In the last two decades it has become increasingly well established that inputs from several cell phenotypes regulate muscle excitability. In a number of organs these cells, known as interstitial cells can act as: (i) pacemakers that generate the underlying electrical activity responsible for phasic contractions of visceral organs, (ii) propagation pathways for regenerative electrical events and co-ordinated contractile activity throughout organs that cannot be propagated actively by muscle cells, (iii) transducers of inputs from motor nerves, (iv) stretch receptors and (v) nerve guidance elements that ensure nerve fibers make appropriate contact with neuroeffector cells. The investigators presenting at this symposium provide evidence related to many of these topics in the regulation of muscle excitability.

To coincide with this meeting we are also honouring the scientific contributions and achievements of Dr. Kenton M. Sanders. Dr. Sanders has made seminal contributions to the field of smooth muscle excitability and enjoys a world class reputation as attested to by his numerous awards, invited seminars, prodigious publication record and highly cited reviews. During his career he has also trained many successful graduate students (10) and post-doctoral fellows (50), and has generously collaborated with numerous visiting national and international research professors. Dr. Sanders has served on a variety of national committees, including the important National Commission on Digestive Diseases that was established by Congress to address the treatments of diseases associated with the GI tract. He continues to be a strong advocate for the advancement of research in the field of digestive diseases by being an active member on a variety of study sections. Over the duration of his career, he has also participated in numerous professional societies as well as serving on national and international editorial boards.
Multicellular Inputs Regulating Muscle Excitability
Granlibakken Tahoe, Tahoe City, California
June 14-17, 2015

Sunday, June 14th 2015

4:00 PM  Check-in
5:00-6:00 PM  Welcome reception
6:00-6:45 PM  Dinner

6:45-7:15 PM  Introductory remarks
   Dr. Kathleen Keef, University of Nevada School of Medicine.
   Dr. David Kreulen, Michigan State University.
   Dr. David Westfall, University of Nevada School of Medicine.

7:15-8:15 PM  Cocktail hour

Monday, June 15th 2015

7:00-8:30 AM  Breakfast

8:30–11:40 AM  Emerging technologies and advances in smooth muscle biology.
   Moderators: Drs. Philip Langton and Satoshi Iino

8:30-8:50 AM  Integrin adhesions and the myogenic response of rat cerebral arteries.
   Dr. William Cole, University of Calgary, Alberta, Canada.

8:50-9:10 AM  Electrical pacemaking in lymphatic vessels.
   Dr. Michael Davis, University of Missouri, Columbia, MO, USA.

9:10-9:30 AM  Cerebrovascular protective effects of TRPA1 channels.
   Dr. Scott Earley, University of Nevada School of Medicine, Reno, NV, USA.

9:30-9:50 AM  Novel cellular pharmacology of prostacyclins.
   Dr. Lucie Clapp, University College London, United Kingdom.

9:50-10:10 AM  CHROMus – a resource for in vivo physiology.
   Dr. Michael Kotlikoff, Cornell University, Ithaca, NY, USA.

10:10-10:20 AM  Break

10:20-10:40 AM  A role for the tyrosine kinase Pyk2 in Ca^{2+}-induced Ca^{2+} sensitization of vascular smooth muscle contraction.
   Dr. Michael Walsh, University of Calgary, Alberta, Canada.

10:40-11:00 AM  Functional effects of Na_{1.5} in airway smooth muscle.
   Dr. Keith Thornbury, Dundalk Institute of Technology, Ireland.

11:00-11:20 AM  Electrical regulation of pulmonary arterial smooth muscle.
   Dr. Alison Gurney, University of Manchester, United Kingdom.

   Dr. Ryuji Inoue, Fukuoka University, Japan.

11:40-12:40 PM  Lunch
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<td>1:00-5:00 PM</td>
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<td>5:00-6:00 PM</td>
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| 6:00-8:20 PM | **Advances in Gastrointestinal Motor Function.**<br>
|              | *Moderators: Drs. Kathleen Keef and Ki Whan Kim*                    |
| 6:00-6:20 PM | **Stem cell therapy for enteric neuropathies.**<br>
|              | *Dr. Conor McCann, University College London, United Kingdom.*      |
| 6:20-6:40 PM | **Serotonin signaling in and from the gut.**<br>
|              | *Dr. Gary Mawe, University of Vermont, Burlington, VT, USA.*        |
| 6:40-7:00 PM | **Therapeutic Epigenetic Modulation of Bacillary Dysentery: A Tale of Two Toxins.**<br>
|              | *Dr. Anthony Bauer, Liberty University College of Osteopathic Medicine, Lynchburg, VA, USA.* |
| 7:00-7:20 PM | Break                                                               |
| 7:20-7:40 PM | **Dangerous Liaisons: Immune Regulation of Gut Smooth Muscle.**<br>
|              | *Dr. Terez Shea-Donohue, University of Maryland, Baltimore, MD, USA.* |
| 7:40-8:00 PM | **Beta-nicotinamide adenine dinucleotide suppresses neurotransmission in the guinea-pig enteric nervous system.**<br>
|              | *Dr. Jackie Wood, The Ohio State University, Columbus, OH, USA.*    |
| 8:00-8:20 PM | **The purine alliance in abdominal viscera.**<br>
|              | *Dr. Violeta Mutafova-Yambolieva, University of Nevada School of Medicine, Reno, NV, USA.* |

**Tuesday, June 16th 2015**

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<tr>
<td>7:30-8:30 AM</td>
<td>Breakfast</td>
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| 8:30-11:40 AM| **Neurons, interstitial cells and smooth muscle integration.**<br>
|              | *Moderators: Drs. James Galligan and Kyu Joo Park*                   |
| 8:30-8:50 AM | **Macrophages and Gastroparesis.**<br>
|              | *Dr. Gianrico Farrugia, Mayo Clinic, Rochester, MN, USA.*           |
| 8:50-9:10 AM | **Identification of the pacemaker cells driving pyeloureteric peristalsis in the mouse renal pelvis using focused ion beam scanning electron microscopy, immunohistochemistry and Ca^{2+} fluorescence.**<br>
|              | *Dr. Richard Lang, Dept. of Physiology, Monash University, Clayton, Victoria, Australia.* |
| 9:10-9:30 AM | **Pacemaker role of pericytes in generating synchronized spontaneous Ca^{2+} transients in the myenteric microvasculature of the guinea-pig gastric antrum.**<br>
|              | *Dr. Hikaru Hashitani, Nagoya City University, Japan.*              |
| 9:30-9:50 AM | **Functional TRPV4-SK3 interaction in Murine Detrusor PDGFRα+ Interstitial Cells: Possible Mechanism of Myogenic Filling.**<br>
|              | *Dr. Sang Don Koh, University of Nevada School of Medicine, Reno, NV, USA.* |
| 9:50-10:10 AM| **The physiological role of PDGFRα+ cells in mouse colonic musculature.**<br>
|              | *Dr. Masaaki Kurahashi, University of Nevada School of Medicine, Reno, NV, USA.* |
| 10:10-10:20 AM| Break                                                              |
10:20-10:40 AM Properties of slow waves recorded from myenteric interstitial cells of Cajal distributed in the rat small intestine.
Dr. Yoshihiko Kito, Nagoya City University, Japan.

10:40-11:00 AM Cholinergic motor innervation of the stomach: role of Ano1.
Dr. Sean Ward, University of Nevada School of Medicine, Reno, NV, USA.

11:00-11:20 AM Epigenetic regulation of ICC turnover.
Dr. Tamas Ordog, Mayo Clinic, Rochester, MN, USA.

Dr. Seungil Ro, University of Nevada School of Medicine, Reno, NV, USA.

12:00-1:00 PM Lunch

1:00–3:00 PM Imaging Techniques in Physiological Systems.
Moderators: Drs. William Gerthoffer and Masatoshi Hori

1:00-1:20 PM Emergent properties of slow waves in the stomach.
Dr. Grant Hennig, University of Nevada School of Medicine, Reno, NV, USA.

1:20-1:40 PM Cooperative Ca\textsubscript{1.3} channel gating controls neuronal and SA node cell excitability.
Dr. Fernando Santana, University of Washington School of Medicine, Seattle, WA, USA.

1:40-2:00 PM K\textsuperscript{+} sensing and electrical signaling by K\textsubscript{ir} channels in the brain endothelium: “The Shadow Nervous System”.
Dr. Mark Nelson, Dept. of Pharmacology, University of Vermont, Burlington, VT, USA.

2:00-2:20 PM Interstitial cells in urethral smooth muscle.
Dr. Gerard Sergeant, Dundalk Institute of Technology, Ireland.

2:20-2:40 PM Ca\textsuperscript{2+} signaling in interstitial cells.
Dr. Salah Baker, University of Nevada School of Medicine, Reno, NV, USA.

2:40-3:00 PM Combining genetic and advanced optical approaches to monitor nerve activity in the intestinal wall.
Dr. Pieter Vanden Berghe, University of Leuven, Belgium.

3:00-4:30 PM Poster Session

5:00-9:00 PM Symposium Banquet (Gar Woods, Carnelian Bay, California)

Wednesday, June 17\textsuperscript{th} 2015

7:30-9:00 AM Breakfast

9:00-11:00 AM Poster Session

12:00-1:00 PM Lunch

1:00 PM Attendees depart
**Integrin Adhesions and the Myogenic Response of Rat Cerebral Arteries**

The Smooth Muscle Research Group, Libin Cardiovascular Institute and Hotchkiss Brain Institute, Faculty of Medicine, University of Calgary, Alberta, Canada, and Department of Physiology, Universidad de Valladolid, Valladolid, Spain*.

The myogenic response of small resistance arteries to changes in systemic blood pressure is an essential determinant of peripheral vasculature resistance, blood pressure regulation and regional blood flow control. Three cellular mechanisms are thought to contribute to the myogenic response: i) Ca2+-dependent activation of myosin light chain kinase (MLCK) and phosphorylation of myosin light chain regulatory subunits (LC20), ii) phosphorylation of the myosin light chain phosphatase (MLCP) targeting subunit MYPT1 that inhibits MLCP activity, and iii) actin dynamics to increase actin polymerization in the cortical cytoskeleton. However, the molecular basis of the mechanotransduction mechanism(s) that detects intravascular pressure and converts this stimulus into biochemical signal to evoke myogenic vasoconstriction has not been defined. We tested the hypothesis that mechanotransduction in the myogenic response involves activation of integrin adhesions. We assessed the role of integrin adhesion signaling in the myogenic response using function-blocking antibodies against alpha-5 or beta-1 integrins, inhibitors of focal adhesion kinase (FAK) and Src family kinase (SFK), and an ultra-high-sensitivity western blotting technique to quantify alterations in phosphoprotein levels and G-actin content in rat cerebral arteries. Pressurization increased tyrosine phosphorylation of key adhesion proteins, including kinases and scaffold proteins that are known to contribute to integrin-mediated mechanotransduction events. Treatment with integrin function-blocking antibodies suppressed the pressure-evoked changes in tyrosine phosphoprotein, phospho-LC20 and phospho-MYPT1T855 content, actin polymerization, and suppressed myogenic constriction. Taken together, our findings support the view that integrins and associated cellular signaling mechanisms contribute to pressure-dependent inhibition of MLCP and stimulation of actin dynamics in the myogenic response of rat cerebral arteries.
Electrical pacemaking in lymphatic vessels

Peichun Gui1, Joshua Scallan1, Scott Zawieja1, Jason Rock2 and Michael Davis1
1Dept. of Medical Pharmacology & Physiology, University of Missouri, Columbia, Missouri, USA
2Depts. of Anatomy and Medicine, UCSF, San Francisco, California, USA.

Spontaneous, propulsive contractions of lymphatic muscle (LM) are required for normal lymph transport and are initiated by an electrical pacemaker within the vessel wall. The established view of the mechanism for lymphatic pacemaking is based on the GI tract, where intracellular Ca2+ events, within LM or from a network of interstitial cells (ICCs), activate a Ca2+-activated Cl- channel, presumably Tmem16a(ANO1), causing small depolarizations (STDs) that summate to trigger an action potential (AP). The evidence derives largely from studies of unstretched LM strips. We explored the basis of electrical pacemaking in isolated lymphatic vessels held at physiologic pressures from rats, mice and humans.

Spontaneous contractions in mouse popliteal and rat mesenteric lymphatics were always preceded by LM action potentials (AP). An AP consisted of a 100 ms spike from -40 to +5 mV, followed by a 0.5-1 sec plateau at -20 mV and ~5 mV after-hyperpolarization. Similarly shaped APs of longer duration were recorded in pressurized human mesenteric lymphatics. AP firing was preceded not by STDs but by a slow, constant diastolic depolarization. The spike component of the AP was sensitive to TTX, mibefradil and nifedipine, and the diastolic depolarization rate was sensitive to pressure and KCNQ channel modulators.

c-Kit is a prototypical marker for ICCs in other hollow organs, where it colocalizes with Tmem16a. Although c-Kit-expressing cells and immune cells have been identified in lymphatic vessels, their functional roles are unknown. Here, c-KitGFP reporter mice revealed that the only c-Kit(+) cells in popliteal lymphatics were mast cells along the adventitial border. Cx3cr1GFP mice revealed GFP(+) dendritic cells present throughout the wall; however, deletion of Cx3cr1 did not interfere with pacemaking. Tmem16aGFP reporter mice revealed GFP expression in every LM cell rather than in an ICC network. Tmem16a inhibition led to inhibition of whole-cell Ca2+-activated Cl- current and to narrowing of the AP plateau phase, rather than changes in AP initiation.

When membrane potential was measured in pairs of LM cells, action potentials fired in both cells, with one cell consistently leading the other. Injection of hyperpolarizing current into the lead cell resulted in a lower pacemaking rate of the entire vessel and reversal of conduction direction. Collectively, these observations are counter to the existence of an ICC-driven pacemaker and suggest instead that the LM cell with the highest intrinsic depolarization rate controls pacemaking. The initiation of pacemaking by progressive diastolic depolarization suggests similarities to the pacemaker in the sino-atrial node.
Cerebrovascular Protective Effects of TRPA1 Channels

Scott Earley
Dept. of Pharmacology, University of Nevada School of Medicine, Reno, Nevada, USA.

Ca2+-permeable transient receptor potential ankyrin 1 (TRPA1) channels are present and colocalized with NADPH oxidase 2 (NOX2), a major source of ROS, in the endothelium of cerebral arteries but not in other vascular beds. We recorded and characterized ROS-triggered Ca2+ signals representing Ca2+ influx through single TRPA1 channels, which we called "TRPA1 sparklets." TRPA1 sparklet activity was low under basal conditions but was stimulated by NOX-generated ROS. Ca2+ entry during a single TRPA1 sparklet was ~200 times that of an L-type Ca2+ channel sparklet. In pressurized cerebral arteries, NOX-induced TRPA1 sparklets activated intermediate-conductance, Ca2+-sensitive K+ channels, resulting in smooth muscle hyperpolarization and vasodilation. NOX-induced activation of TRPA1 sparklets and vasodilation required generation of hydrogen peroxide and lipid-peroxidizing hydroxyl radicals as intermediates. 4-Hydroxy-nonenal, a metabolite of lipid peroxidation, also increased TRPA1 sparklet frequency and dilated cerebral arteries. These data suggest that in the cerebral circulation, lipid peroxidation metabolites generated by ROS activate Ca2+ influx through TRPA1 channels in the endothelium of cerebral arteries to cause dilation. Oxidative degradation of membrane lipids is increased in the vascular wall during a number of cardiovascular diseases, including hypertension. We hypothesized that TRPA1 activity is enhanced in cerebral artery endothelial cells during hypertension and that augmented activity of the channel under these conditions is protective against stroke. Hypertension was induced in control and endothelial cell specific TRPA1 knockout mice (eTRPA1^-/-) by infusion of angiotensin II (1200 ng/kg/min, s.c.) and high salt (8%) diet. After two weeks of this treatment, the nitric oxide synthase inhibitor L-NAME (120 mg/kg/day) was added to the drinking water to further increase mean arterial pressure (MAP). MAP was monitored by radiotelemetry and did not differ between groups under any conditions. All mice suffered fatal stroke. The maximal survival time of eTRPA1^-/- mice (22 days) was significantly less than that of control (52 days) littermates (n = 19-23). Histopathology revealed that eTRPA1^-/- mice develop more intracerebral hemorrhage (ICH) lesions than control mice (75 ± 1 for eTRPA1^-/- vs. 50 ± 9 for control, n = 3-4). However, the volume of each lesion was smaller in eTRPA1^-/- mice (0.03 ± 0.01 mm3 for eTRPA1^-/- vs. 0.12 ± 0.04 mm3 for control, n = 3-4). Together these data provide evidence that TRPA1 channels present in the cerebral endothelium are protective against hemorrhagic stroke associated with hypertension.
**Novel Cellular Pharmacology of Prostacyclins**

*Lucie Clapp*
Department of Medicine, UCL, Rayne Building, London, UK.

Pulmonary arterial hypertension (PAH) is a progressive disease of distal pulmonary arteries in which patients suffer from elevated pulmonary arterial pressure, extensive vascular remodelling and right ventricular failure. The disease is probably initiated by endothelial damage, caused by a combination of shear stress, hypoxia and genetic factors (including mutations in the transforming growth factor family of genes) leading to increased production of vasoconstrictors (endothelin & thromboxane) accompanying the loss of vasodilator and anti-platelet agents, prostacyclin (PGI$_2$) and nitric oxide (NO). This process is exacerbated by ion channel dysfunction, including the loss of potassium channel activity/expression (voltage-gated and the two-pore domain K$^+$ channels) and the upregulation of calcium entry through canonical transient receptor potential cation (TRPC) channels both of which will lead to smooth muscle membrane depolarisation and Ca$^{2+}$ influx. To date prostacyclin therapy remains the most efficacious treatment for PAH. A key thing to note is that improvement exceeds that predicted from vasodilator testing strongly suggesting that additional mechanisms contribute to the therapeutic benefit of prostacyclins in PAH. Given these agents have potent antiproliferative, anti-inflammatory and endothelial regenerating properties suggests therapeutic benefit might result from a slowing, stabilization or even some reversal of vascular remodelling in vivo. Our work provides strong evidence that the pharmacology of each prostacyclin (IP) receptor agonist so far developed is distinct, with non-IP receptor targets clearly contributing to the therapeutic and side effect profile, including prostanoid EP$_1$, EP$_2$, EP$_3$ and DP$_1$ receptors, along with a family of nuclear receptors known as peroxisome proliferator-activated receptors (PPARs), to which prostacyclin and some analogues directly bind. These targets are functionally expressed to varying degrees in arteries, veins, platelets, fibroblasts and inflammatory cells, and are likely to be involved in the biological actions of prostacyclins. Recently, a highly selective IP agonist, selexipag has been developed for PAH. This agent should prove useful in distinguishing mechanisms driven by the IP receptor from those involving other prostanoid receptors and/or direct binding to PPARs.
CHROMus – a Resource for in-vivo Physiology

Michael Kotlikoff
Austin O. Hooey Dean, College of Veterinary Medicine, Cornell University, Ithaca, New York, USA.

Significant progress has been made in the last decade in the development of optogenetic effectors and sensors that can be deployed to understand complex biological signaling in mammals at a molecular level, without disrupting the distributed, lineage specific signaling circuits that comprise nuanced physiological responses. A major barrier to the widespread use of these imaging tools is the lack of readily available genetic reagents that can be easily combined to probe complex biological processes. Our laboratory has recently been funded to create purpose-designed mouse lines expressing optically compatible sensors and effectors, sensor pairs in distinct lineages, and sensor pairs in discrete subcellular compartments, such that they can be crossed to enable in vivo imaging studies of unprecedented scientific power. Such lines could also be combined with mice to determine the alteration in signaling accompanying targeted gene deletion or addition. I will review the scope and plans for the Cornell Heart/Lung/Blood Resource for Optogenetic Mice (CHROMus), which has produced a number of lines to date and will develop at least 50 optically compatible, combinatorial mouse lines.
A role for the tyrosine kinase Pyk2 in Ca\(^{2+}\)-induced Ca\(^{2+}\) sensitization of vascular smooth muscle contraction.

Michael Walsh  
Department of Biochemistry and Molecular Biology, University of Calgary, Alberta, Canada.

Depolarization of the vascular smooth muscle cell membrane evokes a rapid (phasic) contractile response followed by a sustained (tonic) contraction. The phasic contraction is due to Ca\(^{2+}\) entry via voltage-gated Ca\(^{2+}\) channels, leading to activation of calmodulin-dependent myosin light chain kinase (MLCK) and phosphorylation of the 20-kDa regulatory light chains of myosin (LC\(_{20}\)). We showed previously (Mita et al. (2002) Biochem J 364: 431-440) that the sustained contractile response to membrane depolarization involves, in addition to MLCK activation, the activation of RhoA and Rho-associated kinase (ROK), leading to phosphorylation of MYPT1 (the myosin-targeting regulatory subunit of myosin light chain phosphatase (MLCP)), which inhibits the activity of the phosphatase. We went on to show that the sustained contraction involves genistein-sensitive tyrosine phosphorylation upstream of RhoA (Mitas et al. (2013) J Smooth Muscle Res 49: 26-45) and identified the Ca\(^{2+}\)-dependent tyrosine kinase Pyk2 as the major tyrosine-phosphorylated protein in response to membrane depolarization (Mills et al. (2015) J Biol Chem 290: in press (doi: 10.1074/jbc.M114.633107)). The tonic phase of depolarization-induced contraction was inhibited by the Pyk2 inhibitor sodium salicylate, which abolished the sustained elevation of LC\(_{20}\) phosphorylation. Furthermore, membrane depolarization induced autophosphorylation (activation) of Pyk2 at Y402 with a time-course that correlated with the sustained contractile response. Ca\(^{2+}\) entry was shown to activate Pyk2 since the Ca\(^{2+}\) ionophore, ionomycin, in the presence of extracellular Ca\(^{2+}\), elicited a slow, sustained contraction and Pyk2 autophosphorylation, which were blocked by Pyk2 inhibition, and the Ca\(^{2+}\) channel blocker, nifedipine, inhibited peak and sustained depolarization-induced contraction and Pyk2 activation. Inhibition of Pyk2 abolished depolarization-induced translocation of RhoA from the cytosol to the membrane and the phosphorylation of MYPT1. We conclude that depolarization-induced entry of extracellular Ca\(^{2+}\) activates Pyk2 upstream of the RhoA/ROK pathway, leading to MYPT1 phosphorylation and MLCP inhibition. The resulting sustained elevation of LC\(_{20}\) phosphorylation, therefore, accounts for the tonic contractile response to membrane depolarization via a mechanism of Ca\(^{2+}\)-induced Ca\(^{2+}\) sensitization evoked by Pyk2 activation. (Supported by a grant from the Canadian Institutes of Health Research).
Functional effects of NaV1.5 in airway smooth muscle


We have recently reported that isolated rabbit bronchial smooth muscle cells (BSMC) possess a voltage-dependent Na+ current (INa). Comparison of the current with that mediated by NaV1.5 α subunits expressed in HEK 293 cells, revealed similar biophysical properties and sensitivity to tetrodotoxin (IC50 = 1.1 μM for BSMC and 1.2 μM for NaV1.5), strongly suggesting that INa in BSMC was mediated by NaV1.5. RT-PCR for NaV1.2-1.9 in groups of ~20 isolated BSMC, and from whole bronchial tissue, revealed that most transcripts were present in whole tissue, but only NaV1.5 and 1.2 were detected in BSMC.

The functional significance of INa in airways is puzzling, particularly because the V1/2 of inactivation (88 mV) implies that its availability would be negligible at normal physiological potentials. However, we investigated if veratridine, an activator of INa, could evoke contractions in rabbit bronchus. Initially, we found veratridine 10 μM to evoke small contractions (0.3–0.4 mN), but these were abolished by atropine, implying that they were due to ACh release from cholinergic nerves. However, in the presence of atropine, veratridine dramatically altered the response to histamine (1 μM). At this concentration, histamine typically induced an initial tonic contraction that was followed by a series of slow periodic contractions at frequency of 10-12 per hour. When veratridine was applied during the periodic contractions, it converted them back to a tonic response. To verify that this effect was mediated by INa, we examined the effect of TTX on the veratridine-modified histamine response. The TTX-sensitivity of NaV1.5 differs fortuitously from all of the other NaV α subunits, with NaV 1.1-1.4, 1.6 & 1.7 being classified a TTX-sensitive (EC50<12 nM), NaV1.8 & 1.9 as TTX resistant (EC50<40 μM) and NaV1.5 as TTX-insensitive (EC50<1-2 μM). When we applied TTX 100 nM (sufficient to block TTX-sensitive NaV) it had no effect on the veratridine-modified histamine response, which remained tonic. In contrast, TTX 100 nM completely blocked contractions induced by electrical field stimulation, implying that it was sufficient to inhibit nerve-evoked responses. However, when TTX 10 μM (blocks NaV1.5, but not NaV1.8 or 1.9) was applied, the veratridine-modified histamine response reverted to periodic contractions, similar to control histamine responses. These results suggest that when NaV1.5 is activated in BSMC, it can modulate the response to histamine.

Electrical regulation of pulmonary arterial smooth muscle.

Alison Gurney
Faculty of Life Sciences, University of Manchester, UK.

Pulmonary arteries deliver blood from the heart to the gas exchanging surfaces of the alveoli. Blood flow in the lung is regulated by the contractile state of smooth muscle cells (SMC) in the artery wall. These cells are normally fully relaxed, in order to maintain vasodilation and arterial pressure at around 15% of that experienced in the systemic circulation. Potassium channels contribute to the relaxed state of the SMC by promoting membrane hyperpolarisation and suppressing voltage-gated Ca\(^{2+}\) influx. Many K\(^+\) channel genes are expressed in pulmonary artery SMCs, and K\(^+\) currents dominate the voltage-activated currents recorded from these cells. A major role for these channels is probably to limit excitation in response to depolarising stimuli and prevent vasospasm. K\(^+\) channels that are open in the absence of stimulation play an additional role in maintaining a negative resting potential. The K\(^+\) channels underlying the resting potential have yet to be fully elucidated and are the subject of debate. Two strong candidates are the two-pore domain channel TASK-1 and the Kv7 channel family, encoded by the KCNQ genes. This presentation will discuss the involvement of these two channel types in regulating vasoconstriction in healthy pulmonary arteries, their possible roles in the development of pulmonary hypertension and their potential as targets for pulmonary vasodilator drugs.
A FRET- and simulation-based study on the PIP$_2$-mediated regulation of TRPC3/C6/C7 subfamily channels.

*Ryuji Inoue*¹, Kyohei Itsuki¹, Yoko Imai¹, Yasushi Okamura³ and Masayuki X Mori¹,².

¹Department of Physiology, Fukuoka University School of Medicine,
²Department of Synthetic Chemistry and Biological Chemistry, School of Engineering, Kyoto University.
³Department of Integrative Physiology, Osaka University School of Medicine.

TRPC3/C6/C7 subfamily channels are abundantly expressed in the vascular system, and thought to mediate sustained Ca influx during phospholipase C-coupled receptor stimulation. The postulated pathophysiology of these channels includes abnormalities in both vascular response and remodeling. Despite great similarities in sequence and functional properties, however, upregulation of TRPC3 relative to TRPC6 has been found to greatly alter the vascular reactivity.

To explore the mechanism underlying this enigma, we investigated the dynamic regulation of TRPC3/C6/C7 channels by endogenous phosphatidylinositol 4,5-bisphosphate (PIP$_2$) level. To this end, we employed patch clamp technique in combination with FRET measurement in heterologous expression system and an embryonic aortic myocyte cell line A7r5, in which voltage-sensing phosphatase (VSP) was co-expressed to control endogenous PIP$_2$ level in a graded fashion. Further, we numerically simulated the activation time courses of these channels by using the *CellDesigner*, based on an experimental data-adjusted model which describes PIP$_2$ dynamics and TRPC3/C6/C7 channel gating kinetics. The results clearly indicate that only a few-fold change in the channels' PIP$_2$ sensitivity can create remarkably different patterns in the extent and time course of their receptor-mediated activation.

*References*

1. Imai Y et al., *J Physiol (Lond)*, 2012.
2. Itsuki K et al., *J Gen Physiol*, 2014.
Stem Cell Therapy for Enteric Neuropathies

Alan J. Burns and Conor J. McCann
Neural Development and Gastroenterology Units, Birth Defects Research Centre, University College London, UK.

The enteric nervous system (ENS), the intrinsic innervation of the gastrointestinal tract, is critically important for regulating essential gut functions such as peristalsis, blood flow, and fluid exchange. Normal gut contraction and function requires the coordinated interaction of the ENS with other cell types such as interstitial cells of Cajal (ICC) and smooth muscle. Defects in the development of these cells can result in a range of commonly occurring gut disorders/diseases that affect gut motility. Developmental disorders of the ENS (so-called enteric neuropathies) result in life threatening intestinal obstruction at birth. Hirschsprung disease (HSCR) is the commonest enteric neuropathy (1 in 5000 live births) and is characterized by an absence of enteric neurons in a variable length of the distal part of the gut. The cellular basis of HSCR is thought to involve the migration, proliferation, survival, and/or differentiation of the neural crest-derived precursors that ultimately form the ENS.

We are exploring ways to better understand and establish novel treatments for disorders/diseases resulting from abnormal development of the neuromuscular components of the gastrointestinal tract, with a particular focus on the ENS. Specific objectives, using model organisms such as chick and mouse embryos, as well as human embryonic, fetal, and post-natal tissues, are to:
(i) investigate the mechanisms controlling ENS development from neural crest-derived precursors.
(ii) better understand the aetiopathogenesis of enteric neuropathies and myopathies.
(iii) develop novel stem cell-based therapies and tissue engineering approaches for enteric neuropathies and diseased gut.

The integrated methods employed to address these objectives not only give insight to the pathophysiology of developmental disorders of the enteric neuromusculature but also underpin the establishment of novel diagnostic and therapeutic approaches for their treatment.
Serotonin signaling in and from the gut

Gary M. Mawe, Brigitte Lavoie, Stephanie N. Spohn, Rachel Scott, Emily Coffin, Conor O’Neil, Jane Roberts, Jane Lian and Keith A. Sharkey
Department of Neurological Sciences, The University of Vermont, and the Department of Physiology and Pharmacology, University of Calgary.

We are currently working on two projects that are related to serotonin (5-HT) receptors and colitis. One project is testing luminal 5-HT4 agonist administration as a therapy to treat colitis. We have recently demonstrated that 5-HT4 receptors are extensively expressed in the epithelial layer of the colon where their activation leads to 5-HT release from EC cells, Cl- secretion from enterocytes, and mucus discharge from goblet cells. In new studies, we have found that luminal application of 5-HT4 agonist reduced the development of colitis, and accelerated healing in both the DSS and TNBS models. 5-HT4 agonist treatment is protective when administered by enema, but not by intraperitoneal injection. Furthermore, enema treatment with a 5-HT4 antagonist alone increased the disease activity index and histological damage score in control mice indicating that endogenous 5-HT4 activation has a protective effect in healthy mice. In addition to stimulating mucus secretion, 5-HT4 receptor stimulation could be acting via a wound healing mechanism because we have detected an increase in epithelial proliferation in agonist treated mice, and faster migration of cultured colonic epithelial cells in a Caco-2 scratch assay. Furthermore, propulsive motility was improved by daily 5-HT4 agonist enemas in distal colons from guinea pigs with TNBS colitis. We are also testing whether 5-HT entering the blood stream from the inflamed colon contributes to decreased bone density. It has been previously shown that humans with IBD and mice with chronic DSS colitis exhibit decreased bone density. Furthermore, gut-derived 5-HT is a down-regulator of osteoblast formation via activation of 5-HT1B receptors on preosteoblasts. In studies of chronic DSS colitis in mice, we have found that circulating 5-HT levels were elevated, and that bone density was protected by daily treatment of DSS-inflamed mice with a 5-HT1B antagonist. Daily treatment with parachlorophenylalanine, which reduces 5-HT synthesis, also reduced colitis-induced bone loss. Collectively, these findings demonstrate the promise of 5-HT receptors as targets for pharmacotherapeutic treatment of colitis and related symptoms. Supported by DK 62267.
Therapeutic Epigenetic Modulation of Bacillary Dysentery: “A Tale of Two Toxins”

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Despite efforts to improve hygienic conditions and securely regulate food and water supplies, enteric pathogens remain a major public health concern due to emerging multi-antibiotic resistance, widespread outbreaks and disease severity. Of primary importance are the endotoxin (LPS) and Shiga toxin (Stx)–producing bacteria of the *Shigella* genus and enterohemorrhagic *Escherichia coli* species, which infect more than 150 million individuals each year and cause more than a million deaths. In fact, diarrheal diseases are second only to pneumonia in causing pediatric mortality world-wide with Shiga toxin producing E. coli and Shigella intestinal infections being among the top 5 causes of moderate to severe diarrheal disease world-wide. Even in the United States, the estimated incidence of food- and water-borne Shiga toxin producing bacterial infections is approximately 110,000 cases per year with an estimated 700 deaths. Initial common symptoms typically include nausea, vomiting, abdominal pain, hemorrhagic diarrhea, rectal tenesmus spasms and dehydration. If patients survive dehydration, they then are at risk to develop life-threatening renal failure and neurological abnormalities, such as seizures, paralysis, and blindness. Unfortunately, the pathophysiology of shigellosis is incompletely understood, no vaccines exist and treatment options are very limited. In our investigations, we have delineated the specific cellular contributions of endotoxin/TLR4 signaling to the intestinal inflammatory response and demonstrate the remarkable beneficial therapeutic effects of manganese and cellular deacetyylase inhibition on specific pathophysiological mechanisms of endotoxin and Shiga toxin-1 in preventing the organ sequelae and death caused by Shiga toxin-1 and lipopolysaccharide (LPS). Currently, we are exploring the therapeutic potential of these treatments using a unique Shiga toxin-secreting Citrobacter rodentium model of shigellosis. Importantly, our aim is to discover the first therapeutic strategy for the treatment of shigellosis which could save the lives of tens of thousands of children every year.
Dangerous Liaisons: Immune Regulation of Gut Smooth Muscle

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Host defense is an orchestrated response involving changes in the expression of receptors and release of mediators from both immune and structural cells. There is an emerging appreciation of the contribution of innate immune cells such as macrophages to changes in gut function associated with inflammation and infection. These hematopoietic-derived cells are sentinels of innate immunity and are shaped the microenvironment through their ability to both generate, and respond to, immune mediators. Macrophages are divided into classically activated (CAMϕ) or M1, alternatively activated (AAMϕ) or M2 macrophages. Enteric nematode infection induces a type 2 immune response with polarization of macrophages toward the alternatively activated phenotype (M2). The Th2 cytokines, IL-4, and IL-13, induce a STAT6-dependent upregulation of the expression of the number of factors including serpinB2 as well as receptors for protease activated receptors (PARs), and acetylcholine, which allow neuroimmune control of macrophage function. Vagal anti-inflammatory effects were reproduced by cholinergic activation of the nAChRα7 while cholinergic activation of M3 receptors promotes release of proinflammatory mediators. Macrophage interactions with smooth muscle play a key role in regulation of smooth muscle morphology and function.
**Beta-nicotinamide Adenine Dinucleotide Suppresses Neurotransmission in the Guinea Pig Enteric Nervous System**

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Based on reports that Beta-nicotinamide adenine dinucleotide (β-NAD) might be an inhibitory neurotransmitter at motor nerve junctions in smooth muscles of the gastrointestinal tract and that electrical stimulation evokes its released inside ganglia of the enteric nervous system (ENS), we used intracellular microelectrode recording techniques to investigate if it might have a role as a neurotransmitter/neuromodulator at synapses in the neuronal microcircuitry of the ENS. Exposure to β-NAD suppressed slow synaptic excitation and slow synaptic inhibition in the myenteric and submucosal divisions of the ENS. Adenosine mimicked the action of β-NAD. Adenosine A1 receptor antagonists, DPCPX or SLV320 reversed the actions of β-NAD. P2Y1 receptor antagonists, MRS 2500 or MRS2179, did not suppress actions of β-NAD. The data suggest that β-NAD is a ligand for the adenosine A1 receptor subtype expressed by ENS neurons. β-NAD influence on neurogenic motility, glandular secretion or blood flow is likely to emerge from suppression of neurotransmitter release at synapses in the enteric neural microcircuitry, at neuromuscular junctions and by suppression of excitability of enteric neurons, each of which is mediated by stimulation of the adenosine A1 receptor subtype.
**The purine alliance in abdominal viscera**

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Since the early 1970s adenosine 5'-triphosphates (ATP) has been advocated as the universal purinergic neurotransmitter in the body (1). The vast majority of studies proposing ATP as a neurotransmitter are indirect assessments of integral responses to nerve stimulation or to exogenous ATP and synthetic analogs. But in the past decade, new evidence has been accumulated for contribution of other purines, in addition to or instead of ATP, in chemical neurotransmission in the peripheral, enteric and central nervous systems (2). Our group acquired paradigm-shifting observations by using highly-sensitive methodologies for simultaneous evaluation of release and catabolism of purines released in smooth muscles, pheochromocytoma PC12 cells and isolated brain synaptosomes.

In the gut of primates and mice we demonstrated that: i) β-nicotinamide adenine dinucleotide (β-NAD+), ADP-ribose (ADPR), and uridine adenosine tetraphosphate (Up4A) meet key presynaptic and postsynaptic requirements for enteric inhibitory purinergic neurotransmitters (3-8); ii) in contrast to β-NAD+, ADPR, and Up4A, the release of ATP does not depend steeply on the level of neural activity and is not inhibited by neurotoxins, suggesting possible release of ATP from non-neuronal sources; iii) stimulation of serotonergic 5HT3 and nicotinic acetylcholine receptors on myenteric ganglia causes release of ATP, β-NAD+, and ADPR from different sites of enteric neurons: ATP appears to be released from nerve cell bodies and/or enteric glia, whereas β-NAD+ and ADPR are released from nerve varicosities; and iv) like the endogenous neurotransmitter, β-NAD+, ADPR, and Up4A cause smooth muscle cell membrane hyperpolarization and relaxation via activation of P2Y1 receptors (e.g., Up4A>ADPR>β-NAD+ in activating human P2Y1 receptors expressed in human 1321N1 astrocytoma cells) and SK3 channels located on PDGFRα+ cells, the cell type target for purine neurotransmitters in the gut (9). In contrast, the effects of ATP appear to be mediated by multiple receptors in various cell types.

We conclude, therefore, that enteric neurotransmission is likely operated by multiple purine neurotransmitters.

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References:
Cellular and Molecular Basis for Diabetic Gastroparesis

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Diabetes increases the risk of developing gastroparesis by 30 and 8 fold in type I and II diabetes respectively. Diabetic gastroparesis results in significant morbidity for patients and is major economic burden for society. The pathophysiology of diabetic gastroparesis includes damage to intrinsic and extrinsic neurons, smooth muscle, and interstitial cells of Cajal. Abnormally decreased expression of heme oxygenase 1 in the stomach in response to diabetes and resultant increased oxidative stress appears to be the primary insult involved in the pathogenesis of diabetic gastroparesis. There are genetic factors that appear to predispose to diabetic gastroparesis including variants of the calcium activated chloride channel Ano1 and repeat sequences in the promoter of the heme oxygenase 1 gene (HMOX1). There is increasing evidence that in the genetically predisposed, gastric macrophages are central to the development of the cellular damage that leads to gastroparesis. Activation of M2 macrophages with high levels of heme oxygenase 1 expression protects against development of delayed gastric emptying in animal models of diabetes, while activation of M1 macrophages that do not express heme oxygenase 1 are linked to neuromuscular cell injury. Media from cultured M1 macrophages induces loss of ICC while media from M2 macrophage cultures protects against damage from M1 media. Data from human studies also suggest that macrophages are involved in human diabetic gastroparesis with the number of CD206 positive M2 macrophages correlating with the number of interstitial cells of Cajal whose numbers in turn correlate with gastric emptying times. Targeting macrophages and heme oxygenase 1 may therefore be a therapeutic option in diabetic gastroparesis.
Identification of the pacemaker cells driving pyeloureteric peristalsis in the mouse renal pelvis using focused ion beam scanning electron microscopy, immuno-histochemistry and Ca\textsuperscript{2+} fluorescence

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The ultrastructure of the pacemaker cells driving pyeloureteric peristalsis in the mouse upper urinary tract was examined focused ion beam scanning electron microscopy (FIB SEM). Cellular reconstructions of cells within the proximal renal pelvis involved the repeated milling (100 nm thick with the FIB, SEM imaging the surface of each new face revealed, aligning the resulting stack of 500-900 images and volume rendering individual cells within using Avizo 8.1 imaging processing software. The effects of ZD7288, a non-selective blocker of hyperpolarization-activated nucleotide gated cation channel (HCN) channels on the contractile activity of the renal pelvis and the Ca\textsuperscript{2+} signals in typical and atypical smooth muscle cells (TSMCs and ASMCs respectively) was also examined using video microscopy and Fluo-4 Ca\textsuperscript{2+} imaging, respectively. Long darkly-stained TSMCs formed bundles adjacent to the urothelium and displayed Ca\textsuperscript{2+} waves which were associated with muscle wall contraction. ZD7288 significantly reduced contraction frequency and velocity as well as the spontaneous intercellular Ca\textsuperscript{2+} transients recorded in ASMCs. The volume rendered ASMCs, recognised in single sections by their lightly stained long thin processes, were in fact cells with broad cytoplasmic extrusions so that they resembled a irregular-shaped saucer or leaf. These ASMCs formed close appositions with themselves and their neighbouring TSMCs neighbours. As these volume rendered ASMCs occupy the same morphological space as the cells firing spontaneous ZD7288-sensitive Ca\textsuperscript{2+} transients, as well as the cells immuno-positive to antibodies raised against HCN isoform-3 (HCN3), we suggest that they are likely to be the pacemaker cells driving pyeloureteric peristalsis.
Pacemaker role of pericytes in generating synchronized spontaneous Ca\(^{2+}\) transients in the myenteric microvasculature of the guinea-pig gastric antrum.

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The importance of pericytes in regulating capillary blood flow has been recently revealed in several vascular beds, but their functions in the microcirculation of visceral organs remains to be determined. Properties of capillary pericytes and arteriolar smooth muscle cells (SMCs) in the microvasculature of the myenteric layer of the guinea-pig stomach were investigated using fluorescence Ca\(^{2+}\) imaging and immunohistochemistry.

The microvasculature generated spontaneous Ca\(^{2+}\) transients that were independent of both Ca\(^{2+}\) transients in interstitial cells of Cajal and neural activity. Spontaneous Ca\(^{2+}\) transients were highly synchronous along the length of microvasculature, and appeared to be initiated in capillary pericytes and spread to arteriolar SMCs. In most cases, the generation or synchrony of Ca\(^{2+}\) transients was not affected by blockers of L-type voltage-dependent Ca\(^{2+}\) channels (VDCC). In nifedipine-treated preparations, synchronous Ca\(^{2+}\) transients were readily blocked by Ni\(^{2+}\), mibebradil or ML216, blockers for T-type VDCC. Spontaneous Ca\(^{2+}\) transients were also suppressed by caffeine, tetracaine or cyclopiazonic acid (CPA). After the blockade of both L- and T-type VDCC, asynchronous Ca\(^{2+}\) transients were generated in some capillary pericytes but not in arteriolar SMCs, and were abolished by CPA or nominally Ca\(^{2+}\) free solution.

Thus, capillary pericytes may act as the origin of synchronous spontaneous Ca\(^{2+}\) transients in the myenteric microvasculature. Capillary Ca\(^{2+}\) transients primarily rely on spontaneous Ca\(^{2+}\) release from the sarcoplasmic reticulum, but also require Ca\(^{2+}\) influx through T-type VDCC for their synchrony. The opening of T-type VDCC may also contribute to depolarisation and Ca\(^{2+}\) transients propagating into arteriolar SMCs.
Functional TRPV4-SK3 Interaction in Murine Detrusor PDGFRα+ Interstitial Cells: Possible Mechanism of Myogenic Filling

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The functional expression of SK3 channels in detrusor PDGFRα+ cells has been reported. These cells do not display voltage-dependent Ca\(^{2+}\) channels. Among TRP channels, TRPV are relatively more Ca\(^{2+}\) permeable. Previous studies have shown Trpv4\(^{-/-}\) mice displayed an increase in frequency of non-voiding contractions. The mechanisms responsible for this phenotype have not been determined. Since TRPV4 channels are mechanosensitive and Ca\(^{2+}\) permeable, we tested the hypothesis that expression and role of TRPV4 channels in PDGFRα+ cells and serve as an important source of Ca\(^{2+}\) influx to activate SK channels in regulating detrusor stabilization during bladder filling.

C57BL/6, Pdgfra\(^{tm11(EGFP)Sor}\)/J and smMHC/Cre/eGFP mice (~3-12 weeks) were used for this study. We applied qualitative and quantitative PCR analysis, proximity-ligation assay, patch clamp, Ca\(^{2+}\) imaging, isometric force and ex-vivo compliance measurement.

Quantitative analysis in trp transcripts demonstrated trpv4 was highly expressed in PDGFRα+ cells compared to smooth muscle cells (SMCs). In patch clamp experiments, GSK activated non-selective cation currents under Cs\(^{+}\)-rich solution. With K\(^{+}\)-rich solution, GSK activated initial inward currents followed by the outward currents. The GSK-activated currents and hyperpolarization were completely blocked by TRPV4 antagonists and SK channel blockers. Removal of external Ca\(^{2+}\) also abolished GSK-activated outward currents. Detrusor SMCs did not show significant response to TRPV4 agonist. TRPV4 agonist alone did not increase the detrusor contractility. However, TRPV4 agonist decreased the spontaneous contractility and increased the spontaneous contractility in the presence of SK blocker. TRPV4 antagonist and SK blocker increased the non-voiding contractions in ex-vivo compliance test. Furthermore, co-immunoprecipitation and proximity-ligation assay suggested the protein-protein interaction between TRPV4 and SK3 channels.

TRPV4 and SK3 channels are functionally interact in plasma membrane of PDGFRα+ cells. Ca\(^{2+}\) influx through TRPV4 channels directly activates SK3 channels and stabilizes membrane potentials during bladder filling. Defects of this mechanism by loss of PDGFRα+ cells or reduced expression of key proteins may underlie detrusor overactivity. (Supported by NIH/NIDDK 098388)
Role of PDGFRα+ cells in enteric inhibitory motor neurotransmission in the colon

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Electron microscopists described two distinct types of interstitial cells in gastrointestinal (GI) muscles that are closely associated and form gap junctions with smooth muscle cells (SMCs). The interstitial cells have been referred to as interstitial cells of Cajal (ICC) and fibroblast-like cells (FLC). The functions of ICC have been studied extensively because c-Kit was shown to be a good cell marker, and spontaneous loss-of-function mutations in Kit resulted in reduction in certain types of ICC and defects in electrical rhythmicity and motor neurotransmission. The functions of FLC have been more difficult to determine because of a lack of adequate cell markers. Iino et al. (2009) showed that FLC expressed platelet-derived growth factor receptor α (PDGFRα), which can serve as a selective cell marker for these cells. Thus, FLC have been more recently termed PDGFRα+ cells and studied by a variety of techniques all centered around identification by this chemical marker. Mice in which eGFP is expressed in the nuclei of PDGFRα+ cells has been used to make progress in identifying the function of these cells in GI motility. Gene expression studies in PDGFRα+ cells purified from murine colon showed these cells to have very high expression of P2ry1 and Kcnn3 (genes encoding P2Y1 receptors and SK3 channels, respectively). Patch clamp studies demonstrated that PDGFRα+ cells display small conductance Ca2+-activated K+ currents and channels with properties and pharmacology characteristic of SK channels. The SK currents are activated by P2Y1 receptor agonists, blocked by P2Y1 selective antagonists, and inhibited by SK channel blockers. In contrast single smooth muscle cells (SMCs) isolated from colon display very small currents attributable to an SK conductance. SMCs show no outward current response to P2Y1 receptor agonists and ATP elicits depolarization, not hyperpolarization, in these cells. Ca2+ imaging studies have shown an increase in localized Ca2+ transients in PDGFRα+ cells and these events are also activated by electrical field stimulation (EFS) of enteric inhibitory motor neurons. Gap junction inhibitors blocked SMC responses to EFS but did not affect responses in PDGFRα+ cells significantly. Taken together these data suggest a role for PDGFRα+ cells in purinergic inhibitory motor neurotransmission in the colon. (Funded by R01 DK-091336)
Properties of slow waves recorded from myenteric interstitial cells of Cajal distributed in the rat small intestine

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The nature of slow waves recorded in situ from myenteric interstitial cells of Cajal (ICC-MY) in the rat small intestine were investigated using intracellular recording technique in the presence of nifedipine. Rat slow waves consisted of upstroke and plateau components. Ni2+ or ML218, an inhibitor of T-type Ca2+ channel, inhibited the upstroke component of rat slow waves. The plateau component of rat slow waves was inhibited by T16A(inh)-A01, a selective Ano1(TMEM16A) inhibitor, cyclopiazonic acid (CPA), an inhibitor of the internal Ca2+ pump or bumetanide, an inhibitor of Na+-K+-2Cl- cotransporter (NKCC1). NKCC1-like immunoreactivity was detected in ICC-MY in the rat small intestine. ATP-sensitive K channel openers (pinacidil and diazoxide) hyperpolarized ICC-MY and increased the amplitude and dV/dtmax of slow waves without affecting frequency. These results suggest that the upstroke component of rat slow waves is related to Ca2+ influx via T-type Ca2+ channels, whereas the plateau component is formed by Ca2+-activated Cl- efflux. NKCC1 is likely to be responsible for replenishment of intracellular Cl- in ICC-MY.
Cholinergic motor innervation of the stomach: role of Ano1.

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Enteric motor neurotransmission is essential for regulation of gastrointestinal (GI) motility. Controversy exists about the cells and ionic conductance(s) that mediate post-junctional responses to motor neurotransmitters. We utilized gastric fundus muscles to study ionic conductances activated by cholinergic stimulation. Isolated ICC and smooth muscle cells (SMCs) from fundus muscles were studied to determine the conductances activated by the muscarinic agonist carbachol (CCh) in each cell-type. We found Ano1 protein is expressed by ICC but not resolved in SMC. CCh activated a Cl⁻ conductance in ICC and a non-selective cation conductance in SMC. We studied responses to nerve stimulation in fundus muscles from wildtype and mice with genetic deactivation of Ano1. In wildtype mice nerve stimulation activated excitatory junction potentials (EJPs). EJPs were absent in animals with congenital knockout of Ano1 and greatly reduced in animals in which Ano1 was knocked down by the Cre-loxP recombination method. We also found that several inhibitors of Ano1 channels also blocked EJPs. Contractions to cholinergic nerve stimulation were reduced in Ano1 knockouts and by Ano1 blockers. SMCs cells also have receptors and ion channels activated by muscarinic agonists. Blocking acetylcholine esterase with neostigmine revealed a slow depolarization that developed after EJPs in wildtype mice. This depolarization was apparent in mice with genetic deactivation of Ano1 or in the presence of Ano1 blockers. Our data are consistent with the hypothesis that ACh released from motor nerves binds muscarinic receptors on ICC with preference. If extracellular metabolism of ACh is blocked, the neurotransmitter overflows to reach extrajunctional receptors, likely on SMCs.
Epigenetic Regulation of ICC turnover

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Work in our laboratory focuses on dynamics of Kit expression in the ICC/GIST lineage. Genetic lineage-tracing and cell line studies indicate that while mature ICC express high levels of Kit, cellular states characterized by low or no Kit expression (Kit\textsuperscript{low−}) naturally occur in Kit\textsuperscript{low−}Cd34\textsuperscript{+} ICC stem/precursor cells (ICC-SC) and also in Kit\textsuperscript{low−}Cd34\textsuperscript{−} post-functional ICC (post-ICC). These states are regulated, to a great extent, at the transcriptional level in a mitotically heritable manner, i.e., epigenetically. Studies have identified the polycomb histone H3K27 methyltransferase Ezh2 as a key repressor of Kit transcription in both ICC-SC and post-ICC. In-vivo genomic deletion of Ezh2 resulted in stimulation of ICC (re-)differentiation from ICC-SC and post-ICC. In the GIST lineage, KIT blockade by imatinib or other means can also lead to the emergence of KIT\textsuperscript{low−} cells. These KIT\textsuperscript{low−} states permit GIST survival in the presence of continuing KIT blockade. Current studies aim to identify the mechanisms responsible for the epigenetic repression of KIT in imatinib-treated GIST to facilitate re-sensitization to imatinib by pharmacological targeting of the epigenetic regulator(s) involved.
Smooth muscle genome and transcriptome browsers: enabling the identification of novel SIP markers

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Genome-scale expression data on absolute numbers of gene isoforms offer essential clues for cellular functions and biological processes. Gastrointestinal motility is regulated by smooth muscle cells (SMC) closely contacted with interstitial cells of Cajal (ICC) and fibroblast-like PDGFRα+ cells, forming an electrical syncytium (SIPs cells). To uncover genetic identifies and cellular functions of SIPs cells, we isolated these cell populations from mouse small intestine and colon, obtained the transcriptome for each type of cells, and built Smooth Muscle Transcriptome and Genome Browsers. To our knowledge, this is the first genetic resource providing a comprehensive reference for all transcriptional variants expressed in these unique gastrointestinal cell populations. Integration of these data with the UCSC genome browser (called UCSC Smooth Muscle Genome Browser) revealed novel cell-specific genes and unique cellular and biological functions of these cells in gastrointestinal physiology. Furthermore, we built Smooth Muscle Transcriptome Browser that offers references with expression profiles for all expressed genes. Our Smooth Muscle Genome and Transcriptome Browsers bring new insight into the alternative expression of genes in different types of SIPs cells and provides references for future functional studies.
Emergent properties of slow waves in the stomach

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The first recordings of gastric slow waves (SW’s) were performed over 90 years ago (Alvarez & Mahoney, 1922) allowing the basic waveform to be described. Since then, a wealth of information regarding cell types and ionic currents involved in the generation and propagation of SW’s has been generated. Some of the first experiments designed to examine the spatial spread of gastric SW’s were performed by Kent Sanders in the 1980’s using multiple electrodes. More recent experiments with fluorescent Ca2+ indicators and arrays of extracellular electrodes, have purportedly characterized SW propagation in ICC-MY - however the ability to resolve SW’s at cellular resolution has not been achieved. Over the last 2 years, using transgenic mice in which the genetically encoded Ca2+ sensor (GCaMP3) can be induced specifically in ICC, we have visualized SW’s in intact and flat-sheet stomach preparations and have characterized how cell-to-cell communication affects their generation and propagation. The most surprising and consistent observation from this work is that the majority of SW’s propagate as “spirals”. This has far-reaching implications for how we presume smooth muscle is activated by SW wave fronts. Spontaneous and pharmacologically-induced disruptions of cell-to-cell communication produce extremely complex patterns of SW’s including multiple initiation sites, unstable velocities and directions of propagation and spatial asymmetries that likely impair gastric mixing and emptying. So finally, some 30 years after Kent’s initial studies, we are able to visualize SW’s directly in ICC-MY at µm resolution, allowing the full complexity of how different patterns of SW’s underlie normal and abnormal mixing and emptying in the intact stomach.
Ca\textsuperscript{2+} entry into neurons is facilitated by cooperative gating of clustered Cav1.3 channels

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Cav1.3 calcium channels play a critical role in regulating excitability in many central neurons. As is the case for all voltage-gated channels, it is widely assumed that individual Cav1.3 channels behave independently with respect to voltage-activation, open probability, and facilitation. Here, we report the results of super-resolution imaging, optogenetic, and electrophysiological measurements that refute this long-held view. We found that Cav1.3 channels in hippocampal neurons associate in functional clusters of two or more channels that open cooperatively, facilitating Ca\textsuperscript{2+} influx. Cav1.3 channels are coupled via a C-terminus-to-C-terminus physical interaction that requires binding of the incoming Ca\textsuperscript{2+} to calmodulin (CaM) and subsequent binding of CaM to the pre-IQ domain of the channels. Physically coupled channels facilitate Ca\textsuperscript{2+} currents as a consequence of their higher open probabilities, leading to increased firing rates in hippocampal neurons. We propose that cooperative gating of Cav1.3 channels represents a new general mechanism for the regulation of Ca\textsuperscript{2+} signaling and electrical activity in neurons.
Sensing and Electrical Signaling by Kir Channels in the Brain Endothelium: “The Shadow Nervous System”.

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Brain energy substrates are extracted from the blood on an as-needed basis, and to ensure that the metabolic requirements of neurons are consistently satisfied, local cerebral blood flow (CBF) is increased within seconds of a rise in neuronal activity. Pial arteries on the brain's surface give way to a limited number of penetrating parenchymal arterioles which in turn feed a vast capillary network, with capillary endothelial cell (EC) density matching neuronal density on a ~1:1 ratio in cortical structures. This angioarchitecture implies that capillary ECs play an essential role in the signaling mechanisms controlling CBF, in particular in communicating the occurrence of neuronal activity from deep within the cortex to upstream arterioles and arteries, which can dilate to deliver more blood into the local vascular network. However, little is known of the environmental sensing or signaling properties of native brain capillaries, or their role in CBF regulation. We recently discovered that capillaries are capable of detecting increases in external K⁺—which is a feature of neuronal activity—through Kir channels. A rise in K⁺ initiates a Kir channel-mediated regenerative hyperpolarization that rapidly propagates to upstream arterioles, driving vasodilation and producing hyperemia in the capillary bed. Our findings reveal that brain capillaries constitute an active sensory web, converting changes in the external milieu into rapid ‘inside-out’ electrical signals that regulate blood flow into the brain. In the context of these results, brain capillaries can be viewed as a series of electrical wires—or a nervous system within the nervous system—facilitating the long distance communication of neuronal activity to the surface vasculature.
Interstitial cells in urethral smooth muscle

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The smooth muscle layer of the urethra generates spontaneous myogenic tone that makes a major contribution to urinary continence. Urethral tone is associated with the occurrence of spontaneous transient depolarisations (STDs) and slow waves (Hashitani et al., 1996). Studies from our laboratory highlighted a role for a specialised population of interstitial cells (IC) in the initiation of this activity. Urethral IC possess an electrical pacemaker mechanism characterised by rhythmic activation of Ca2+-activated Cl- channels (CACCs) leading to spontaneous transient inward currents (STICs) under voltage clamp and spontaneous transient depolarisations (STDs) under current clamp conditions (Sergeant et al., 2000). ANO1, which is known to encode CACCs in a range of cell types, including interstitial cells of Cajal in the GI tract (Sanders et al., 2012) is highly expressed in urethral smooth muscle and both STICs and STDs in freshly isolated urethral IC are inhibited by the novel ANO1 inhibitors, T16AinhA01 and CACCinhA01. These agents also reduce spontaneous and nerve-evoked contractions of rabbit urethral smooth muscle, suggesting that ANO1 channels are important regulators of urethral tone.

Simultaneous recordings of STICs and intracellular Ca2+ in freshly isolated IC revealed that STICs were activated by propagating Ca2+ waves. Inhibiting Ca2+ influx by removing extracellular Ca2+ or by applying the reverse mode NCX blockers KBR-7943 or SEA0400, reduced the propagation of Ca2+ waves and unmasked localized Ca2+ events. Thus it was concluded that Ca2+ influx via reverse mode NCX was involved in the propagation of Ca2+ waves, but not their initiation. Similar results were achieved with the IP3R blocker, 2-APB, suggesting that IP3Rs were also involved in the propagation of Ca2+ waves rather than their initiation. On the other hand, the RyR blockers tetracaine and ryanodine abolished all activity suggesting that RyRs were responsible for initiation of Ca2+ waves in urethral IC. In summary, pacemaker activity in urethral IC is brought about by activation of ANO1 channels by underlying Ca2+ waves that result from a complex interplay between Ca2+ release from intracellular stores, via RyRs and IP3Rs and Ca2+ influx via reverse mode NCX.

Sanders et al., 2014 Physiol Rev. 2014 Jul;94(3):859-907
Calcium signaling in Interstitial Cells

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Interstitial cells of Cajal (ICC) are essential regulators of smooth muscle excitability and motility in the gastrointestinal (GI) tract. ICC located in the deep muscular plexus (ICC-DMP) are closely associated with varicosities of enteric motor neurons and are suggested to mediate neuronal responses to smooth muscle. The ability of ICC-DMP to act, as neuromodulators is believed to rely on the firing of intracellular Ca\(^{2+}\) signals, however, to date these signals have not yet been described in detail. To address this, we have monitored Ca\(^{2+}\) activity in ICC-DMP using confocal imaging. We revealed spontaneous Ca\(^{2+}\) transients in ICC-DMP and we characterized the nature of these Ca\(^{2+}\) signals and examined their source in situ.

ICC-DMP fired a range of Ca\(^{2+}\) events from discrete, localized Ca\(^{2+}\) signals to propagating events that could spread over entire regions of the cell (0.27 to 57 µm). These Ca\(^{2+}\) events also demonstrated variable frequency and amplitude. Ca\(^{2+}\) events fired stochastically and there was no correlation between the firing of Ca\(^{2+}\) events within a single cell or between multi ICC-DMP. Ca\(^{2+}\) transients in ICC-DMP were suppressed during nerve stimulation and these Ca\(^{2+}\) transients were apparent in the presence of LNNA.

This study reveals spontaneous Ca\(^{2+}\) activity in ICC-DMP and describes the nature of the localized-stochastic Ca\(^{2+}\) transients. Activation of Ca\(^{2+}\) release from the ER is likely to be the signaling mechanism in ICC-DMP responsible for transduction of enteric neuronal input in small bowel muscles. Excitatory and inhibitory neurotransmitters can modulate Ca\(^{2+}\) firing in ICC-DMP. These findings provide information on Ca\(^{2+}\) handling at the sub-cellular level and link cellular Ca\(^{2+}\) release mechanisms to the genesis of spontaneous Ca\(^{2+}\) activity in the ICC-DMP.
Combining genetic and advanced optical approaches to monitor nerve activity in the intestinal wall

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Gastrointestinal functions, such as motility, absorption and secretion, and to some extent host defense are controlled by different cell types of cells in the gut wall. Some of these are organized in concentric layers (epithelium, muscle, ICC, nerves…) while others (like some glial cells and immune cells) are more scattered and tend to migrate more within the wall. Several techniques have been used to understand how these cells all connect together to form a functional units. Imaging techniques have proven to be powerful tools in order to link spatial organization with functionality, as they can, in contrast to electrophysiological techniques, simultaneously report from many different cells. Synthetic activity reporters (voltage sensors, membrane labels and Ca indicators) have been successfully used to monitor activity in individual neurons, glia, interstitial cells of Cajal and muscle cells. Dye loading has however the disadvantage that tissue dissection or local injections are required to assure that dyes make it into the relevant cells. With the advent of genetically encoded fluorescent proteins and reporter systems and especially the improved control over their expression (inducibility and promoter driven cell specificity) it has become feasible to express reporter molecules specifically in time and space in tissues that do not need to be peeled. Genetically encoding of fluorescent proteins do not only overcome the problem of dissection, but also produce high contrast which in combination with advanced optical techniques (2-photon, spinning disk confocals) yields high quality information from deeper layers. Since the integrity of the tissue can be maintained this approach allows one to study cell activity in undamaged integrated circuits. We have used the genetically encoded GCaMP3 reporter to study neurons and glial cells in intact intestines to understand how they develop and connect to produce functional units. Moreover since the mucosa can remain in place, this technique can be used to investigate what specific populations of neurons respond to the luminal applications of drugs and food related molecules. During this presentation, we will show evidence that indeed the combination of genetic and advanced optical tools is an essential next step to interrogate nerve circuits in the intact intestinal wall.
Role of Post-Stimulus Responses (3rd component) in the colonic migrating motor complex.

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Gastrointestinal (GI) motility patterns are accomplished, in part, by coordination of contractions by enteric motor neurons. Both excitatory and inhibitory motor neurons are intrinsic to the enteric nervous system and outputs are coordinated to generate specific motor patterns in GI organs. In the colon a prominent motor pattern, known as colonic migrating motor complexes (CMMC) provides propulsive forces to move fecal material in the anal direction. CMMC are found in all mammalian species, including humans, and the mechanism for this motor pattern has been discussed for many years. At present CMMC are thought to be based on the peristaltic reflex in which local stimulation can generate an ascending contraction and a descending inhibition. CMMC result from migration of this complex from oral to anal sites.

In fact there is a 3rd phase of the peristaltic reflex that is poorly understood and infrequently mentioned in reviews on peristalsis. In many parts of the gut, including the colon, the response to electrical field stimulation (EFS) is followed by a post-stimulus response (also referred to as rebound excitation). EFS, however, does not clearly simulate the peristaltic reflex because in the physiological response motor nerves are activated with both spatial and temporal specificity to generate ascending and descending components of the response. Nevertheless, in many published records of peristaltic responses, periods of stimulation are followed by post-stimulation responses (PSR).

PSR is actually an extremely important component of the propagating contractile event known as CMMC. CMMC are somewhat reduced, but not blocked by antagonists to excitatory motor neurotransmitters, but CMMC are eliminated by inhibition of nitric oxide synthase including nNOS KO mice in the proximal colon or antagonism of P2Y1 receptors including P2TY1 KO mice in the distal colon. These data suggested that inhibitory responses, that are essential for PSR, are the propagating component of CMMC and PSR is the source of much of the propulsive force developed during CMMC. Further, we dissected the role of the cell-specific contributions of ICC, platelet-derived growth factor α receptor positive (PDGFRα+) cells and smooth muscle cells to PSR. These data provided a novel explanation of why slow transit constipation develops when ICC are lost or defective in the colon. (Supported by NIH/NIDDK P01-DK-41315)
Biomagnetic field measurement of propagating electric activity in musculatures of functional syncytium using amorphous metal-based magneto sensor

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Excitable cellular organs and tissues employ propagating electric current to execute their functions. In the heart, pacemaker potentials are amplified and forwarded from the atrium to ventricle muscle, thus synchronizing heart beats. Also, in the gut, coordinated motions, such as peristalsis, require electric activities to organize smooth muscle segments. Magnetic detection would provide a non-invasive and aseptic estimate of their functional states. To date, SQUID and atomic magnetometers, such as magnetoencephalography and magnetocardiography, have been used to measure biomagnetic fields. However, because these magnetometers require difficult conditions for small labs and hospitals, biomagnetic field measurement has not become a popular method.

We thus applied an amorphous metal-based gradio-magneto sensor, which is operated at room temperature without any magnetic shield, in order to measure biomagnetic vector fields in the musculatures of functional syncytium. Smooth muscles were isolated from gastrointestinal and urinary systems of guinea-pigs. Also, right ventricular musculature along with the right atrium was isolated from the heart of mice. The gradio-magneto sensor was fabricated with a single amorphous metal wire with a pair of transducer coils in both ends. The performance of differentiation was largely improved, resulting in well-balanced induction potentials in the paired coils. The distance between the sample and the wire was ~1 mm in 'normal' measurements. When musculature samples were incubated at 32-35 °C, spontaneous magnetic waves of up to several nT were recorded. The frequency and time course of biomagnetic waves reflected the cellular tissue-dependent excitability. For instance, in stomach samples only several magnetic waves occurred in a minute, while in cardiac samples magnetic transients occurred at 1-2 cycles/s at the beginning of recording after preparing samples. Since the thickness of right ventricular musculature was < 1 mm in mice, it is considered that our sensor would be able to measure magnetic activity of cardiac muscle sheets in regenerative medicine, if available. In musculatures, biomagnetic fields were further characterized by changing the direction and distance of the sample. The polarity of magnetic waves was altered depending on the relative angle of the muscle layer and magneto sensor, indicating the existence of propagating intercellular currents. Also, the magnitude of biomagnetic waves was rapidly decreased by a separation of 1-2 mm between the sample and the sensor, reflecting bioelectric circuits constructed by a propagating intercellular current and extracellular return currents separated by a small distance.

We also hope to show some results using our latest amorphous metal-based sensors.
Interactions of spermine with TRPC4

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Spermine is one of endogenous polyamines and positively charged (4+) at physiological pH. Therefore, it is suggested to participate in many cellular processes through their binding to various negatively charged molecules such as DNA, RNA, phospholipids, etc. The role of spermine in the control of gastrointestinal motility has been poorly investigated. Spermine added extracellularly to intestinal and vascular smooth muscle cells causes relaxation through inhibition of Ca2+ channel activity, but the detailed inactivation mechanisms are still unclear. The muscarinic receptor-mediated cationic current (mICAT) is cationic currents carried through transmembrane channels with less selectivity for Na+, K+, Cs+ and Ca2+. The cationic channel activation, of mICAT, is taken as the primary and most important mechanism underlying the contractile response, for it results in increased Ca2+ influxes. PAs inhibit mICAT in single guinea-pig ileal myocyte which TRPC4 and TRPC6 channels are responsible for. TRPC4 underlies 70-80% of the mICAT, however, the effects of PAs on TRPC4 have not been investigated.

In this study, we performed conventional whole-cell recordings in HEK293 cells transfected with mTRPC4β, changing the concentration of intracellular spermine. Spermine inhibited mTRPC4 in dose-dependent manner. In deletion mutant model, Δ(720-740) was less inhibited by spermine. To find out more specific spermine-binding sites, cytosolic aspartate/glutamate residue between 720 and 740 of TRPC4 were substituted with noncharged alanine. Mutants with E728A showed relatively bigger current at positive potential. D629 residue which is known as Mg2+ binding site was substituted with alanine; TRPC4 was blocked only in half by internal spermine. However, the current of D629A/Δ(720-740) was greatly suppressed by spermine. In cultured interstitial cell of Cajal, pretreatment of spermine blocked the membrane depolarization induced by external CCh, and repolarized CCh-induced membrane depolarization.

In conclusion, spermine may suppress gastrointestinal motility by inhibition of TRPC4 and amino acid (720-740) is thought to be one of the binding sites.
The expression of small conductance calcium-activated potassium (SK3) channel is specifically concentrated at interstitial cells of the spaces between detrusor smooth muscle cells in the guinea pig urinary bladder.

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Aims of study
Small conductance calcium-activated potassium channels consist of three subtypes, SK1, SK2 and SK3. Above all, SK3 is thought to be involved in the suppression of detrusor smooth muscle excitability and contractility. Recently, it was reported that SK3 transcript and protein in the bladder wall are highly expressed in PDGFRα+ interstitial cells and these cells may particpate in the control of detrusor excitability during the filling phase. In this study, we investigated the distribution and structural feature of SK3 expressing interstitial cells in the bladder wall.

Materials and methods
Male guinea pigs weighting 500 to 700 mg were anesthetized and urinary bladder was extirpated and opened longitudinally from neck to dome. The specimen was pinned to a Sylgard dish and fixed in Zamboni solution for 5hr. After several rinses, the urinary bladder was incubated in the 0.1 M phosphate buffer containing 30% sucrose for cryoprotection. Five μm bladder wall section and 50 μm detrusor layer plate (semi-whole mount preparation) was made by cryostat. These sections were processed immunohistochemistry and examined by confocal microscopy. These sections were labeled with anti-SK3 antibody, and also double-immunolabeled with antibodies to αSMA, vimentin, platelet-derived growth factor receptor-α (PDGFRα), tyrosine hydroxylase (TH) and vesicler acetylcholine transporter (vAchT), respectively.

Results
The expression of SK3 was observed in interstitial cell in the detrusor layer. SK3 immunolabeled cell was not observed in the lamina propria. SK3 immunolabeled interstitial cell had a branched stellate or spindle morphology and connected each other like a mesh network. This network existed in the spaces between detrusor smooth muscle. Double labeling revealed that SK3 immunolabeled interstitial was co-labeled by vimentin and PDGFRα. The axon immunolabeled by TH and vAchT ran the side of this network.

Conclusion
SK3 positive interstitial cells are distributed in the detrusor smooth muscle layer. These cells are subfamily of mesenchymal cells, and might be involved in the regulation of motility of the urinary bladder detrusor smooth muscle. The structural feature (closeness to detrusor smooth muscle) of those SK3 positive interstitial cells might be advantageous to modulate the electrical activity of the urinary bladder detrusor smooth muscle.
In Vivo Enteric Nervous System Stem Cell Transplantation Restores Functional Responses In nNOS Deficient Mouse Colon

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Enteric neural stem cells (ENSC) have recently been identified as a possible treatment for enteric neuropathies following successful colonization of recipient gut after transplantation. However, the ability of ENSC to rescue pathophysiological conditions remains unclear. Interestingly, loss of neuronal subtypes including neuronal nitric oxide synthase (nNOS) has been implicated in enteric neuropathies. nNOS-/ mice display slow colonic transit providing a model to test ENSC rescue in a pathological setting.

Our aim was to assess the functional integration of transplanted ENSC within recipient nNOS-/ colon.

Donor ENSC were obtained from Wnt1-cre;YFP transgenic mice for fluorescent labeling and fate mapping of cells. Integration and functionality were assessed using immunolabeling and in vivo and organ bath physiology.

At 1 month, transplanted cell networks were identified in recipient nNOS-/ colon with a maximal spread of 6.6±0.8mm2; n=3. YFP+/nNOS+ neurons were identified and transcriptional analysis showed upregulation of nNOS in recipient nNOS-/ colon. In vivo analysis showed significant recovery in total gastrointestinal (GI) transit time in transplanted nNOS-/ (114.8±2.9 mins; n=5), similar to wildtype C57 controls (117.4±2.6 mins; n=5), compared to nNOS-/ non-transplanted (177.4±8.6 mins; n=5). This was combined with a significant increase in faecal output in transplanted nNOS-/ (44.9±5.3mg/h; n=5) versus non-transplanted nNOS-/ (21.±4.1mg/h; n=5).

In NANC (non-adrenergic non-cholinergic) conditions, organ bath physiology revealed significant increases in stimulation-induced relaxation (Area under curve; AUC) in transplanted nNOS-/ (-0.82±0.12g.s; n=3) compared with non-transplanted nNOS-/ (-0.26±0.15g.s; n=3).

Our experiments show integration of transplanted ENSC and for the first time provide restoration of function, at the organ level, in a pathological GI disease model.
Modeling of stochastic behavior of pacemaker potential in interstitial cells of Cajal

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It is widely accepted that interstitial cells of Cajal (ICCs) generate pacemaker potentials to propagate slow waves along the whole gastrointestinal tract. Previously, we constructed a biophysically based model of ICCs in mouse small intestine to explain the pacemaker mechanism. Our previous model, however, could not explain non-uniformity of pacemaker potentials and random occurrence of unitary potentials, thus we updated our model. The inositol 1,4,5-trisphosphate (IP3)-mediated Ca2+ mobilization is a key event to drive the cycle of pacemaker activity and was updated to reproduce its stochastic behavior. The stochasticity was embodied by simulating random opening and closing of individual IP3-mediated Ca2+ channel. The updated model reproduces the stochastic features of pacemaker potentials in ICCs. Reproduced pacemaker potentials are not uniform in duration and interval. The resting and peak potentials are -75.5±1.1 mV and -0.8±0.5 mV, respectively (n=55). Frequency of pacemaker potential is 14.3±0.4 min-1 (n=10). Width at half-maximal amplitude of pacemaker potential is 902±6 ms (n=55). There are random events of unitary potential-like depolarization. Finally, we compared our updated model with a recently published model to speculate which ion channel is the best candidate to drive pacemaker depolarization. In conclusion, our updated mathematical model could now reproduce stochastic features of pacemaker activity in ICCs.
Angiotensin II/AT1 signaling upregulated in gastric antrum of STZ-induced diabetic mice

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Objectives: Angiotensin II (AngII), as local hormone in gastrointestinal tract, plays an important role in regulation of smooth muscle motility and smooth muscle proliferation. However, it is not clear whether Ang II is involved in diabetics-induced loss of gastric ICC. The present study was designed to investigate the relationship between diabetics-induced loss of gastric ICC and Ang II signaling pathway upregulation in streptozotocin (STZ)-induced diabetic mice.

Methods: The angiotensin converting enzyme (ACE) expression in gastric mucosa and Ang II type 1 receptor (AT1R) expression in gastric smooth muscle tissue were observed by western blot method in normal and diabetic mice. The effect of Ang II on the mSCF expression of gastric smooth muscle cells was investigated by western blot. Isometric muscle recording was used to assess contraction of gastric circular muscle strip. Cell viability was assessed using cell counting kit-8 (cck-8).

Results: The results indicated that the protein expressions of c-Kit and membrane-bound stem cell factor (mSCF) in gastric smooth muscle were decreased in STZ-induced diabetic mice. However, the expressions of AT1R in gastric smooth muscle and ACE in gastric mucosa were increased. The effect of Ang II on the gastric smooth muscle tonic contraction was potentiated in diabetic mice. Ang II increased the mSCF expression in cultured gastric smooth muscle cells (GSMCs) and promoted cell proliferation in cultured GSMCs, and stimulated the phosphorylation of Akt-Ser473 in a dose-depend manner. Ang II-induced increase of mSCF and phosphorylation of Akt-Ser473 were reduced by specific inhibitors ZD7155 (an AT1R antagonist) and LY294002 (a PI3-kinase inhibitor).

Conclusions: These results suggest that up-regulation of Ang II/AT1 signaling may be a protective factor against ICC loss which is mediated by PI3K/Akt signaling pathway in STZ-induced diabetic mice.

Keywords: Angiotensin II; Diabetic gastroparesis; Interstitial cell of cajal (ICC); Membrane-bound stem cell factor (mSCF); Gastric smooth muscle cells (GSMCs); Phosphoinositide -3 kinase (PI3k)

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The excitatory effect of hydrogen sulfide on gastric fundus smooth muscle tension in mice

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Objectives: Hydrogen sulfide (H2S) has been proposed as a gasotransmitter similar to nitric oxide (NO) and carbon monoxide (CO). It has been proved to play an important biologic roles in the regulation of gastrointestinal motility. The present study was to explore the effect of hydrogen sulfide on smooth muscle motility in the gastric fundus.

Methods: The immunocytochemistry technique was used to characterize the expression of cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) in cultured smooth muscle cells from the gastric fundus. The smooth muscle motility was recorded by isometric force transducers. Intracellular recordings, whole-cell patch clamp technique and calcium imaging were used to investigate the mechanism by which hydrogen sulfide regulates gastric fundus motility.

Results: We found that both CBS and CSE were expressed in the cultured smooth muscle cells from the gastric fundus and that H2S increased the smooth muscle tension of the gastric fundus in mice at a low concentration. In addition, AOAA, a CBS inhibitor, reduced the tension, whereas L-NAME, a nonspecific nitric oxide synthase (NOS), increased the tension. The AOAA-induced relaxation was significantly recovered by H2S, and the NaHS-induced increase in tonic contraction was blocked by 5 mM 4-aminopyridine (4-AP) and 1 μM nicardipine. NaHS significantly depolarized the membrane potential and inhibited the voltage-dependent potassium currents. Moreover, NaHS increased L-type Ca2+ currents and caused an elevation in intracellular calcium ([Ca2+]i).

Conclusions: These findings suggest that H2S may be an excitatory modulator in gastric fundus in mice. The excitatory effect is mediated by voltage-dependent potassium and L-type calcium channels.

Keywords: Hydrogen sulfide (H2S); cystathionine β-synthase (CBS) ; cystathionine γ-lyase (CSE); gastric fundus

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Abstract #38
H2S enhances the tension of gastric fundus smooth muscle via inhibition of PI3K/Akt/eNOS pathway

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H2S has been considered to be the third gaseous signaling molecule, following nitric oxide (NO) and carbon monoxide (CO). It has been confirmed that endogenous H2S can be produced in gastrointestinal tissue. Therefore, it is crucial to clarify the effect of H2S on the regulation of gastrointestinal motility.

In our study, immunohistochemical results show that the H2S synthase protein CBS and CSE, NO synthase protein eNOS and nNOS are expressed in cultured gastric smooth muscle cells in mice. The electrochemical method is used to detect the generation of H2S and NO in smooth muscle tissue. We evaluate the effect of NaHS and SNP on the tension of strips in mice smooth muscle. NaHS increases the tension of gastric fundus, SNP decreases the tension of gastric fundus; NaHS can reverse the effect of SNP. HNO decreases the tension of gastric fundus. NO synthase inhibitor, L-NAME, can reduce the effect of NaHS -increased tension. It is found that the NO generation can be significantly increased by AOAA. Western blot method shows that NaHS can reduce the phosphorylation of eNOS synthase at serine 1177 site, the phosphorylation of Akt at threonine 308 site and the phosphorylation of Akt at serine 473 site; PI3K inhibitor, LY294002, can block NaHS-induced decrease of phosphorylation of eNOS synthase at serine 1177 site; AOAA can increase the phosphorylation of eNOS synthase at serine 1177 site, the phosphorylation of Akt at threonine 308 site and the phosphorylation of Akt at serine 473 site; Given CBSsiRNA interference for 48 hours, the results show that CBSsiRNA can increase the phosphorylation of eNOS synthase at serine 1177 site, the phosphorylation of Akt at threonine 308 site and the phosphorylation of Akt at serine 473 site; CBSsiRNA interference increases the total eNOS protein expression, but it does not change the total amount of Akt kinase significantly.

It is concluded that H2S and NO synthase are expressed in the gastric fundus smooth muscle, and the cells can generate H2S and NO stably; H2S can increase gastric tension and NO can reduce gastric tension; H2S decreases the eNOS enzyme activity via PI3K / Akt pathway thereby increases muscle tension.

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A novel stromal cell network visualized by FIB/SEM tomography

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Fibroblasts or stromal cells are a dominant cell type in the connective tissue of various organs, and are connected to one another by gap junctions to form cellular networks. However, it is not easy to understand the whole shape of these cells nor their spatial relationships by either light microscopy because of thinness of their processes or by electron microscopy as these processes extend well beyond a single thin section. The recent development of a novel electron microscopic technology, FIB/SEM tomography, enables us to establish the three dimensional (3D) ultrastructure of these cells. Using FIB/SEM, we can obtain over 1000 serial images which are then aligned and the 3D cell structures within reconstructed using computerized segmentation instrumentation.

In the present study, we have concentrated on visualizing stromal cells in the smooth muscular tissue of the seminal vesicle. We report here that stromal cells, or fibroblasts, which have previously been considered as bipolar or stellate in shape by their long thin cytoplasmic processes in two-dimensional single sectional images, were in fact cells with broad, thin and wavy “sheet-like” cytoplasmic processes. They approached one another and appose each other at several points along their edge to form an incomplete wall in the connective tissue beneath the epithelial cell layer. In the muscular layer, stromal cells constructed a honeycomb-like structure that encircled each smooth muscle bundles. Thus these stromal cell structures seem to create spatial divisions to form functional units within the tissues.
The role of Ca2+ influx in spontaneous Ca2+ wave propagation in interstitial cells of Cajal from the rabbit urethra

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Interstitial cells of Cajal (ICC) are putative pacemaker cells in the rabbit urethra. Pacemaker activity in ICC results from spontaneous propagating Ca2+ waves that are modulated by [Ca2+]o and whose propagation is inhibited by inositol tri-phosphate receptor (IP3R) blockers. The purpose of this study was to further examine the role of Ca2+ influx and Ca2+ release in the propagation of Ca2+ waves. Intracellular Ca2+ was measured in fluo-4 loaded ICC using a Nipkow spinning disc confocal microscope at fast acquisition rates (50 fps). We identified previously undetected localised Ca2+ events originating from ryanodine receptors (RyRs). Inhibiting Ca2+ influx by removing [Ca2+]o or by blocking reverse mode sodium/calcium exchange (NCX) with KB-R 7943 or SEA-0400 abolished Ca2+ waves, while localised Ca2+ events persisted. Stimulating RyRs with 1mM caffeine restored propagation. Propagation was also inhibited when Ca2+ release sites were uncoupled by buffering intracellular Ca2+ with EGTA-AM. This was reversed when Ca2+ influx via NCX was increased by reducing [Na+]o to 13 mM. Low [Na+]o also increased the frequency of Ca2+ waves and this effect was blocked by tetracaine and ryanodine but not 2-aminoethoxydiphenyl borate (2-APB). RT-PCR revealed that isolated ICC expressed both RyR2 and RyR3 subtypes. We conclude: i) RyRs are required for the initiation of Ca2+ waves, but wave propagation normally depends on activation of IP3Rs; ii) under resting conditions, propagation by IP3Rs requires sensitisation by influx of Ca2+ via reverse mode NCX; iii) propagation can be maintained by RyRs if they have been sensitised to Ca2+. 
ANO1 and CavL activity underlies slow wave and tone generation in the internal anal sphincter.

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Objectives: The internal anal sphincter (IAS) is a phasic muscle which develops tone; a property that is essential for maintaining resting anal pressure. Alterations in anal pressure contribute to fecal incontinence and other defecatory disorders. The mechanisms underlying slow wave (SW) and tone generation in the IAS are still largely unknown. Previously we have shown that the IAS is divided into a number of electrically isolated muscular bundles each containing interstitial cells of Cajal (ICC). SW are present in each muscular bundle but the SW of adjacent muscle bundles are not synchronized (Hall et al, 2014). The present study further examines the role of ICC in tone generation by investigating the hypothesis that tone results from an increase in L-type calcium channel (CavL) activity in smooth muscle occurring in response to depolarization via SW initiated by calcium activated chloride channels (ANO1) in ICC.

Methods: Quantitative PCR was used to compare Ano1 and Cacna1c gene expression levels in FACS sorted ICC and smooth muscle cells (SMC) dispersed from the IAS of KitcopGFP/+ and smMHCcre-egfp mice. Protein expression of ANO1 was examined in cryostat sections from wild-type and Ano1eGFP mouse whole tissue preparations using dual labeling immunohistochemical techniques. The functional role of ANO1 and CavL in SW and tone generation in whole tissues was also examined by recording membrane potential with intracellular microelectrodes and contractile activity with force transducers.

Results: Ano1 gene expression was 11x greater in ICC than in SMC. Likewise, immunohistochemical studies revealed that ANO1 was predominantly localized in Kit+ ICC. Cacna1c gene expression was 6x greater in SMC than in ICC. The ANO1 antagonists CaCCinh-A01 and T16Ainh-A01 (10 µM) and the CavL antagonist nifedipine (0.1 µM) almost completely eliminated SW and tone.

Conclusion: These data support a central role for ANO1 and CavL in the generation of SW and tone in the IAS. They further identify ICC as the predominant cell type expressing ANO1 and therefore the most likely candidate for SW generation. We propose that SW originate in ICC via the activation of ANO1 followed by transmission to SMC where CavL amplifies and conducts SW to adjacent SMC as well as delivering calcium to the myofilaments. As previously described, the asynchronous nature of SWs in adjacent muscular bundles is a key factor translating phasic SW activity into tone in the intact IAS.

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Aqueous extracts of Liriope platyphylla induced significant laxative effects on normal and two models of experimental constipated mice

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Background/Aims
Constipation is a worldwide functional gastrointestinal disorder. Suaeda asparagoides (Miq.) has been used as Korean folk herbal medicine for the treatment of functional gastrointestinal disorders. The aim of this study was to evaluate the laxative effects of Suaeda asparagoides on loperamide- and clonidine- induced constipation mice.

Methods
ICR mice were administrated intragastrically (i.g.) by gavage with water extracts of the root, stem, branch, leaf and shoot of SA while the control mice were received saline. The constipated mice were induced by two types of drugs, loperamide (5 mg/kg BW, i.g.) and clonidine (200 μg/kg BW, i.g.), after SA aqueous extracts treatment while the control mice were received saline. Number, weight and water content of feces were subsequently measured.

Results
The extracts of the leaf and shoot of SA significantly increased the number and weight of feces in normal mice, but the extracts of other parts of SA did not. The leaf extract showed dose-dependently restored the fecal number and fecal weight in the clonidine-induced constipation model. Low dose of the shoot extract (10 mg/kg) significantly restored the fecal number and fecal weight in the clonidine-induced constipation model. Low dose of leaf extract (10mg/kg) significantly restored the fecal number and fecal weight in the loperamide-induced constipation model. The shoot extracts significantly restored the fecal number and fecal weight in the loperamide-induced constipated mice.

Conclusions
These results suggest that Suaeda asparagoides has a potential to be used as a hydrophilic laxative for constipation.
Development of coordinated motor patterns, ICC, and enteric neurons in zebrafish

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Coordinated muscle contractions are regulated by enteric neurons, interstitial cells of Cajal (ICC), and smooth muscle cells in the gastrointestinal (GI) tract. Regulatory inputs external to the GI tract also influence smooth muscle motor patterns. Therefore it is important to assess the functional role for each regulatory component in intact tissues in a physiological setting. The zebrafish model system offers this opportunity because larvae are transparent and GI function can be directly observed. ICC development can be delayed or eliminated using a tyrosine kinase inhibitor or a morpholino oligonucleotide designed to block Kit expression. The role of enteric neurons can be examined using the Sox10 mutant zebrafish. Development of coordinated motor patterns and GI transit is dependent on ICC and enteric neurons. Specific effects for ICC and enteric neurons will be presented using spatiotemporal maps and GI transit data.
Characterization of spontaneous Ca2+ transients in interstitial cells of Cajal located in the deep muscular plexus of the murine small bowel

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Introduction: Interstitial cells of Cajal (ICC) are essential regulators of smooth muscle excitability and motility in the gastrointestinal (GI) tract. ICC located in the deep muscular plexus (ICC-DMP) are closely associated with varicosities of enteric motor neurons and are suggested to mediate neuronal responses to smooth muscle. The ability of ICC-DMP to act as neuromodulators is believed to rely on the firing of intracellular Ca2+ signals, however, to date these signals have not yet been described in detail.

Aim: To characterize Ca2+ transients in ICC-DMP and examine their source.

Methods: Jejunum tissues were taken from c-kit-iCre mice crossbred with GCaMP3 mice. Ca2+ transients in ICC-DMP were recorded in situ, using confocal imaging with a 60x magnification.

Results: ICC-DMP fired a range of Ca2+ events from discrete, localized Ca2+ signals to propagating events that could spread over entire regions of the cell (0.27 to 56.9 μm). These Ca2+ events also demonstrated variable frequency, ranging from 12 to 376 events min⁻¹ with an average of 134.3 ± 15.3 events min⁻¹. Ca2+ events fired stochastically and there was no correlation between the firing of Ca2+ events within a single cell or between multi ICC-DMP. Pretreatment of jejunum tissues with tetrodotoxin (TTX; 1 μM) significantly increased the firing frequency of Ca2+ events in ICC-DMP (82.9 ± 10.37 to 151.3 ± 20 min⁻¹) suggesting their involvement in tonic inhibition. Ca2+ transients in ICC-DMP were not inhibited by Ca2+ free solution (frequency changed from 64.86 ± 9.3 to 66.3 ± 8.1 min⁻¹), indicating that Ca2+ influx is not the major source of these events. Instead, ICC Ca2+ transients appeared to rely on intracellular store release as depletion of intracellular calcium stores by thapsigargin (10 μM) and CPA (10 μM) abolished ICC-DMP Ca2+ transients. Ca2+ transients were also blocked by inositol triphosphate (IP3) receptor antagonists (2-APB 100 μM, xestospongin C 10 μM), and ryanodine (100 μM), indicating that Ca2+ release from the endoplasmic reticulum (ER) store via both IP3Rs and RyRs was necessary for their generation.

Conclusions: This study reveals spontaneous Ca2+ activity in ICC-DMP and describes the nature of the localized-stochastic Ca2+ transients. Activation of Ca2+ release from the ER is likely to be the signaling mechanism in ICC-DMP responsible for transduction of enteric neuronal input in small bowel muscles.
Rho Kinase Regulates Airway Smooth Muscle Contraction by Regulating Actin Polymerization but not Myosin Light Chain Phosphorylation

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Rho kinase (ROK), a target of RhoA GTPase, is known to play a role in the regulation of airway smooth muscle (ASM) contraction. ROK has been proposed as a potential target for asthma therapy. However, the mechanism by which ROK regulates ASM contraction is not fully understood. Studies of several smooth muscles have shown that ROK enhances smooth muscle force by inhibiting myosin light chain phosphatase activity, resulting in an increase in myosin light chain phosphorylation. However, in vitro studies of non-muscle cells have shown that ROK can regulate non muscle cell motility and migration by regulating actin filament dynamics. Actin polymerization plays a critical role in tension development in ASM; and we have previously shown that RhoA regulates ASM contraction through its role in regulating actin polymerization rather than MLC phosphorylation (Zhang, et al. AJP, 2010; Zhang et al, JBC 2012). We hypothesized that ROK may also regulate ASM contractility by regulating actin dynamics.

Canine tracheal smooth muscle strips were attached to force transducers and stimulated with 10-5 M acetylcholine (ACh) for 5 minute either before or after ROK was inhibited in ASM tissues. ROK was inhibited by three approaches; (1) Small molecule ROK inhibitor (H-1152) (2) By transfecting ASM tissues with kinase inactive ROK mutant (ROK K121G) or (3) By transfecting with ROK2 isoform specific ShRNA plasmids and incubating for 2 days for protein expression. Tissues were snap frozen after ACh stimulation and processed for ROK activation, actin polymerization, and MLC phosphorylation assays. Statistical significance was determined using one-way ANOVA.

ROK inhibition by small molecule inhibitor (H-1152), kinase inactive ROK mutant, and ROK2 shRNA significantly reduced ACh induced contractile force in ASM tissues to 48%, 43%, and 45% respectively. All three approaches also completely inhibited ACh stimulated actin polymerization. ACh stimulation caused an increase in phosphorylation of the myosin binding subunit of myosin light chain phosphatase (MYPT) at the Thr696 and Thr853 sites, and these phosphorylations were inhibited by ROK inhibition. However, the increase in MLC phosphorylation induced by ACh stimulation was not significantly affected by ROK inhibition, suggesting ROK regulated MLC phosphatase activity does not have a significant effect on ACh stimulated contraction in ASM. We conclude that ROK regulates force generation in airway smooth muscle primarily through its role in regulating actin dynamics. ROK may regulate critical intermediate steps leading to the catalysis of actin polymerization in ASM.

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Nitric oxide modulates the release of purine neurotransmitters in the murine large intestine

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Introduction
Enteric inhibitory motor neurotransmission in the gastrointestinal tract is mediated primarily by nitric oxide (NO) and purines released from motor nerve terminals. Neurotransmitter release sites in the gut are closely apposed to smooth muscle cells (SMC), interstitial cells of Cajal (ICC) and platelet-derived growth factor receptor α-expressing (PDGFRα+) cells, the SIP syncytium (Koh et al., NMO 2012). ICC appear to be the target for neurally released NO whereas PDGFRα+ cells mediate the responses to purine neurotransmitters. Interestingly, the purinergic component of inhibitory neurotransmission is enhanced in mice with reduced expression of ICC (W/Wv) (Burns et al., PNAS 1996; Sergeant et al., Physiol Genomics 2002). One possibility is that the purine release is augmented in ICC-deficient colons to compensate for diminished nitrergic neurotransmission, but direct quantification of purine release in the W/Wv intestine has not been done previously.

Aims
The present study was undertaken to determine if neural release of purine neurotransmitters, nicotinamide adenine dinucleotide (NAD+), uridine adenosine tetraphosphate (Up4A) and ATP, are altered in colons from W/Wv mice.

Methods
A small-volume superfusion assay and HPLC with fluorescence detection (HPLC-FLD) were used to evaluate spontaneous and nerve-evoked release of NAD+, Up4A, ATP, and metabolites, in colons isolated from C57BL/6 (wildtype) and W/Wv mice. To determine whether modified extracellular catabolism contributes to enhanced extracellular purines in W/Wv colons, degradation of fluorescent substrates 1,N6-etheno-NAD (eNAD), eUp4A and eATP was also quantified.

Results
Spontaneous overflow of NAD+, Up4A and ATP was similar in wildtype and W/Wv colonic smooth muscle, however, nerve stimulation-evoked release was significantly increased in W/Wv colons. The increase in purine release was not attributed to reduced metabolism. Inhibition of action potential firing using the fast Na+ channel blocker, tetrodotoxin, significantly reduced purine release in W/Wv mice indicating release was neuronal. Interestingly, the nitric oxide synthase inhibitor, N(ω)-nitro-L-arginine (L-NNA), and the inhibitor of soluble guanylyl cyclase, 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) , further augmented purine release in W/Wv mice suggesting that NO normally inhibits release of purines in the colon. Moreover, in W/Wv mice the NO donor, (±)-S-Nitroso-N-acetylpenicillamine (SNAP), reduced the release of purines to wildtype levels.

Conclusion
These results suggest that NO presynaptically inhibits release of purine neurotransmitters in the colon via a guanylyl cyclase pathway. Enhanced purinergic signaling provides a safety factor for inhibitory responses to occur in the absence of ICC or when nitrergic signaling is compromised.
THBS4 is a new diagnostic and phenotypic marker for ICC in intestinal injuries

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Previously, we isolated primary interstitial cells of Cajal (ICC) from the jejunum and colon of mice, identified the genome-wide transcriptome, and built an ICC transcriptome browser. Using this browser we analyzed the transcriptome and identified Thrombospondin 4 (THBS4), an extracellular glycoprotein, as a new marker for ICC.

We used an intestinal partial obstruction (PO) model of mice that express GFP exclusively in ICCs, smooth muscle cells (SMCs), and/or PDGFRα+ cells. PO was surgically induced by placing a plastic ring around the distal ileum for approximately 2 weeks. After development of dilation and hypertrophy in the small intestine, the ring was surgically removed to study the recovery process for up to 8 weeks. Hypertrophic and recovering smooth muscles were collected at different time points and examined for: 1) population changes in ICC, SMCs, and PDGFRα+ cells; 2) population changes in ICC subtypes, and 3) changes in expression of THBS4, KIT, and ANO1 using GFP labeled cells, immunohistochemistry, and/or Western blot.

During the development of hypertrophy, KIT+ ICCs became significantly reduced, SMCs became dedifferentiated, and PDGFRα+ cells became PDGFRαlow/βhigh cells. Populations of both KIT+ ICC-DMP and ICC-MY gradually decreased in hypertrophic muscle. However, expression of THBS4 and KIT was differentially regulated in ICC. KIT was down-regulated while THBS4 was dramatically up-regulated (especially in ICC-SS). During the recovery process, ICCs, SMCs, and PDGFRα+ cells gradually returned to near baseline levels. However, THBS4 remained in the extracellular space of ICC for up to 8 weeks of recovery. THBS4 protein appeared to localize to the extracellular region, plasma membrane, and ER of ICC-MY, ICC-DMP, and ICC-SS. Furthermore, THBS4 expression corresponded with KIT expression, except in ICC-SS, which expressed ANO1. Induction of THBS4 in ICC-SS correlated with outgrowth of undifferentiated ICC during hypertrophy, of which some became differentiated ICC (ANO1+) during recovery. Our data suggest THBS4 plays an important role in ICC development and serves as a novel marker for ICC in intestinal injuries.
Adeno-associated virus mediated gene transfer to intestinal smooth muscle

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Adeno-associated virus (AAV)-based vectors are attractive for tissue-specific gene transfer since they are nonpathogenic and can establish stable long-term gene expression with broad tropisms in both dividing and non-dividing cells. However, hitherto there has been no significant investigation on targeted AAV transduction of specific intestinal cell types. We have examined the ability of various AAV serotypes to transduce cells within the mouse intestine in vivo and human intestine ex vivo.

We examined the transduction efficiencies of AAV-1, AAV-2, and AAV-DJ expressing RFP in HEK-293 and PAC1 cell lines. Our laboratory has previously obtained or generated the transgenic GFP labeled mice line including the smooth muscle cells (SMCs)-eGFP mouse line, the platelet-derived growth factor receptor α (PDGFRα+)-eGFP mouse line, and the interstitial cells of Cajal (ICC)-copGFP mouse line. Thus, we directly injected AAV-DJ into the muscularis of colon and/or ileum of these transgenic GFP model mice during partial obstruction surgeries. By using immunohistochemistry (IHC) and confocal microscopy, we identified the cell types that were transduced by AAV-DJ in the mouse and human intestine. We validated the identity of the transduced cell types by IHC using cell-specific markers including smooth muscle myosin heavy chain 11 (MYH11) for SMCs; c-KIT for ICCs; PDGFRα for PDGFRα+ cells; pan-protein gene product 9.5 (PGP9.5) for neurons; and CD31 for microvascular cells.

The IHC data indicated that the AAVs expressing the RFP reporter gene transduced MYH11 labeled SMCs of the longitudinal and circular layers. The AAVs also transduced the PGP9.5 labeled neurons and sympathetic nerves in the epithelium, submucosal perivascular area, and myenteric plexus in both human and mouse intestinal samples. Moreover, the intestinal microvessels were clearly transduced by AAV-DJ. Therefore, this study for the first time establishes a foundation for cell-specific AAV-mediated delivery into the intestines in vivo and provides a unique platform for treating intestinal disorders.
The angiotensin II type-1 receptor is a mechanosensor in cerebral parenchymal arteriole smooth muscle cells

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Angiotensin II type 1 receptors (AT1R) have been linked to mechanosensitivity in cardiomyocytes, mainly through activation of a β2-arrestin signaling pathway. AT1Rs are also involved in sensation of intraluminal pressure by vascular smooth muscle cells. However, it is not known if the AT1R is a mechanosensor in cerebral parenchymal arterioles (PA), a highly specialized vasculature in which small alterations in myogenic reactivity can have profound effects on blood flow and neurovascular coupling. We hypothesized that AT1Rs are mechanosensors in cerebral arteries and arterioles, acting through β-arrestin signaling to cause myogenic constriction. Anterior cerebellar arteries (ACA) from C57Bl/6J mice were isolated and pressurized. Myogenic reactivity was reduced after exposure to the AT1R antagonist losartan. The biased AT1R agonist peptide TRV120023, a selective activator of the β-arrestin pathway, caused a small yet significant constriction (-5.9±2.9 μm, p<0.05) that was inhibited by pre-incubation with losartan. Incubation with a scrambled peptide had no effect. Myogenic reactivity of PA was also reduced after exposure to losartan. To assess if local production of angiotensin II plays a role in AT1R-dependent myogenic reactivity, PA were incubated with the angiotensin-converting enzyme inhibitor captopril. There was no difference in myogenic reactivity of PA after incubation with captopril. These data suggest that: 1) The AT1R is a smooth muscle cell mechanosensor in large cerebral pial arteries and parenchymal arterioles, through a mechanism that is not dependent on local angiotensin II production; and 2) AT1R-dependent activation of β-arrestin signaling causes a small constriction of the ACA but does not fully account for myogenic reactivity.

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Unitary TRPV3 Channel Ca2+ Influx Events Elicit Endothelium-Dependent Dilation of Cerebral Parenchymal Arterioles

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Elementary Ca2+ influx events through single transient receptor potential vanilloid 4 (TRPV4) channels, called TRPV4 sparklets, mediate endothelium-dependent dilation of mesenteric arteries. TRPV3 channels are present in the cerebral artery endothelium and have larger unitary conductance and Ca2+ permeability compared with TRPV4 channels. Therefore, we attempted to use total internal reflection fluorescent microscopy (TIRFM) to record TRPV3 sparklets from primary cerebral artery endothelial cells. Carvacrol, a monoterpenoid compound derived from oregano, increased the frequency of TRPV3 sparklets in a concentration-dependent manner (EC50 = 8.5 µM). Carvacrol-induced TRPV3 sparklets were inhibited by the selective TRPV3 blocker isopentenyl pyrophosphate (IPP). TRPV3 sparklets have a greater unitary amplitude (ΔF/F0 = 0.20), spatial spread (0.74 µm2), and duration (70 ms) than TRPV4 sparklets, suggesting that TRPV3-mediated Ca2+ influx could have a robust influence on cerebrovascular tone. In pressure myograph experiments, carvacrol caused dilation of cerebral parenchymal arterioles that was blocked by IPP, in an endothelium dependent mechanism. Carvacrol-induced dilation was also inhibited by block of intermediate and small conductance Ca2+-activated K+ (IK and SK) channels. Together, these data suggest that TRPV3 sparklets cause dilation of cerebral parenchymal arterioles by activating SK and IK channels in the endothelium.
Disruption of gastrointestinal PDGFRα+ cells leads to loss of post-junctional inhibitory motor responses

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Fibroblast-like cells (FLCs) exist in smooth muscles of visceral organs, yet their functional role is unclear. FLCs express PDGFRα and are a novel class of excitable cells recently described in gastrointestinal muscles. Crenolanib, a new potent inhibitor of PDGFRα, was developed to treat certain cancers with PDGFRα over-expression. Here, Crenolanib was used to disrupt PDGFRα expression and signaling in the GI tract following administration in BALB/c mice. Intra-peritoneal injections of Crenolanib (µg/g body weight) or control vehicle were given to littermates from postpartum days 1 through 25. Mice were analyzed for differences in gross anatomy and weight, PDGFRα protein and gene expression. Western analysis and immunofluorescence demonstrated significant decreases in PDGFRα protein expression and qPCR revealed down-regulation of PDGFRα gene expression. Stomachs, small intestines, and colons of Crenolanib injected mice were also subject to electrophysiological examination. Stomach and small intestine electrical slow waves were not affected by Crenolanib administration. However, enteric inhibitory motor responses were greatly attenuated throughout the GI tract, with highly significant decreases in purinergic inhibitory motor activity in response to nerve stimulation. These data provide the first evidence for a functional role of PDGFRα+ FLCs in inhibitory motor responses in the tunica muscularis.
miR-10b suppresses expression of KIT by NCOR2 in diabetic interstitial cells of Cajal in the gastrointestinal tract

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Diabetes gradually increases the risk of problems in the gastrointestinal (GI) tract. The long-term high blood sugar (hyperglycaemia) in diabetes can lead to specific GI complications, resulting in dysmotility syndromes (DS). Such GI motility disorder, characterized by depletion of interstitial cells of Cajal (ICC), is the most common pathophysiological abnormality in patients with long-standing diabetes (both type 1 and type 2). In recent years, it has become apparent that an abnormal modulation of epigenetic mechanisms has been highlighted as a crucial part in the pathogenesis, progress, and evolution of this disorder. However, a molecular and epigenetic mechanism of ICC depletion in DS has proved elusive. MicroRNAs (miRNAs) are a new class of transcriptional regulators that are involved in nearly all developmental and pathological processes in animals. Here we report miR-10b plays an important role in the phenotypic change of intestinal ICC during the development of diabetes. miR-10b was abundantly expressed in healthy ICC in the small intestine, but it was dramatically depleted in diabetic ICC. miR-10b targets an epigenetic repressor, nuclear receptor corepressor 2 (NCOR2), which negatively regulates expression of tyrosine-protein kinase KIT. Expression of NCOR2 and KIT proteins was significantly reduced in diabetic intestine where KIT+ ICC were disappeared. Our findings on KIT regulation by miR-10b offer a new insight into how ICC phenotype is changed and become non-functional in diabetic conditions. Furthermore, our data suggest miR-10b-5p may be potentially an attractive therapeutic target to restore KIT expression in diabetic ICC of patients with DS.
Influence of intracellular Ca2+ and alternative splicing on the pharmacological profile of ANO1 channels

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Anoctamin-1 (ANO1) encoded by (Ano1, aka Tmem16a) is a Cl- channel gated by cytoplasmic Ca2+ and responsible for the Cl- conductances in many types of cells. Several splice variants of Ano1 with differing properties are expressed naturally by cells, affecting the ultimate biophysical properties of the conductance. Recent high throughput screening studies have identified new compounds that can block ANO1 with relatively high affinity and selectivity and the druggability of this conductance has been considered for therapeutic purposes. We investigated the effects of intracellular Ca2+, over a range (100-1000 nM) that might be accomplished during physiological activation of ANO1 channels, on the block of ANO1 channels expressed in HEK 293 cells. Tests were performed using a variety of the naturally occurring splice variants of Ano1. We found that with most of the splice variants tested, channel block is highly dependent upon [Ca2+]i, and increasing [Ca2+]i dramatically reduced the potency of the blockers. Experiments on excised, inside-out patches showed that the diminished potency of the blockers occurs by the actions of Ca2+ at the cytoplasmic surface of the channels, involving possibly a competitive interaction for a common binding site or steric hindrance or repulsion of the blocking drugs by electrostatic forces. The degree of interaction between the channel blockers and [Ca2+]i depended upon the splice variant expressed. Our findings suggest caution when using pharmacological tests to determine the role of ANO1 in physiological functions or when considering ANO1 as a target for channel antagonists.
Role of Na+/Ca2+ exchange in the pacemaker activity of interstitial cells of Cajal

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Interstitial cells of Cajal (ICC) are the pacemaker cells that generate electrical slow waves in gastrointestinal (GI) smooth muscles that organize the motor patterns in many regions of the GI tract into phasic contractions. Slow waves result from activation of Ca2+-activated Cl- channels (CaCC) encoded by Ano1 and depolarize ICC to the Cl- equilibrium potential (ECl). How activation of CaCC is sustained through the duration of slow waves is unknown. We tested the role of the Na+/Ca2+ exchanger (NCX) in regulating pacemaker activity in ICC. ICC of small intestine and colon expresses NCX isoforms. NCX3 co-immunoprecipitated with ANO1 and these proteins were co-localized in ICC using a proximity ligation assay. Inhibitors of NCX increased CaCC current in ICC, suggesting that NCX, acting in Ca2+ exit mode, helps to regulate basal [Ca2+]i in these cells. Shifting NCX into Ca2+ entry mode by replacing extracellular Na+ with Li+ increased spontaneous transient inward currents (STICs) recorded under voltage clamp. The STICs were due to activation of CaCC. Stepping ICC from -80 to -40 mV activated slow wave currents. These events were reduced in amplitude and duration by KB-R7943 and SN-6 and enhanced in amplitude and duration by increasing the driving force on NCX. Our results suggest that NCX regulates the activation of ANO1 in ICC. Dynamic changes in ionic gradients during slow waves may flip the directionality of the exchanger, allowing it to remove Ca2+ during the inter-slow wave period and to sustain activation of ANO1 during the slow wave plateau phase.
The expression of small conductance calcium-activated potassium (SK3) channel is specifically concentrated at interstitial cells of the spaces between detrusor smooth muscle cells in the guinea pig urinary bladder.


Aims of study
Small conductance calcium-activated potassium channels consist of three subtypes, SK1, SK2 and SK3. Above all, SK3 is thought to be involved in the suppression of detrusor smooth muscle excitability and contractility. Recently, it was reported that SK3 transcript and protein in the bladder wall are highly expressed in PDGFRα+ interstitial cells and these cells may particpate in the control of detrusor excitability during the filling phase. In this study, we investigated the distribution and structural feature of SK3 expressing interstitial cells in the bladder wall.

Materials and methods
Male guinea pigs weighting 500 to 700 mg were anesthetized and urinary bladder was extirpated and opened longitudinally from neck to dome. The specimen was pinned to a Sylgard dish and fixed in Zamboni solution for 5hr. After several rinses, the urinary bladder was incubated in the 0.1 M phosphate buffer containing 30% sucrose for cryoprotection. Five μm bladder wall section and 50 μm detrusor layer plate (semi-whole mount preparation) was made by cryostat. These sections were processed immunohistochemistry and examined by confocal microscopy. These sections were labeled with anti-SK3 antibody, and also double-immunolabeled with antibodies to αSMA, vimentin, platelet-derived growth factor receptor-α (PDGFRα), tyrosine hydroxylase (TH) and vesicler acetylcholine transporter (vAchT), respectively.

Results
The expression of SK3 was observed in interstitial cell in the detrusor layer. SK3 immunolabeled cell was not observed in the lamina propria. SK3 immunolabeled interstitial cell had a branched stellate or spindle morphology and connected each other like a mesh network. This network existed in the spaces between detrusor smooth muscle. Double labeling revealed that SK3 immunolabeled interstitial was co-labeled by vimentin and PDGFRα. The axon immunolabeled by TH and vAchT ran the side of this network.

Conclusion
SK3 positive interstitial cells are distributed in the detrusor smooth muscle layer. These cells are subfamily of mesenchymal cells, and might be involved in the regulation of motility of the urinary bladder detrusor smooth muscle. The structural feature (closeness to detrusor smooth muscle) of those SK3 positive interstitial cells might be advantageous to modulate the electrical activity of the urinary bladder detrusor smooth muscle.
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