may inadvertently be less effective compared to situations where slow wave frequency is maintained within the normal range.

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Role of Ca^{2+} influx and diffusion in the initiation and propagation of calcium waves in ICC freshly isolated from the rabbit urethra

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Interstitial cells of Cajal (ICC) act as pacemaker cells in the rabbit urethra to stimulate smooth muscle to contract and maintain myogenic tone during bladder filling (Sergeant *et al* 2000). Pacemaker electrical activity in urethral ICC results from propagating Ca^{2+} waves that stimulate Ca^{2+} activated Cl^- channels. The purpose of the present study was to examine the role of Ca^{2+} influx and diffusion in the initiation and propagation of the waves. All the experiments described were approved by DkIT Animal Care and Use Committee. New Zealand white rabbits were humanely killed by lethal injection of pentobarbitone (i.v), and strips of urethral smooth muscle were dissected and enzymatically dispersed to release single ICCs. These single cells were allowed to settle on a glass bottomed dish, loaded with Fluo - 4 AM (500nm) and imaged using an EMCCD (iXon) attached to a spinning disk confocal microscope. Cells were superfused with Hanks solution at 37^{o}C. Under control conditions spontaneous increases in fluorescence (indicating calcium release from the endoplasmic reticulum, ER) occurred at various distances along the cell length. These varied in magnitude (from 1.28 to 14.02 F/F_0, mean 5.49 ± SEM 0.23, n=146 events in 6 cells). The length of spread varied in a continuum from 1.64 μm in the case of transient events to 183.3 μm in the case of full propagating waves with a mean value of 17.62 ± 2.35. Similarly velocity ranged from 69.6 μm s^{-1} (for events showing minimal spread) to 1.39 (for fully propagating waves) with a mean 21.6 ± 1.38. Sometimes waves arose simultaneously at opposite ends of the cell and when they collided this caused mutual annihilation.

The development of propagating waves depended on three factors: influx of calcium from the extracellular medium; diffusion of calcium

within the cell and the level of IP_3 within the cell. Thus in a cell that was firing regular propagated waves, removal of extracellular calcium abolished these leaving only short-lived calcium transients which did not propagate. Similarly when diffusion of calcium within the cell was inhibited, by adding 3μM EGTA-AM to the external solution, propagated waves were blocked, leaving short-lived calcium transients. Conversely in cells that exhibited only transient calcium increases these events developed into propagated waves when agonists (such as phenylephrine, which increased intracellular IP_3 levels) were added. Furthermore increasing the sensitivity of calcium release from ryanodine receptors (RyR) by adding 1mM caffeine to calcium-free external solution caused transient calcium events to develop into propagated waves. We conclude from these results that transient calcium events result from spontaneous release of Ca^{2+} from the ER. These can develop into propagated waves when sufficient cytoplasmic calcium and IP_3 exists to cause sensitization of adjacent release sites. Wave propagation occurs when calcium diffuses to an adjacent sensitized release site and stimulates regenerative release.

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Pacemaker activity, c-kit positive cells and contractility in the prostate gland

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Introduction and objective: We have previously reported the presence of distinct electrical activities and cell types in the guinea-pig prostate and speculated as to their functional roles. Of primary importance in regulating prostatic smooth muscle activity are the prostatic interstitial cells (PIC). These specialised c-Kit immunoreactive cells lie between the glandular epithelium and smooth muscle stroma and are likely to have a similar role to intestinal interstitial cells of Cajal (ICC), generating the pacemaker signals that manifest as slow wave activity and contractility in the smooth muscle cells of the prostate. Since changes in smooth muscle tone are involved in the aetiology of age-dependent conditions such as benign prostatic hyperplasia (BPH), the aim of this study was to investigate how age affects the spontaneous pacemaker and contractile activity in the guinea-pig prostate gland.

Methods: Prostate glands were taken from both younger (~ 300 - 500g) and older guinea pigs (~ 900 - 1000g); standard tension-recording and intracellular micro-electrode recording techniques were used.

Results: Slow waves recorded within the guinea pig prostatic stroma had similar frequencies to that of the spontaneous contractions within both age groups of animals. In addition, older prostates (n=78) had a higher basal tension of 6.0 ± 0.3mN than the younger prostates (n=51) 4.7 ± 0.66mN. Pacemaker activity was recorded in approximately 10% of all electrical recordings in the younger guinea-pig prostate (n=>100). In the older guinea-pig prostate, pacemaker activity was not recorded (n=84), however cells exhibiting large-amplitude spontaneous transient depolarisations (STD) were recorded in 9% of all electrical recordings. In a subset of experiments, we examined the effects of the tyrosine kinase inhibitor, imatinib mesylate, on spontaneous slow wave and contractile activities. Slow wave activity was insensitive to imatinib mesylate (1-10μM) across both age groups,

although contractile activity was more significantly reduced in the prostates of younger animals (n=5).

Conclusion: The significant reduction in contractile activity but persistence of slow wave activity suggests that imatinib mesylate may affect the smooth muscle contractile mechanism in addition to tyrosine kinase inhibition. Imatinib mesylate reduced the spontaneous contractile activity in the younger to a greater extent than the aging guinea-pig prostate, which is consistent with the notion that the aging guinea-pig prostate is less reliant on the tyrosine-dependent pacemaker ability of PIC. Similarly, older prostates exhibited an increased level of smooth muscle tone which may also be linked to the change in the proportion of cells exhibiting the various electrical activities with age. Overall, interventions comprising targeted modulation of the electrical activities may well provide novel, and perhaps more selective avenues for controlling prostatic excitability and smooth muscle tone.

P-6

Identification of telocytes in the upper lamina propria of the human urinary tract

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The upper lamina propria (ULP) area of interstitial cells (IC) has been studied extensively in bladder, but is rather unexplored in the rest of the urinary tract. This cell layer is intriguing because of the localization directly underneath the urothelium, the intercellular contacts and the close relation with nerve endings and capillaries.

In the present study we examine the ULP layer of IC in human renal pelvis, ureter and urethra, and we make a comparison with ULP IC in bladder. Tissue was obtained from normal areas in nephrectomy, cystectomy- and prostatectomy-specimens, and processed for morphology, immunohistochemistry and electron microscopy. A morphological and immunohistochemical phenotype for the ULP IC was assessed and region-dependent differences were looked for.

The ULP IC in renal pelvis, ureter and urethra had a similar ultrastructural phenotype, which differed somehow from that of bladder IC i.e.: thinner and longer cytoplasmic processes, no peripheral actin filaments, and presence of dense core granules and microtubules. Together with their immunohistochemical profile, these features are most compatible with the phenotype of telocytes, a recently discovered group of stromal cells. Based on their global ultrastructural and immunohistochemical phenotype ULP IC in human bladder might also be classified as telocytes. The most striking immunohistochemical finding was the variable expression of estrogen receptor (ER) and progesterone receptor (PR). The functional relevance of ULP telocytes in the urinary tract remains to be elucidated, and ER and PR might therefore be promising pharmacological research targets.

IL-10 Reverses Delayed Gastric Emptying, Slow Wave Abnormalities and Smooth Muscle Membrane Potential Gradient changes in Diabetic NOD/ShiLtJ Mice

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Background: In non-obese diabetic (NOD/ShiLtJ) mice, development of delayed gastric emptying (GE) is associated with a loss of diabetes-induced up-regulated heme oxygenase-1 (HO1) in M2 macrophages, resulting in increased oxidative stress, loss of interstitial cells of Cajal (ICC) and abnormalities in slow wave frequency and regularity. IL-10 induces HO1 in vivo and therefore may be an option for the pharmacological treatment of gastroparesis. The aim of the study was to determine if IL-10 can reverse delayed GE, abnormal slow waves and smooth muscle resting membrane potential (RMP) gradient changes in diabetic NOD/ShiLtJ mice. Methods: Mice were included in this study if they developed delayed GE ($T_{½}$ >118 min) within 10 weeks of the start of hyperglycemia (glucose>250mg/dl). GE was measured every week. Mice with delayed GE were treated with either vehicle (n = 4) or IL-10 (1 μg ip twice daily, n = 4). Smooth muscle membrane potential and electrical slow waves were recorded from the circular muscle layer of the stomach at 12 regions distributed evenly from the proximal body to distal antrum. Results: Prior to treatment, the mean $T_{½}$ value was180±19 min (n = 8, all delayed). While GE remained delayed in mice treated with vehicle ($T_{1/2}$ = 157±11 min, n = 4 mice) GE returned to normal after 3.1 ± 0.9 weeks in IL-10-treated mice ($T_{1/2}$ = 106 ± 4 min, n=4). Peak amplitude (PA) and frequency of the slow wave were not different between vehicle- and IL-10-treated mice in the proximal body. In contrast, in the 3 sites recorded from in the distal antrum of IL-10-treated mice (see table), the slow waves frequencies were higher than vehicle controls ($P < 0.05$ indicated by*). Slow wave amplitude variability was assessed as variance in PA/amplitude (VPA), and showed significant difference between vehicle and IL-10 (table). The difference in RMP between the

proximal body and distal antrum was greater in IL-10-treated mice compared to vehicle controls (-11.6±3.2 mV vs -9.5±3.3 mV).

Distal Antrum	Posterior		Greater Curvature		Anterior	
Region	10		11		12	
Treatment	Veh	IL-10	Veh	IL-10	Veh	IL-10
Variance of PA (mV)	0.33±0.09	0.49±0.18	3.85±0.71	0.58±0.41*	0.27±0.06	0.29±0.17
RMP (mV)	-60.7±4	-63.3±6.8	-68.6±3.8	-64.9±2.6	-64.3±4.4	-57.9±2.2
Frequency (Hz)	0.03±0.01	0.06±0.02	0.04±0.01	0.07±0.02*	0.03±0.01	0.07±0.01*

Conclusion: These data suggest that the treatment of delayed GE with IL-10 normalizes delayed GE, slow wave abnormalities and smooth muscle membrane potential gradients.
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In situ calcium responses of ICC populations in the guinea-pig bladder to electrical field stimulation suggests functional innervation

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Introduction
Interstitial cells of Cajal (ICC) in the bladder are classified into distinct sub-populations: interconnected networks of stellate shaped ICC-LP in the lamina propria region and intramuscular ICC-IM which are elongated with branched processes tracking detrusor smooth muscle bundles (1). Immuofluorescence and electron microscopy studies show that both ICC-LP and ICC-IM are found in close proximity to nerves; moreover they respond to exogenous agonists by firing Ca^{2+} transients (2). While this implies neuronal regulation of ICC activity, there is currently no direct evidence of functional innervation. The aim of this study was to determine whether bladder ICC are functionally innervated.

Methods
Bladders were removed from male Dunkin-Hartley guinea-pigs (200-500g) which had been killed humanely by cervical dislocation. The protocols were in accordance with Schedule 1, Animal Scientific Procedures Act, 1986, UK and were approved by the local ethics committee, Queen's University Belfast. Bladders were opened longitudinally and the mucosa removed from the underlying detrusor by sharp dissection. Mucosal preparations were pinned over a pair of parallel silver electrodes embedded in a Sylgard recording chamber and loaded with the calcium indicator fluo-4 AM. Tissues were imaged with a Nikon 80i upright epifluorescent microscope using a water dipping objective lens. Fluo-4AM was excited with a mercury lamp which was attenuated with neutral density filters to minimize sample photobleaching. Filter sets appropriate for fluo-4 imaging were

selected with the resulting fluorescence imaged with an electron multiplying charged coupled device camera imaging system and recorded to a personal computer running WinFluor software. Images were captured at a frame rate of 20 frames per second (20 fps) using 2x2 binning from the software which represented an acceptable compromise between acquisition speed and image resolution.

Results

Tissues loaded with fluo-4AM contained urothelial cells, ICC-LP, ICC-IM and smooth muscle cells (SMC), readily identified by their distinctive morphologies when the plane of focus was adjusted. Microvessels with associated perivascular ICC (PICC) were also visible. SMC, ICC-IM, ICC-LP and perivascular ICC (PICC) displayed spontaneous Ca^{2+}-oscillations. Electrical field stimulation (EFS; 0.5Hz, 2Hz, 10Hz) evoked tetrodotoxin (1μM)-sensitive Ca^{2+} transients in ICC-LP, ICC-IM and PICC associated with mucosal microvessels. SMC and vascular SMC (VSM) also responded to EFS.

ICC-LP networks are interconnected with CX43 gap junctions (Sui et al, 2002). Prior to EFS, ICC-LP exhibited spontaneous Ca^{2+}-oscillations which occurred asynchronously. Ca^{2+} signals could be seen travelling from the branch of one cell to a neighbouring cell, suggesting the presence of a functional cellular network, although this transmission was not always evident. These events became coordinated following EFS.

ICC-IM and adjacent SMC which had been exhibiting non-synchronous spontaneous oscillations responded to EFS with simultaneous neurogenic-evoked Ca^{2+}-transients. Spontaneous Ca^{2+}-oscillations in PICC were little affected by EFS, whereas large Ca^{2+}-transients were evoked in pre-EFS quiescent PICC with simultaneous responses in vascular SMC.

Conclusions

These results demonstrate that ICC-LP, ICC-IM and PICC respond to neurogenic stimulation, providing novel evidence that bladder ICC sub-populations are under direct control of the complex innervations that governs normal bladder function. Similar studies in neurogenic bladder could help elucidate the contribution of impaired nerve-ICC communication to bladder pathophysiology.

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Cellular location and nature of the ion channel responsible for nitrergic inhibitory junction potential (IJP) in the smooth muscle cells

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Cellular location and nature of the ion channel responsible for the nitrergic, slow inhibitory junction potential (sIJP) are controversial. It has been proposed that these channels are located on the intermediary cells such as interstitial cells of Cajal (ICC), where the active nitrergic potential may be generated and then transferred as passive potentials to smooth muscles cells. Accordingly, the IJP recorded from the smooth muscle cells may represent passive potential in the smooth muscles. Alternatively, the ion channels may be located directly on the smooth muscle cells and the IJP may be generated because of active conductance changes in the smooth muscle membrane. We reasoned that if the sIJP was a passive potential conducted from a coupled ICC, it will not be associated with significant change in membrane conductance. On the other hand, an actively generated potential will be associated with change in membrane conductance. Moreover, the direction of change in conductance may signify whether the IJP was due to opening of channel such as K+ or closure of channel such as Cl-. Changes in ion conductance can be estimated by monitoring changes in the amplitude of electrotonic potentials (ETP) which are inversely related to membrane conductance. We recorded changes in ETP during the sIJP in the same circular muscle cell in the guinea pig ileum, using a modified Tomita Bath Technique. IJP was elicited by transmural stimulation under NANC conditions and the nitrergic IJP was isolated by blocking the purinergic IJP. The nitrergic IJP (~8 mV) was associated with doubling of the amplitude of the ETP, suggesting a decrease in net conductance of the smooth muscle cell. These observations suggested that the nitrergic IJP: 1) the IJP was due conductance changes generated with the smooth muscle cell was not a passive potential conducted from an neighboring ICC; 2) may be associated with a decrease in conductance of ion such as Cl-. This was supported by the fact that Cl- channel blocker, niflumic acid (NFA) also produced smooth muscle membrane hyperpolarization

associated with decreased conductance and suppressed the nitrergic IJP. Further studies on the nature of the Cl- channels showed that CaMKII inhibitor, KN93, but not KN92 blocked the nitrergic IJP but not the purinergic IJP. These studies show that nitrergic IJP: 1) is not a passive potential that is conducted from the ICC and is actively generated in the smooth muscle cell; 2) is due to closure of Cl- channels in the smooth muscle cells; 3) These channels may belong to CaMKII activated ClC3 molecular form.

Interstitial cells of Cajal in c-Kit mutant mice

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Interstitial cells of Cajal (ICC) generate electrical rhythmicity and transduce neural signals in the gastrointestinal musculature. ICC express the proto-oncogene *c-kit*, a receptor tyrosine kinase, and are identified morphologically by c-Kit immunoreactivity. The *c-kit* gene is allelic with the murine white-spotting locus *W*, and mutations of *c-kit* are known as *W* mutations. *W* mutations affect various developmental aspects of hematopoietic cells, germ cells, melanocytes, mast cells and ICC. There are several types of *W* mutations, such as *W* that shows the lack of transmembrane region of c-Kit protein, W^{v} that has an amino acid substitution from Thr to Met in c-Kit. We examined W^{jic} that has an amino acid substitution from Gly to Arg and W^{sh} that have an inversion mutation in the transcriptional regulatory elements of the *c-kit* transcription start site. Both W^{jic}/W^{jic} homozygotes and W^{sh}/W^{sh} homozygotes exhibited white coats and black eyes. The gross morphology of the gastrointestinal tract showed no abnormality in both mutant mice.

W^{jic}/W^{jic} homozygotes have a mutation in c-Kit tyrosine kinase domain resulting in severe loss of protein function. In the stomach, intramuscular ICC (ICC-IM) were missing, and myenteric ICC (ICC-MY) were reduced in number. In the small intestine, the number of ICC-MY was severely reduced; however there was a normal distribution of deep muscular plexus ICC (ICC-DMP). In the cecum, the numbers of ICC-IM and ICC-MY were severely depleted. ICC-IM were almost entirely absent in the colon, whereas ICC-MY loss was restricted to the distal colon. Among the W^{v}/W^{v}, W/W^{v} and W^{jic}/W^{jic}, W^{jic}/W^{jic} mice retained the lowest number of ICC.

In W^{sh}/W^{sh} homozygotes, the *c-kit* gene and c-Kit protein were not observed in both embryos and adults. To determine ICC in W^{sh} mice, we used antibody to another ICC marker TMEM16A (transmembrane protein 16A, anoctamin-1). In the stomach, all ICC-IM were missing, and ICC-MY were reduced in number. In the small intestine, the number of ICC-MY was severely reduced; however

there was a normal distribution of ICC-DMP. In the cecum, the numbers of ICC-IM and ICC-MY were severely depleted. In the colon, ICC-IM were almost entirely absent, whereas ICC-MY loss was restricted to the distal colon. Using electron microscope, ICC in W^{sh}/W^{sh} had characteristic morphological features as observed in normal mice. ICC were characterized by numerous mitochondria, caveolae, a basal lamina, and gap junctions. The ICC-DMP were connected to smooth muscle cells of the circular muscle layer, as well as to each other, by gap junctions. ICC-IM and ICC-DMP were frequently associated with nerve fibers and nerve terminals. The enteric nervous system of the mutant mice appeared normal.

From these findings, we conclude that W^{jic}/W^{jic} and W^{sh}/W^{sh} mutants are lacking several types of ICC in gastrointestinal tract. The subtypes of ICC that are deficient in these mutant mice are consistent with other *W* mutant mice such as W/W^{v}. These types of ICC are considered to develop and survive without c-Kit signal.

Effects of Transient Receptor Potential Channel Blockers on Pacemaker Activity in Interstitial Cells of Cajal from Mouse Small Intestine

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The interstitial cells of Cajal (ICCs) are pacemakers in the gastrointestinal tract and transient receptor potential melastatin type 7 (TRPM7) is a candidate for pacemaker channels. The effect of the 5-lipoxygenase (5-LOX) inhibitors NDGA, AA861, MK886 and zileuton on pacemaking activity of ICCs was examined using the whole cell patch clamp technique. NDGA and AA861 decreased the amplitude of pacemaker potentials in ICC clusters, but the resting membrane potentials displayed little change, respectively. Also, perfusing NDGA and AA861 into the bath reduced both inward current and outward current in TRPM7-like current in single ICC, respectively. But, they had no effects on Ca^{2+} activated Cl^- currents. The 5-LOX inhibitors MK886 and zileuton were, however, ineffective in pacemaker potentials in ICC clusters and in TRPM7-like current in single ICC, respectively. A specific TRPC3 inhibitor, pyrazole compound (Pyr3), and a specific TRPM4 inhibitor, 9-phenanthrol, had no effects in pacemaker potentials in ICC clusters and in TRPM7-like current in single ICC. These results suggest that, among the tested 5-LOX inhibitors, NDGA and AA861 modulate the pacemaker activities of the ICCs, and that the TRPM7 channel can affect intestinal motility.

Key Words: Interstitial Cells of Cajal, pacemaker, TRPM7, 5-lipoxygenase inhibitor

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Molecular screening of chloride channels in single interstitial cells of cajal

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Introduction: The interstitial cells of Cajal (ICCs) are responsible for the pacemaker activity in the gut (Huizinga et al., 1995). The motor patterns developing in the intestine are driven by rhythmic membrane potential changes in ICCs known as slow waves. The slow waves are very likely the product of coordinated action of ion channels, exchangers and transporters (Barajas-Lopez et al., 1989, Koh et al., 2002, Takaki, 2003, Liu et al., 2005, Parsons and Huizinga, 2010). To date, several conductances have been identified to play a role in the generation of slow waves, including chloride currents generated by a channel of unidentified molecular nature (Huizinga et al., 2002, Parsons and Sanders, 2008, Parsons and Huizinga, 2010). Very recently it has been proposed that the chloride channel could be actually a maxi anion channel, given its properties of multiple subconductances, activation by patch excision and by protein kinase A (Parsons et al., 2011, Wright et al., 2012). **Objective:** Given the importance of the maxi channel to understand the function of ICC and the generation of the pacemaker activity, we proposed to perform a chloride channel profiling in these cells using the RT-PCR. The different ICC populations are accompanied by several other cell types like neurons, smooth muscle cells and more composing the different layers of the small intestine and colon (Ordog et al., 2004), therefore a pure culture of ICC has been proven difficult to obtain. To solve this problem our objective was to perform single cell PCR. **Methods:** In this work we report the validation of a RT-PCR essay that enables the molecular profiling of the ICC transcriptome at the single cell level. Based on a microarray set of data from enriched ICC populations (Chen et al., 2007) and bioinformatics analysis, we decided to profile several chloride channels as maxi anion channel candidates in primary cultures of ICC. **Results:** Of all collected cells, we chose those expressing c-kit, a specific marker for ICC, to look for chloride

channels. Here we report the presence of the Calcium Activated Chloride Channel (Clca1), Chloride Channel 2 (Clcn2) and Chloride Channel 3 (Clcn3) in single ICC in culture, whereas Chloride Channel 5 (Clcn5) was not detected. **Conclusions:** Several families of chloride channels remain to be tested by this technique. The complete profiling of chloride channels in ICC will allow studies of gene knockdown to pinpoint the molecular identity of the maxi anion channel.

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Primary cilia in interstitial cells of Cajal (ICCs) and gastrointestinal stromal tumor (GIST)

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Single non-motile 9+0 cilia, which exist in a single copy on the surface of several cell types, are called primary cilia (1). In the last decade, the functional importance of primary cilia has been established. They function as the unique cellular site for mechano-, chemo- and osmo-sensation to regulate cellular processes during development and tissue homeostasis (2). The structural and signaling proteins required for cilia function are synthesized in the cell body and transported into the cilium by a mechanism known as intraflagellar transport (IFT; 3). IFT is required by the formation and maintenance of all mammalian cilia, and defective IFT is associated with several diseases and developmental defects collectively called Ciliopathies (4).

The relationship between PC and proliferation and differentiation during development, has led to investigation about the role of PC in cancer. While cilia have been referred so far absent in most cancers, primary cilia are being increasingly found harboring in tumors (5). It is now believed that gastrointestinal stromal tumors (GIST) originate from ICCs, that control gut motility, or from a precursor of these same cells. We have previously reported the presence of primary cilium in ICC (6).

In this study we analyze the ultrastructural features presented by the single cilium of ICCs and we compare them with those found in cilia located in different GIST tumor cells.

Methods: Transmission electron microscopy (TEM). For the present study we obtained samples corresponding to duodenum from four adult Wistar rats and 5 gastric GISTs, provided in accordance with institutional guidelines.

The experimental study was carried out on serial ultrathin sections belonging to different cells. We checked the whole thickness of each cell in order to locate the presence of a basal body or ciliary

structure. From these structures, the reconstruction of the cilium has been carefully performed.

Results: The primary cilium was projected into the extracellular space and had a length of about 1-2 μm. The most characteristic structural element was the axoneme, consisting of nine outer doublet microtubules that originated from the basal body and extended through the length of the cilium, differing from the conventional motile cilia as they possessed a pair of centrioles. The parental and daughter centrioles displayed peripheral satellites. The daughter centriole was close to the Golgi complex. The axonema was surrounded by a bilayer lipid membrane that was continuous to the plasma membrane. The proximal region of the cilium could remain partially intracellular within a membrane invagination, the ciliary pocket. Ciliary pocket represents an endocytic domain for endocytosis by vesicles formation. Distal end of the basal body contacted with the plasma membrane through transitional fibres (alar sheets). The region separating the basal body and cilium proper, transition zone, contained a moderately opaque structure: the terminal plate. This point of contact defines the boundary between the plasma membrane and ciliary membrane. These ultrastructural features are common in both ICCs and tumor cells. We observed no differences in the frequency of occurrence of single cilium in both cellular types.

Conclusion: Thus, at present, new lines of research have branched off to investigate the role of primary cilia in ICCs signaling, adult homeostasis, and GIST tumor formation.

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Telocytes in normal and scleroderma skin

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Objective: Telocytes are a distinct population of interstitial (stromal) cells which have been recently identified in a wide variety of mammalian tissues and organs (www.telocytes.com). Telocytes are ultrastructurally characterized by a small cell body and extremely long prolongations (telopodes) with a moniliform aspect alternating thin segments (podomeres) with dilations (podoms). Moreover, telocytes may display different immunohistochemical markers, such as CD34 and c-kit/CD117, among organs and even in the same organ examined [1]. Telocytes have also been recently described in human dermis, where they may be involved in skin homeostasis and remodeling [2,3]. Systemic sclerosis (SSc, scleroderma) is a complex connective tissue disease characterized by immune dysfunction, widespread small-vessel vasculopathy and progressive fibrosis of the skin and multiple internal organs. The purpose of our study was to investigate the presence, distribution and ultrastructural features of telocytes in the skin of patients with SSc compared with healthy subjects.
Methods: Full-thickness skin biopsies were obtained from the clinically involved skin of one-third of the distal forearm of 20 patients with SSc classified according to disease subset (limited cutaneous SSc [lcSSc] and diffuse cutaneous SSc [dcSSc]) and stage (early- and late-stage SSc). Skin samples from the same forearm region of 10 age- and sex-matched healthy subjects who underwent surgery for traumatic lesions were used as controls. For immunohistochemical analysis, the specimens were fixed in 10% buffered formalin, dehydrated in a graded alcohol series, and embedded in paraffin. Sections (5 micron thick) were subjected to both double immunoenzymatic labeling (with alkaline phosphatase and peroxidase) and double immufluorescence labeling using a mouse monoclonal anti-human CD34 antibody and a rabbit polyclonal anti-human c-kit/CD117 antibody or a rabbit polyclonal anti-human

CD31/pan-endothelial cell marker. For transmission electron microscopy (TEM), biopsies were immediately fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide and embedded in epoxy resin. Semithin sections (2 micron thick) were cut and stained with toluidine blue-sodium tetraborate and observed under a light microscope. Ultrathin sections (70 nm thick) were obtained from the pathological areas chosen after observation by light microscopy and stained with uranyl acetate and alkaline bismuth subnitrate for examination under a transmission electron microscope.
Results: In normal skin, immunostaining revealed numerous CD34-positive cells throughout the dermis. Many CD34-positive cells were CD31-expressing endothelial cells, but, consistent with previous findings [3], there was also a large population of telocytes, which appeared as spindle-shaped cells with very long and thin prolongations forming an interstitial network in both the papillary and reticular dermis. CD34-positive telocytes were scattered between dermal collagen bundles, retinacula cutis and adipocytes in the hypodermis, and appeared concentrated around dermal capillary vessels and arterioles, nerves, and adnexal structures, including hair follicles, arrector pili muscle bundles, sebaceous and eccrine sweat glands. Double immunostaining for CD34 and c-kit/CD117 clearly showed that skin telocytes expressed CD34 but not c-kit/CD117, while many c-kit/CD117-positive mast cells were observed. A striking reduction in CD34-positive telocytes was found in the skin of patients with SSc. In early-stage lcSSc skin, telocytes were reduced throughout the papillary dermis and in some areas of the reticular dermis, with a patchy distribution. In late-stage lcSSc, the loss of CD34-positive telocytes extended to affect more severely the reticular dermis and the connective tissue around adnexal structures in the deep reticular dermis. In patients with dcSSc, skin telocytes were severely reduced in both the papillary and reticular dermis starting from the early-stage. A few CD34-positive telocytes were still present around adnexal structures in the deep reticular dermis and hypodermis of early-stage dcSSc, while they almost completely disappeared in late-stage dcSSc. TEM analysis confirmed that telocytes were severely reduced in the skin of SSc patients. In both lcSSc and dcSSc skin, telocytes exhibited ultrastructural features of degenerating cells, such as cytoplasmic vacuolization and loss of organelles. Moreover, in the fibrotic dermis of SSc patients, abundant elastic and collagen fibres enveloped telopodes thereby separating them from nerve fibres and microvessels.
Conclusions: Our study is the first to demonstrate a severe reduction in telocytes in the skin of patients with SSc. Because telocytes have been suggested to be implicated in several processes, such as mechanical support, neurotransmission, intercellular signaling and

connective tissue repair and regeneration, the progressive loss of telocytes might have important pathogenetic implications in SSc.

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Muscarinic neuromuscular transmission amplification by adenosine released from interstitial cells of Cajal of the rat ileum

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Gastrointestinal motility is chiefly coordinated by interstitial cells of Cajal (ICCs) in the tripartite myenteric synapse. Despite the close apposition of ICCs, motor neurons and smooth muscle fibers [1], there is a lack of information regarding the putative neuromodulatory role of ICCs activation. Confocal microscopy studies demonstrate that c-kit positive intramuscular ICCs (ICC-IM) exhibit high immunoreactivity against muscarinic M_3 receptors. Results from our group indicate that activation of muscarinic M_3 receptors in the longitudinal muscle-myenteric plexus (LM-MP) facilitates nerve-evoked acetylcholine (ACh) release, through a mechanism that depends on adenosine outflow leading to A_{2A} receptors activation [2].
This study was designed to investigate the link between muscarinic M_3 receptors on ICCs, adenosine outflow and subsequent facilitation of transmitter release from motoneurons in the LM-MP of the rat ileum.
Stimulation-evoked (5Hz, 200-3000 pulses) release of [^{3}H]-ACh and adenosine (ADO plus inosine) was measured by liquid scintillation spectrometry and HPLC-DAD, respectively (see [2]). Muscarinic-induced contractile responses of the LM-MP were continuously monitored via a PowerLab data acquisition system. Immunolocalization of muscarinic M_3 and A_{2A} receptors was performed by confocal microscopy (FV1000, Olympus) using specific cell markers.
Results indicate that adenosine A_{2A} receptors are predominantly localized in VAChT-positive cholinergic nerve terminals, in contrast with muscarinic M_3 receptors which are mainly localized on c-kit positive ICCs. The muscarinic receptor agonist, oxotremorine (Oxo, 300 μM), increased the release of both [^{3}H]-ACh and ADO from stimulated LM-MP of the rat ileum; the facilitatory effect of Oxo was prevented by dipyridamole (0.5 μM), an inhibitor of the equilibrative nucleoside transport system [2]. Blockade of muscarinic M_3 receptors

with J104129 (6 nM) prevented Oxo (300 μM)-induced facilitation of [^{3}H]-ACh and ADO release and competitively antagonized Oxo (0.003-300 μM)-induced contractions of the LM-MP. The facilitatory effect of Oxo (300 μM) on evoked [^{3}H]-ACh release was prevented by the selective adenosine A_{2A} receptor antagonist, ZM241385 (50 nM), while the ADO outflow was kept unchanged. Blockade of nerve action potentials generation and smooth muscle contraction, respectively with tetrodotoxin (1 μM) and nifedipine (1 μM), failed to modify Oxo (300 μM)-induced facilitation of ADO outflow. Implication of ICC-IM on M_3-receptor mediated facilitation of ADO outflow was confirmed since mibefradil (3 μM, a blocker of T-type Ca^{2+} channels located predominantly in ICC-IM) substantially decreased Oxo-induced ADO outflow. Mibefradil (3 μM) attenuated the spontaneous motor activity and the maximal tension produced by Oxo (0.003-300 μM) in the LM-MP. Mibefradil (3 μM) also decreased Oxo (300 μM)-induced facilitation of [^{3}H]-ACh release by a similar amount to that observed with J104129 (6 nM) and ZM241385 (50 nM).
Data suggest that activation of muscarinic M_3 receptors mediate a positive feedback mechanism on evoked ACh release by increasing ADO outflow from ICC-IM leading to the activation of facilitatory presynaptic A_{2A} receptors on myenteric motoneurons.

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Functional characteristics of submucosal venule in rat distal colon

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Objective: The functional properties of the venule in the gastrointestinal tract have not been well understood as compared with the arteriole. Here we report the spontaneous rhythmic constrictions of the submucosal venule of the rat distal colon. We also examined whether neurotransmitters evoked constriction or dilation of the venule.
Methods: The mucosa and the muscle layers of the rat distal colon were removed, and remained submucosal preparation containing microvasculature was pinned flat. Changes in the diameter of submucosal venule were recorded with video camera and analyzed using the edge-tracking software Diamtrak.
Results: The submucosal venule but not the arteriole exhibited spontaneous constrictions at about 6 min^{-1}. The spontaneous constrictions were abolished by cyclopiazonic acid (CPA, 10 μM) or 2-aminoethoxydiphenyl borate (2-APB, 10μM) suggesting the involvement of sarcoplasmic reticulum Ca^{2+} stores. The L-type Ca^{2+} channel blocker nicardipine (1 μM) or nifedipine (1 μM) also abolished the spontaneous activity. Niflumic acid (100 μM) or 4,4′-diisothiocyanatostilbene 2,2′-disulfonic acid (DIDS, 100 μM) abolished the spontaneous constriction and dilated the venular wall. The venular dilation evoked by acetylcholine (1 μM) was blocked by the nitric oxide synthase inhibitor N-ω-nitro-L-arginine (LNA, 100 μM) while calcitonin gene-related peptide (CGRP, 10 nM)-induced dilation was LNA-insensitive. The sustained constriction of venule evoked by transmural nerve stimulation or noradorenaline (1 μM) was inhibited by the alpha-adrenoceptor antagonist phentolamine (1 μM). Immunohistochemical experiments revealed that perivascular adrenergic sympathetic nerves and CGRP-containing nerves were sparsely distributed around the submucosal venules.
Conclusions: The spontaneous constrictions of the venular smooth muscle appear to depend on Ca^{2+} release from sarcoplasmic reticulam that activates Ca^{2+} activated Cl^- channels to induce Ca^{2+} influx through L-type Ca^{2+} channels. Noradrenaline released from perivascular sympathetic nerves constrict the venule. The spontaneous constrictions of the submucosal venule may contribute to maintain blood flow in the microcirculation even when a fecal pellet in the lumen strongly distends the wall of the distal colon.

Spatial Characterization of Spontaneous Electric Activity in the Ileum of Mice Using Arrayed Microelectrodes with Increased Surface Area

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Smooth and elaborate gut motility is based on cellular cooperation, including smooth muscle, enteric neurons and special interstitial cells acting as pacemaker cells. Therefore, spatial characterization of electric activity in tissues containing these electric excitable cells is required for a precise understanding of gut motility. Furthermore, tools to evaluate spatial electric activity in a small area would be useful for the investigation of model animals. We thus employed a microelectrode array (MEA) system (MED system: Alpha Med Scientific, Ibaraki, Japan) to simultaneously measure a set of 8 × 8 field potentials in a square area of ~1 mm^2. A multi-channel AC amplifier was operated at 0.1 Hz. The size of each recording electrode was 50 × 50 μm^2, however the surface area was increased to ~200-folds by fixing platinum black nano-particles. The capacitance and resistance measured by an LCR meter were 0.052 μF and 15 kΩ, respectively. The impedance of the recording electrode at 0.1 Hz was thus small enough [~31 MΩ = $\sqrt{\{1/(2\pi \times 0.1\ \text{Hz} \times 0.052\ \mu\text{F})^2 + (15\ \text{k}\Omega)^2\}}$] to follow oscillating potentials, compared to the input impedance of the multi-channel amplifier (100 MΩ at 0.1 Hz). The efficacy of electric signal transmission was ~95% at 0.1 Hz: 100 MΩ / $\sqrt{\{(100\ \text{M}\Omega)^2 + (31\ \text{M}\Omega)^2\}}$. Mapping of spectral power, and auto-correlation and cross-correlation parameters characterized the spatial properties of spontaneous electric activity in the ileum of wild-type (WT) and W/W^v mice, the latter serving as a model of impaired network of pacemaking interstitial cells. Namely, electric activities were varied in both size and cooperativity in W/W^v mice, despite the small area. In the ileum of WT mice, procedures suppressing the excitability of smooth muscle and neurons altered the propagation of spontaneous electric activity, but had little change in the period of oscillations. MEA with low impedance electrodes enables to measure slowly oscillating electric activity, and is useful to evaluate both histological and functional changes in the spatio-temporal property of gut electric activity.

Characterization of ascending and descending colonic neuromuscular reflexes in wild type and interstitial cell of Cajal (ICC)-deficient mice

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Background: Ascending and descending neuromuscular reflexes induced by distension play an important role in gastrointestinal motility. However, mechanisms underlying this reflex in colon are incompletely understood.
Methods: Nerve stimulation (NS) and balloon distension (BD)-mediated ascending and descending responses were investigated using conventional intracellular recordings from distal colonic circular and longitudinal smooth muscle (CSM; LSM) in W/W^v wild-type and mutant mice, which lack intramuscular ICCs.
Results: *Ascending responses:* In the CSM, NS evoked mono-phasic inhibitory junction potentials (IJP), whereas BD induced depolarization with superimposed action potentials that reached a peak in 4 – 7 s and was maintained until the termination of the BD. Atropine (3 μM) significantly increased the NS-induced IJP from 35.4 ± 1.8 mV to 38.5 ± 1.8 mV, but abolished the BD-induced depolarization. In LSM, NS produced a biphasic IJP (fast IJP following by slow IJP (sIJP)). BD evoked an initial IJP following by a train of APs. MRS-2500 (1 μM), a P2Y1 antagonist, abolished the initial IJP, whereas atropine abolished the action potentials. In CSM from W/W^v mutant mice, responses to NS were no different from controls. However, the depolarization in response to BD was attenuated and delayed in onset until after the balloon was deflated. In LSM from W/W^v mutant mice, NS-induced ascending sIJPs were diminished and BD-evoked action potentials were absent. *Descending responses:* NS produced comparable descending monophasic IJPs in distal colonic CSM and LSM, while BD induced transient descending monophasic IJPs as well. These IJPs were abolished by MRS-2500. Unlike ascending excitation in W/W^v wild-type and mutant mice, there was no difference in the descending IJPs evoked by NS and BD between the wild and mutant types.

Summary & Conclusions: These data indicate that in the murine distal colon: 1) Ascending response induced by BD differs from that induced by the NS, while NS and BD produce similar descending inhibition; 2) Ascending response evoked by the BD in CSM differs from LSM, In contrast, descending inhibition is similar in both CSM and LSM; 3) Only cholinergic nerves are involved in ascending excitation to both CSM and LSM and purinergic nerves play a dominant role in descending inhibition; 4) In distal colonic CSM and LSM of mice deficient in intramuscular ICCs, ascending excitation to BD is impaired whereas descending responses to BD and NS are intact.

Myogenic origin of rhythmic propulsion of the murine jejunum *in vitro*

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In the colon a low frequency motor pattern generates large pressure gradients and is responsible for content propulsion. This pattern of propulsion has been suggested to be attributed to a secondary interstitial cell of Cajal (ICC) pacemaker network located at the myenteric plexus (Huizinga et al., 2011). In the small intestine, contractions associated with slow waves generated at the ICC of the myenteric plexus have been well described (Huizinga et al., 1995). Although these contractions can propel content over short areas, their primary function may have more to do with mixing of luminal content. It has been suggested that propulsion in the small intestine follows a model known as the "peristaltic reflex" (Bayliss and Starling, 1899). This model has been inadequately assessed in more physiologically relevant systems. Content propelling motor patterns of the small intestine have been reported, however explanation and acknowledgment of their importance is often lacking (Trendelenburg, 1917). Using a *Trichinella spiralis* model of inflammation, it was demonstrated that damage and loss of neurons and ICC at the level of the deep muscular plexus (DMP) led to abolishment of rhythmic propulsion, while the slow wave associated contractions (SWAC) were unaffected. Propulsion in small intestinal segments returned upon resolution of the infection and recovery of the DMP (Wang et al., 2005).

Our objective was to further describe the characteristics of the propulsive motor pattern (PMP) of the jejunum. Secondly, we wanted to further the mechanism of propulsion, specifically focusing on the physiological mechanism underlying the rhythmicity of this pattern.

Propulsion was studied using segments of the jejunum from CD1 mice employed in an organ bath apparatus, using an *in vitro* organ bath technique employing intraluminal pressure recording and spatiotemporal mapping.

We have shown that the PMP of the jejunum has a rhythmic low frequency pattern (Table 1) associated with aboral content outflow when distended with 1-3 cm H_2O pressure. The PMP is an inducible pattern which can often be stimulated by addition of

carbachol (5 μM) or intraluminal distention. The pattern relies on unknown preconditions as seen by the fact that cholinergic stimulation or distention does not always activate propulsion. Carbachol was in contact with the tissue at a constant concentration and thus the rhythmicity of the pattern is likely not dependent on rhythmic release of acetylcholine from neural sources which would be overcome by carbachol. Carbachol increased the frequency of the PMP but the duration, velocity, and resulting intraluminal pressures generated were unchanged (Table 1), suggesting that these parameters are defined by the pacemaker activity, and not the stimulatory cholinergic input.

Unlike the stable slow wave, the frequency of the PMP can be altered by varying the degree of distention of the jejunum. Increasing the levels of distention increases the frequency of the propulsive motor complexes. We also demonstrated a threshold where by lowering distension abolished the PMP; perhaps governed by stretch sensitive neurons.

Neural involvement in rhythmic propulsion potentially plays a sensory role in initiating the pattern. Blocking of enteric nervous system input using tetrodotoxin (0.1 μM) abolished the pattern by overcoming it with a large increase in muscle tone or shifting the motility to a more segmentation like pattern. Addition of carbachol was unable to restore rhythmic propulsion, suggesting a neural requirement of the pattern, perhaps a sensory or stimulatory component as the effect with carbachol suggests. Interestingly, block of nitric oxide production with L-NNA (0.2 mM) also led to a tonic increase in muscle tone; however rhythmic propulsion persisted in the absence of nitric oxide with unaltered parameters in frequency, amplitude, and duration (Table 1). This further confirms the TTX sensitivity of the PMP as the pattern was actually blocked and not simply overcome, since the PMP can persist in an increased muscle tone environment.

Propulsion is not as neurally mediated as the peristaltic reflex model predicts, thus our results suggest a myogenic origin of rhythmic propulsion in the murine jejunum. We show that excitatory neurotransmitters can induce (cholinergic) the observed pattern but it is not required in a rhythmic fashion. Since the propulsive pattern of contractions is highly rhythmic, its origin must be a pacemaker of the small intestine. Like research on the colon, where it has been suggested to have two contractile governing pacemakers, the ICC-DMP may govern the rhythmicity of propulsion in the small intestine. The ICC-DMP, unlike the ICC-MP of the small intestine are highly innervated (Ward et al., 2006) and thus provided an anatomical basis for a neurally activated, but ICC driven motor pattern of propulsion as we have demonstrated.

Table 1

	Control	Carbachol (5 μM)	Tetrodotoxin (0.5 μM)	L-NNA (0.2 mM)
Frequency (PMC/min)	1.0 ± 0.1	1.8 ± 0.1*	Pattern Abolished	1.3 ± 0.03
Amplitude (% SWAC)	11.8 ± 1.4	17.8 ± 3.7	Pattern Abolished	7.7 ± 1.7
Duration (s)	26.2 ± 2.1	20.4 ± 1.5	Pattern Abolished	22.4 ± 5.6
Velocity (cm/s)	1.4 ± 0.3	0.7 ± 0.1	Pattern Abolished	-

Mean ± SEM; * $P < 0.05$, paired t-test with corresponding control

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Development of c-kit-immunopositive interstitial cells of Cajal in the human digestive tract

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Although the exact role of interstitial cells of Cajal (ICC) is still controversial[1], it is well known that these specialized network-forming cells are involved in the control of digestive motility[2]. It has been also confirmed that ICC are reduced or otherwise affected in several dysmotilities[3].
ICC depend on signaling via Kit receptors for development and maintenance of phenotype[4]. A cytokine, termed steel factor or stem cell factor (SCF), has been identified as a *c-kit* ligand[5]. Recent studies have shown that ICC are not derived from the neural crest, but rather are mesodermal in origin[6]. However, some findings suggest that the cells present in the inception myenteric plexus (MP) and submucous plexus (SMP) ganglia are "responsible" for ICC differentiation, representing the source of SCF[5].
At the beginning of week 4 of embryonic development, the neural crest cells (NCC) enter the foregut and migrate rostrocaudally to reach the terminal hindgut by week 7. These cells are coalesced to form ganglia along a rostrocaudal gradient of maturation; the myenteric plexus (MP) developed primarily in the foregut, then in the midgut and finally in the hindgut[7]. SMP formed approximately 2-3 weeks after MP, arising from cells that migrated centripetally through the circular muscle layer from the myenteric region[7]. Whether or not ICC differentiation requires NCC has not been clearly established, although some recent studies have identified ICC in the absence of neural crest cells[8].
Numerous data have confirmed that ICC appear in the digestive tract along a rostrocaudal gradient of differentiation. However, recent research has demonstrated that in addition to the time of appearance, patterns of development of ICC in particular portions of the human digestive tract significantly differ too[9].
Development of ICC in the foregut. At the end of the embryonic period of development, in weeks 7 and 8, c-kit immunoreactive (IR) cells are present in the esophagus, stomach and first portion of the

small bowel corresponding to the proximal duodenum[9]. They are distributed in the form of a wide belt of densely-packed cells, encircling completely the inception of the MP ganglia (Fig. 1). Thus, c-kit IR cells form an uninterrupted wide belt extending throughout the parts of the digestive tube originating from the foregut. All of the described cells are morphologically very similar, pleomorphic with a small body and numerous thin processes. In the same period of development, c-kit IR cells are absent in other parts of the gut originating from the midgut and hindgut[7,9].

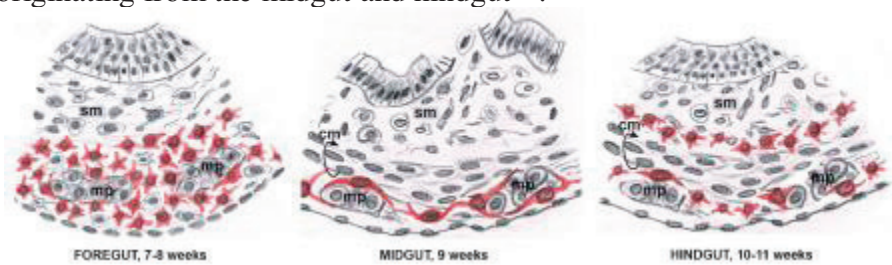

Figure 1. Development of c-kit immunoreactive cells (red colour) in human embryonal and foetal digestive tract. cm, circular muscle layer; mp, myenteric plexus; sm, submucosa.

Development of ICC in the midgut At the beginning of the fetal period of development, in week 9, c-kit IR cells appear in the distal duodenum, jejunum and ileum i.e., the parts of the digestive tube originating from the midgut[9,10]. c-kit IR cells were present in the form of a narrow band of cells, located at the level of the MP (Fig. 1). Some c-kit-IR cells are located at the border of the MP ganglia, but neither these cells nor their processes are present inside the ganglia. In addition to the already described multipolar c-kit IR cells, spindle-shaped cells also appeared with two long processes originating from the opposite ends.

Development of ICC in the hindgut. In contrast to the foregut and midgut in which ICC appear at the MP level, around the inception of the MP ganglia, ICC in the hindgut are characterized by a different appearance pattern. At 10-11 weeks, c-kit IR cells appear in the distal colon in the form of two parallel belts of cells extending at the SMP and MP level[11] (Fig. 1). These two belts of cells are separated by a circular muscle layer, already clearly differentiated in this period. C-kit IR cells are pleomorphic, with a small body and numerous thin processes.

Simultaneous appearance of ICC at the SMP and MP level in the terminal portion of the colon can be explained by the fact that there are differences in the migration of NCC in particular portions of the

digestive tube. In the foregut and midgut of NCC migrate through the outer part of the mesenchyme, just under the serosa, where the MP will form[6,7]. However, in the hindgut NCC are scattered widely throughout the mesenchyme, although the presumptive submucosal region was sparsely populated[7]. Such a distribution is possible since in this developmental period muscle layers are still undifferentiated in the hindgut. After the gut colonization is completed, NCC are differentiated into neurons and glial cells, which join together and form ganglia[7]. Neurons are one of the sources of SCF and are able to induce ICC differentiation[5]. This could be the reason why this different migratory route of NCC in the distal colon leads to a different model of ICC differentiation.

A conclusion may be drawn that ICC develop in the human digestive tract following different patterns, and that their appearance topographically matches the migratory route of NCC.

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Morphological features of the interstitial cells of Cajal associated with the submucosal plexus

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The subtypes of ICC distributing within the tunica muscularis have been extensively studied up to now, while only limited number of studies focused on ICC subtypes outside muscle layer including ICC in the subserosal layer (ICC-SS) and ICC associated with the submucosal plexus(ICC-SP). The present study revealed the morphological characteristics of ICC-SP in the caecum and proximal colon in the guinea-pig by c-Kit immunohistochemistry and electron microscopy.

In the guinea-pig caecum, c-Kit positive network was observed around the submucosal plexus which was clearly distinguished from the myenteric plexus in three-dimensional observation. The guinea-pig caecum appears to be useful to study functional aspect of ICC-SP because whole-mount specimen is easy to handle due to their specific wall structure containing only thin circular muscle layer except at the portions of taenia caeli (coli).

In the proximal colon, there were abundant c-Kit positive cells namely, ICC-SP. In this region, ICC-SP were found not only in the close association with the submucosal ganglia and but also distributed in the wide connective tissue space. Some ICC-SP were observed adjacent to muscularis mucosae in double staining section with c-Kit and α-smooth muscle actin. Electron microscopic observation revealed that this ICC-SP represent typical ultrastructural features of ICC; the presence of caveolae, abundant mitochondria, intermediate filament, basal lamina and gap junctions.

Since ICC-SP were especially abundant in the proximal colon where absorption of water and electrolytes occur, and located near to the submucosal plexus which were generally believed to control the mucosal function such as secretion and absorption of fluids, they may contribute to the regulation of water and electrolytes.

Insulin-like Growth Factor 1 (IGF1) Encodes Memory of Past Food Intake via Pleiotropic Effects on the Gastric Neuromuscular Apparatus

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Objective: Gastric dysfunction is common in protein-energy malnutritions and interferes with nutritional rehabilitation.[1-3] Conversely, food intake is often reduced in gastroparesis.[4] We found dystrophy of interstitial cells of Cajal (ICC) and enteric neurons in young female mice exposed to chronic dietary restriction.[5] Patients with severe weight loss had similar deficits. Caloric restriction reduces IGF1, a growth/survival factor for smooth muscle cells (SMC) and, indirectly, for ICC. Here we studied whether weight gain after dietary restriction is limited by gastric neuromuscular dystrophy and whether it can be restored by IGF1.
Methods: Female BALB/c mice aged 4, 8 or 13 weeks (n=120) were exposed for 170-224 days to dietary restriction that prevented natural weight gain. Subsets of mice were allowed to feed ad libitum starting at 0 (AL controls), 83, 96, or 152 days of limited feeding and/or were treated with R^3LONG-rhIGF1 (150 μg/kg sc BID) starting with dietary restriction or refeeding. Solid gastric emptying was measured by ^{13}C breath test. Estrous cyclicity was assessed by vaginal cytology. Blood glucose, ketones, serum malondialdehyde, insulin, IGF1 and corticosterone were measured by commercial kits. ICC-, SMC- and neuron-specific gene expression was studied by Western immunoblotting.
Results: Weight rose logarithmically in AL mice. Mice on restricted diet had lower ketones (P=0.002) and IGF1 (P=0.016) and 81% were in anestrus. Gastric emptying was delayed (P=0.016), and Kit (ICC), Myh11 (SMC), Uchl1 and Nos1 (neurons) protein were significantly reduced. Weights of mice restricted from 13 weeks of age normalized in 18 days upon ad-libitum refeeding. In contrast, in mice first exposed to restricted diet at 4 or 8 weeks of age, weights only increased to 88±2% and 86±3% of AL weights, respectively (P<0.01), and this difference was maintained for >60 days. In these

cohorts, gastric emptying was further delayed (P<0.001). IGF1 normalized gene expression and gastric emptying in both restricted and refed mice and also improved weight gain in refed mice.
Conclusions: Chronic dietary restriction reduced functional capacity of the gastrointestinal neuromuscular apparatus likely through the dystrophic effects of low circulating IGF1. In young mice these changes persisted after refeeding but could be minimized by IGF1 treatment.
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ANO1, a marker for interstitial cells of cajal in the zebrafish intestine

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Over the last decade, the zebrafish has emerged as a leading model organism to study vertebrate development. Molecular pathways, organ systems and physiology are well- conserved between zebrafish and mammals. Most major organ systems are present in the larvae within 5 days post fertilization (dpf). In addition to studies of vertebrate development the zebrafish is now used to investigate human disease conditions including functional gastrointestinal (GI) disorders. Interstitial cells of Cajal (ICC) are specialized cells that generate electrical slow waves initiating GI motility. In mammals, c-kit expression has been found to be a valid marker for ICC in mammals. Presence, distribution patterns and expression features of these iCC are known to be affected in various GI motility disorders. Anoctamin 1 (ano1, also known as DOG1), a Ca^{++}-activated Cl^{-}channel, has been shown to be a useful additional ICC marker in mice, primates and humans[1-3].
This study aimed at testing the validity of ano1 as ICC-marker in zebrafish.
Using multiple immunofluorescent staining methods, sections and whole mounts of adult zebrafish intestine as well as isolated intestines of zebrafish embryos and larvae (3 to 6 dpf) were analyzed for the expression of ano1, along with general markers human neuronal protein HuC and acetylated tubulin (α-tub).
In adult zebrafish intestine, ano1-immunoreactive particles revealed ICC-like cells in two distinct layers forming a three-dimensional network, i.e., an intramuscular (ICC-IM) layer located within the circular muscle layer and an intermuscular layer (ICC-MP) situated between the two muscle layers. ICC-MP were found to be intertwined with neuronal fibers and close associations between α-tub immunoreactive fibres and the ano1 immunoreactive network were

observed.
Zebrafish embryos lacked ano1 immunoreactivity at 3 dpf. From 4 dpf on, ano1 immunoreactivity appeared in the more distal parts of the intestine, whereas very faint immunoreactivity was observed in the proximal intestine. From 5 dpf on, the whole intestine showed clear ano1 immunoreactivity, forming a cellular network.
The present study shows the presence of an ano1 immunoreactive network in the proximity of the myenteric plexus of zebrafish from 4 dpf on, coinciding with the first spontaneous intestinal contractions. This network becomes more pronounced in time as GI contractility becomes more established. In adult zebrafish, an ano1 immunoreactive three-dimensional network was observed in close proximity to enteric neuronal fibers, pointing to putative cell-to-cell contacts between neurons and ICC. In conclusion, the present study demonstrates that ano1, just like in rodents and humans, is a useful marker for ICC-like cells in the zebrafish intestine.

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Distribution of pacemaker cells and peristaltic coordination in human gastro-duodenal junction

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Background: Disordered gastro-duodenal motor coordination is one of the mechanisms responsible for gastroparesis. In animal models, studies have shown that the slow wave was absent at the pylorus and this was concordant with the ICC pacemaker network interruption. Even though ICC were detected in human pylorus, a quantified study was not done, and most investigations focused not only on ICC but also on the enteric nervous system. The aim of this study was to evaluate ICC distribution and contractile activity in the gastro-duodenal junction to better understand the peristaltic coordination in human pylorus.
Methods: Twenty surgical gastro-duodenal specimens were obtained from adult patients with pancreatic cancer. ICC distribution and their relationship with enteric nerves in antrum, pylorus and duodenum were determined by immunohistochemistry and electron microscopy. Ten healthy volunteers were studied with videofluoroscopy and video images were acquired. Spatiotemporal maps were generated by image analysis to calculate the frequency, velocity and direction of contractions.
Results: There was no dense cellular network of ICC but there were networks of PDGFR-α positive cells at the level of myenteric plexus in human antrum. The total densities of c-Kit and PDGFR-α positive cells were significantly higher in antrum and duodenum than pylorus. Many ultrastructurally defined fibroblast-like cells were found connecting to each other around the myenteric plexus in antrum and duodenum but not pylorus. Close associations were present between ICC-MP and intrinsic sensory neurons in duodenum. Videofluoroscopic study showed independent contraction frequencies

and propagating velocities in antrum and duodenum. Gastric contractions propagated up to the pylorus. Most duodenal peristaltic contractions originated before gastric contractions reached pylorus. The diameter of duodenal bulb was significantly increased before duodenal contraction. Pyloric opening was frequently followed by antegrade propagating contraction in duodenum.
Conclusions: Despite of the lack of pacemaker network, strong rhythmic contractions were detected in human antrum. PDGFR-α positive cells may be the potential governors of antral phasic contraction. ICC-MP and intrinsic sensory neuron were closely associated and may function as distension receptor in duodenum. Time lapse between antral and duodenal contractions suggested that antral contraction increased luminal pressure to open pyloric sphincter, which allowed the gastric luminal content passing through the gastro-duodenal junction, distending the duodenal wall and initiating duodenal contractions. Duodenal peristalsis is therefore triggered by duodenal distension and not by propagation of antral contractions.

Colonic interstitial cells of Cajal associated with the myenteric plexus harbour ion channels regulated by cholinergic neurotransmission

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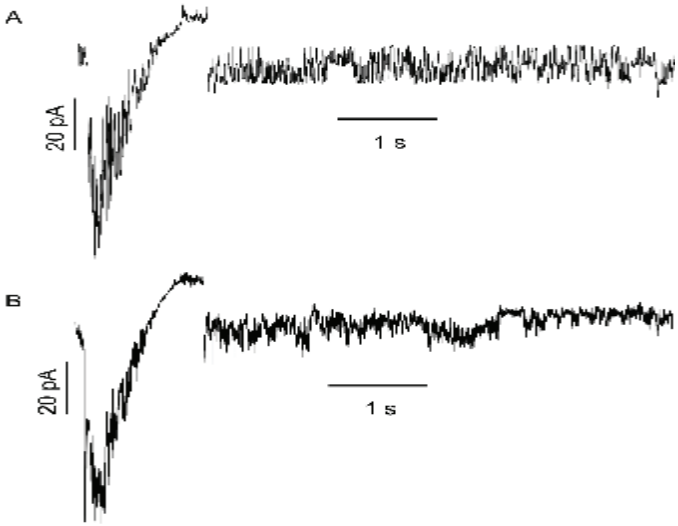
Introduction: Little is known about the colonic population of interstitial cells of Cajal (ICC) associated with the myenteric plexus (ICC-MP). Evidence is mounting that ICC-MP harbour an inducible pacemaker responsible for generating slow waves associated with propulsion of luminal content (Huizinga, et al., 2011; Pluja, et al., 2001). This pacemaker is not constitutively active but may require activation by excitatory neurons. Studies on small intestinal ICC-MP have shown that pacemaker activity is regulated by muscarinic acetylcholine receptor (mAChR) M_3 stimulation of non-selective cation channels (So, et al., 2009). Similar mechanisms may be present in ICC-MP from the colon. The mechanism of slow wave generation certainly involves ion channels which depolarize the ICC, but the identities and properties of these channels remains unknown.
Objective: The objective of this study was to identify and characterize ion channels involved in ICC-MP pacemaker response to excitatory neurotransmitters.
Methods: We recorded currents from colonic ICC-MP using cell-attached patch clamping with -80 to +80 mV voltage ramps using 150 mM KCl or 140 mM NMDG chloride pipette solution. Cells were either cultured or exposed *in situ* using techniques, as previously described (Parsons and Huizinga, 2010; Wang, et al., 2008).
Results: Two types of currents were observed. The first was active and inward between -20 to +20 mV and outward above +20 mV in KCl pipette solution (n=22). It was insensitive to 5 mM TEA (n=4), but turned off after patch excision into the inside-out configuration (n=11). It was similar to the transient outward potassium currents from ICC-MP of the small intestine (Parsons and Huizinga, 2010).
In experiments where 1 μM CCh was applied to cultured cells, the transient K^+ current was inhibited (n=7). This suggests that mAChR signaling mediates the inhibition of these often termed M-currents, which are carried by channels from the $K_v7.2$-5 family (Jepps, et al., 2009). To test the hypothesis that the K^+ currents were K_v7 channels,

we used the selective K_v7 blocker XE991. In experiments with KCl pipette solution, K^+ currents were observed in 18/28 patches (0.643). There was a significant reduction in the proportion of patches exhibiting K^+ currents when 20 μM XE991 was added to the pipette solution; currents were active and not blocked in 9/30 patches (0.3) ($z=2.616$, $\alpha=0.01$, c.v.=2.575). Thus, K_v7 channels are present in colonic ICC-MP as part of a population of K_v channels, some of which are insensitive to XE991.

The second current was sporadically active and inwardly rectifying (n=22). It was very "flickery" and high magnitude, often ~100 pA at -80 mV. The current was insensitive to 5 mM TEA (n=5) and did not inactivate on patch excision. One micromolar carbachol may be able to activate or increase the activity of inwardly rectifying currents while cell-attached (n=10). Upon excision of patches to the inside-out configuration, currents became outwardly rectifying, similar to those recorded from cultured ICC-MP of the jejunum (Wright, et al., 2012).

Conclusions: Colonic ICC-MP harbour several channels requiring further characterization. Two currents were active without pharmacological stimulation. The small conductance K^+ channel may be involved in setting resting membrane potential. It is suppressed by carbachol and is blocked by XE991. The large conductance chloride channel may be involved in neuron-activated ICC-MP pacemaker activity. It was activated by carbachol and similar to maxi chloride currents from small intestine ICC-MP. Supported by CIHR and NSERC.



gure 1: Inwardly tifying chloride rrents recorded from ll attached patches of lonic ICC-MP. A) rrents spontaneously tive *in situ*. B) Currents m cultured cell tivated by 1 µM rbachol.

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