INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

POSGRADO EN CIENCIAS EN BIOLOGIA MOLECULAR

SIGNALING MODULATION IN CELLS THAT CONTROL GASTROINTESTINAL MOTILITY

Tesis que presenta

Raúl Loera Valencia

Para obtener el grado de

Doctor en Ciencias en Biología Molecular

Codirectores de la Tesis:

Dr. Carlos Barajas López

Dr. Jan D. Huizinga

Constancia de aprobación de la tesis

La tesis "**SIGNALING MODULATION IN CELLS THAT CONTROL GASTROINTESTINAL MOTILITY**" presentada para obtener el Grado de Doctor en Ciencias en Biología Molecular fue elaborada por **Raúl Loera Valencia** y aprobada el **17 de febrero de 2014** por los suscritos, designados por el Colegio de Profesores de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C.

_____
Dr. Carlos Barajas López
(Director de la tesis)

_____
Dr. Jan D. Huizinga
(Codirector de la tesis)

_____
Dra. Irene Castaño Navarro
(Miembro del Comité Tutoral)

_____
Dr. Juan Francisco Jiménez Bremont
(Miembro del Comité Tutoral)

Créditos Institucionales

Esta tesis fue elaborada en el Laboratorio de Neurobiología la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C., bajo la dirección del Dr. Carlos Barajas López y el Dr. Jan D. Huizinga con fondos del Consejo Nacional de Ciencia y Tecnología (Proyecto 48297).

Durante la realización del trabajo el autor recibió las becas académicas No: 204088 (doctoral) y 290618 (mixta) del Consejo Nacional de Ciencia y Tecnología, así como de apoyo especial (interna) del Instituto Potosino de Investigación Científica y Tecnológica, A. C.

IPICYT

Instituto Potosino de Investigación Científica y Tecnológica, A.C.

Acta de Examen de Grado

El Secretario Académico del Instituto Potosino de Investigación Científica y Tecnológica, A.C., certifica que en el Acta 065 del Libro Primero de Actas de Exámenes de Grado del Programa de Doctorado en Ciencias en Biología Molecular está asentado lo siguiente:

En la ciudad de San Luis Potosí a los 28 días del mes de febrero del año 2014, se reunió a las 12:00 horas en las instalaciones del Instituto Potosino de Investigación Científica y Tecnológica, A.C., el Jurado integrado por:

Dra. Irene Beatriz Castaño Navarro	**Presidenta**	**IPICYT**
Dr. Juan Francisco Jiménez Bremont	**Secretario**	**IPICYT**
Dr. Carlos Barajas López	**Sinodal**	**IPICYT**
Dr. Luis M. Montaño Ramírez	**Sinodal externo**	**UNAM**
Dr. Ricardo Espinosa Tanguma	**Sinodal externo**	**UASLP**

a fin de efectuar el examen, que para obtener el Grado de:

DOCTOR EN CIENCIAS EN BIOLOGÍA MOLECULAR

sustentó el C.

Raúl Loera Valencia

sobre la Tesis intitulada:

Signaling Modulation in Cells that Control Gastrointestinal Motility

que se desarrolló bajo la dirección de

Dr. Carlos Barajas López

El Jurado, después de deliberar, determinó

APROBARLO

Dándose por terminado el acto a las 14:00 horas, procediendo a la firma del Acta los integrantes del Jurado. Dando fe el Secretario Académico del Instituto.

A petición del interesado y para los fines que al mismo convengan, se extiende el presente documento en la ciudad de San Luis Potosí, S.L.P., México, a los 28 días del mes de febrero de 2014.

Dr. Marcial Bonilla Marín
Secretario Académico

Mtra. Ivonne Lizette Cuevas Vélez
Jefa del Departamento del Posgrado

INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.
IPICYT
SECRETARIA ACADEMICA

DEDICATORIA

A mis padres, Raúl Loera García y Beatriz Esmeralda Valencia Valencia.

A mi esposa Alma Berenice Jasso Salcedo.

A mi familia, especialmente a la familia Carrillo-Valencia, a Imelda Valencia Valencia y a Eva Valencia Valencia.

AGRADECIMIENTOS

Al Dr. Carlos Barajas López, al Dr. Jan D. Huizinga y a la Enf. Rosa Espinosa Luna por su mentoría, paciencia e invaluables enseñanzas.

A mis colegas y amigos Néstor Nivardo Jiménez Vargas, Egina Criseida Villalobos Hernández y al Dr. Esri Hazael Juárez por su gran apoyo en la realización de esta tesis. Asimismo a los colegas, ahora amigos, del laboratorio de Neurobiología del IPICYT.

A los doctores Sean Parsons, Xuan Yu Wang, Yong Fang Zhu, Waliul Khan y al M. en C. George W.J. Wright por su apoyo en la realización de mi estancia en el extranjero. Asimismo a Marc Pistilli y Andrew Pawelka por su apoyo personal en el laboratorio de ICC del Instituto Farncombe de Investigación en Salud Digestiva.

A las grandes amistades que formé en la generación de maestría en Biología Molecular del IPICYT de 2006.

INDEX

RESUMEN

MODULACIÓN DE SEÑALES EN CÉLULAS REGULADORAS DE LA MOTILIDAD GASTROINTESTINAL

La vida es sustentada en parte por la actividad motora del tracto gastrointestinal. Los patrones de motilidad en dicho tracto son orquestados por la interacción de mecanismos reguladores neurogénicos y miogénicos. Las neuronas mientéricas modulan el reflejo peristáltico a través de circuitos neurales, mientras que las células intersticiales de Cajal (ICC) controlan la generación de oscilaciones rítmicas de voltaje en el músculo liso conocidas como ondas lentas. Las neuronas mientéricas y las ICC modulan su actividad por la señalización de neurotransmisores y su acción sobre la apertura/cierre de canales iónicos. En este trabajo usamos técnicas moleculares para tratar problemas particulares en la señalización de neuronas mientéricas y las ICC. Las neuronas mientéricas usan el ATP como neurotransmisor excitatorio a través de receptores P2X, sin embargo la presencia de $P2X_3$ no parece intervenir en la fisiología normal de la motilidad en el intestino delgado de cobayos. Proponemos que su función se da durante el desarrollo postnatal. Nuestros resultados muestran una reducción en el número de neuronas mientéricas positivas a $P2X_3$ durante el desarrollo postnatal y el incremento de neuronas $P2X_5$ positivas en los adultos. La aparición de neuronas $P2X_2$ y $P2X_4$ positivas permaneció estable. La sensibilidad al ATP de neuronas neonatales y adultas no cambió. Las ICC de ratón pueden participar en la modulación de la motilidad colónica a través de señalización muscarínica colinérgica. Nuestros resultados indican que dicha señalización bloqueó la actividad de canales K^+ en cultivos primarios de ICC. La aplicación de un bloqueador de Kv7 también bloqueó la actividad de estos canales y activó un maxicanal en las ICC. RT-PCR de célula única y doble inmunotinción de cólones de ratón revelaron ICC-IM Kv7.5 positivas inervadas por fibras colinérgicas. Sugerimos ampliar el uso de las técnicas moleculares para tratar problemas básicos en la motilidad gastrointestinal.

Palabras clave: $P2X_3$; ATP; neuronas mientéricas, canales Kv7, ICC, motilidad.

ABSTRACT

SIGNALLING MODULATION OF CELLS THAT CONTROL GASTROINTESTINAL MOTILITY

Life is sustained in part by the motor activity of the gastrointestinal tract. The motility patterns in the gastrointestinal tract are orchestrated by the interplay of neurogenic and myogenic governing mechanisms. The myenteric neurons modulate the peristaltic reflex through circuits of sensory, motor and interneurons, while interstitial cells of Cajal (ICC) pace the generation of rhythmic voltage oscillations in the smooth muscle known as slow waves. Both systems act together to generate diverse motility patterns in the gut. Myenteric neurons and interstitial cells of Cajal modulate their activity through signaling of neurotransmitters and its effect on the gating of ionic channels. In this work we use diverse molecular techniques to address particular problems in the signalling of myenteric neurons and interstitial cells of Cajal. Myenteric neurons use ATP as excitatory neurotransmitter through P2X receptor signalling, however the presence of $P2X_3$ does not play a role in the normal physiology of guinea pig small intestinal motility. We propose this subunit has a function in postnatal development. Our results show reduction of $P2X_3$ positive myenteric neurons during postnatal development and increase of $P2X_5$ positive neurons to adulthood, whilst appearance of $P2X_2$ and $P2X_4$ remained unchanged. This regulation does not alter ATP sensibility in cultured neurons from neonatal or adult guinea pigs. Murine colonic ICC could participate in the modulation of colon motility through muscarinic cholinergic signalling; we provide experimental evidence showing that muscarinic stimulation of ICC blocked K^+ channel activity in primary cultures. The application of a Kv7 blocker also abolished this activity and activated a maxi channel in ICC. Single cell RT-PCR and double immunostaining of colons revealed Kv7.5 positive ICC-IM and its dense cholinergic innervation. We suggest expanding the use of molecular techniques to approach basic physiological problems in gut motility.

Keywords: $P2X_3$; ATP; myenteric neurons; Kv7 channels; ICC; colonic motitlity.

CHAPTER 1. INTRODUCTION.

Life is sustained in part by the motor activity of the gastrointestinal (GI) tract. Intestinal failure in humans is cause of death (Goulet and Ruemmele, 2006) and shows the importance of gastrointestinal motility. Together with its primordial role in physiology, there is also a complexity associated to the motor patterns in the gut (Huizinga and Lammers, 2009). The esophagus, for example, is controlled by the central nervous system (particularly the swallowing center of the brain). However, it is innervated by autonomic neurons organized in ganglia, which can take control of peristalsis if the vagus nerve is severed (Preiksaitis and Diamant, 1999). In the same way, gastric and intestinal movements have both central and enteric controls that overlap and interact with each other but are not directly orchestrated by the central nervous system (Langley and Magnus, 1905, Langley, 1922).

While motor patterns exist in the whole digestive system, there is a large body of research on the function of the intestine at multiple levels (Furness and Costa, 1987, Kunze and Furness, 1999, Thuneberg, 1999). Particularly, the small intestine and colon have been the center of recent research and the models to study the motility patterns that allow digestion to take place (Lentle et al., 2008, Huizinga et al., 2011, Angeli et al., 2013, Janssen and Lentle, 2013). In the next chapters we will focus on the small intestine and colon´s anatomy and physiology to understand the problems addressed in this thesis.

1.1. Anatomy of the small intestine and colon. Localization of myenteric neurons and interstitial cells of Cajal.

The small intestine and colon are organized in layers (Figure 1A). The more external layer is the serosa and it's a thin film of connective tissue that surrounds the gut external wall. The next layer is the *muscularis externa* and it is responsible for most contractions in the GI tract, which is divided in the longitudinal and circular muscle layers; between them, the myenteric plexus (MP) is located forming a network of neurons that innervate both muscle layers (Heanue and Pachnis, 2007, Furness, 2008a). This plexus is organized in ganglia (group of myenteric neuronal

bodies inside a connective tissue capsule) connected through internodal nerves (Furness, 2008a). Deeper than the circular muscle, the submucosal layer can be found with its own embedded neuron network named submucosal plexus (SMP) with a lower number of ganglions than the MP (Furness, 2007). Then the mucosa represents the final layer before the intestinal lumen. This layer represents the barrier to the outside of the body and it is in charge of nutrient exchange and secretion.

The interstitial cells of Cajal are pacemaker cells organized in networks located in all layers of the intestines (Figure 1B). A network of interstitial cells of Cajal (ICC) can be found in close relationship with the myenteric plexus (ICC-MP) (Komuro, 2006). An additional network has been identified in the small intestine between the circular muscle layer and the submucosal layer; these cells are termed the deep muscular plexus interstitial cells of Cajal (ICC-DMP)(Komuro, 2006). In the colon, there are no ICC-DMP, and the pacemaker function belongs to ICC of the submucosal plexus (ICC-SMP) (Rae et al., 1998, Yoneda et al., 2004). A fourth population of ICC permeates both muscle layers, these are known as intramuscular ICC (ICC-IM)(Komuro, 2006). Both networks of ICC and enteric neurons control the movements of the small intestine and colon and generate the group of motility patterns commonly known as *peristalsis* (Huizinga, 1999, Huizinga and Lammers, 2009, Zhu et al., 2013).

1.2. Peristalsis

The movements of the gastrointestinal tract are usually identified by the term *peristalsis;* however, it involves a variety of motor patterns that ultimately allow digestion and nutrient absorption in the GI tract (Huizinga and Lammers, 2009). The classical peristalsis is commonly identified as the *peristaltic reflex* and can be seen in the whole digestive system, it produces the movement of the intestinal content in the aboral direction through the contraction of the intestine in the oral section and relaxation in the anal portion of a given segment of the gut located around the bolus (Figure 2A) (Bayliss and Starling, 1899).

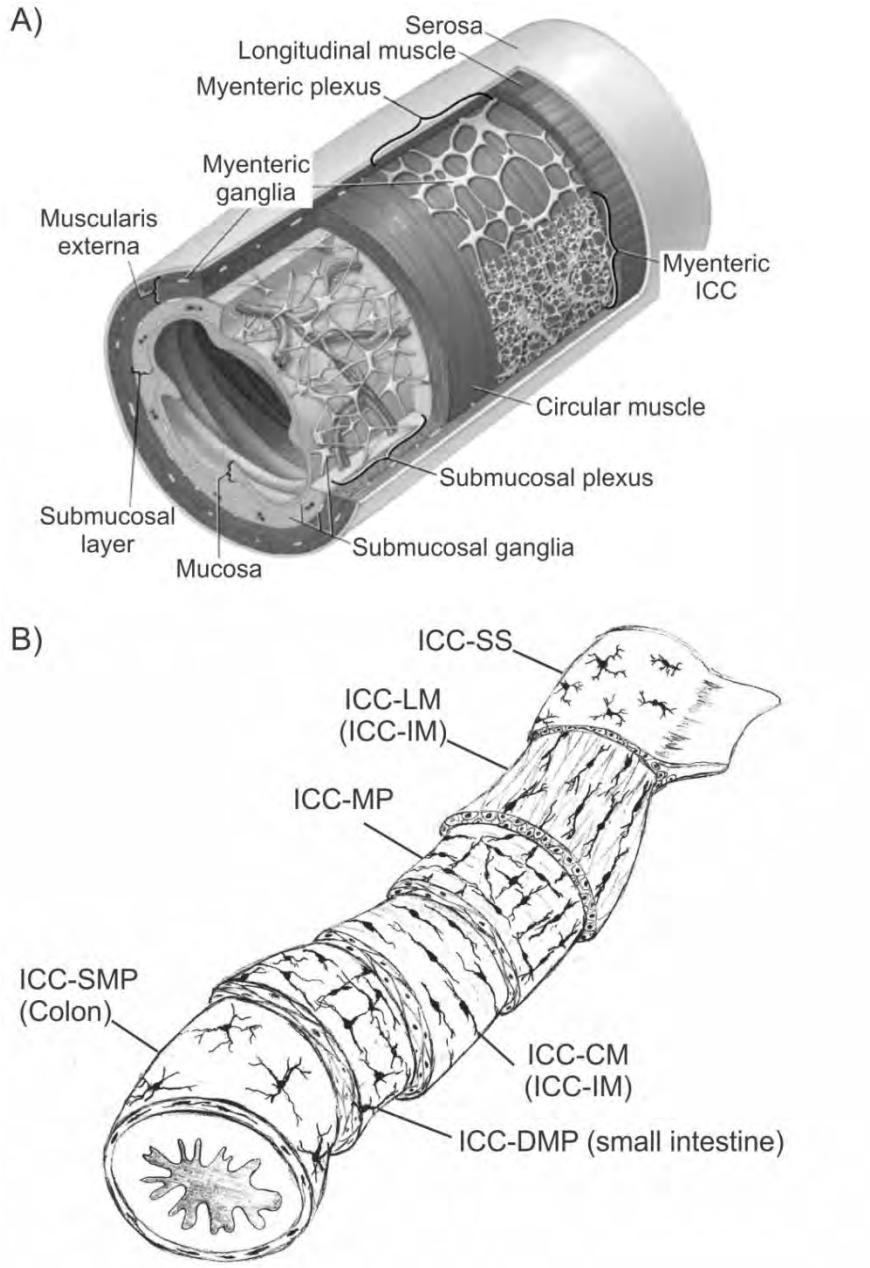

Figure 1. A) Organization of the small intestine and location of myenteric plexus. The serosa is the most external layer of the gut, while the mucosa comes in contact with the intestinal content. The myenteric plexus is located sitting atop of the circular muscle layer, while the submucosal plexus is within the submucosal layer. The ICC-MP network runs closely related to the myenteric plexus and blood vessels of this layer. Modified from (Low and Benarroch, 2008) **B) Location of gastrointestinal cells of Cajal.** Interstitial cells of Cajal are present in the serosa (SS), longitudinal muscle (LM), along the myenteric plexus (MP, both in small intestine and colon), embedded in the circular muscle (CM), in the deep muscular plexus (DMP, only in the small intestine), and in the submuscular plexus (SMP, only in the colon). Figure references from (Komuro, 2006).

The circular muscle layer plays a central role in this reflex and its physiological basis will be discussed later in this work. Even when this reflex has been demonstrated *in vitro*, the movement of the intestine *in vivo* is far more complicated since the distention of the circular muscle does not occur in a single focalized point but in large section of the organ. Thus, all of intestinal motility cannot be the product of repeated, summed peristaltic reflexes (Waterman et al., 1994, Spencer et al., 1999, Huizinga and Lammers, 2009). A rhythmic contraction of the circular and longitudinal muscle layers is present in the small intestine and can propagate in both directions (oral and aboral) of the intestine, this pattern was termed *pendulum oscillation* and its nature is myogenic (Bayliss and Starling, 1899). In the colon, a coupling between the peristaltic reflex and the rhythmic oscillations of the muscle layers has been proposed. In this model, distension sensing, ascending excitatory reflexes above the bolus and descending inhibitory reflexes below it are neurogenic; whilst rhythmicity of contractions and their propagation along the colon is myogenic (Costa and Furness, 1976, Huizinga et al., 2011, Costa et al., 2013). Other types of propulsive motility patterns are observed in the gut and are named *migrating motor complex* (MMC). The term points to the propagating coordinated contraction patterns allowing the intestinal content to be moved in the anal direction through large portions of the intestine presented also with mixing contractions (Grivel and Ruckebusch, 1972). Additional to the peristaltic reflex, the rhythmic peristaltic movements (with their neural and myogenic components) and MMC, a local movement in the small and large intestine exist called *segmentation* (Figure 2B). During segmentation, a ring of circular muscle contracts while muscles in both sides relax. Then, the contracted ring of circular muscle relaxes and the side rings (previously relaxed) contracts. In this particular portion of the intestine (2 to 5 cm long), contraction and relaxation of circular muscle oscillate, generating a mixing effect on the bolus (Wood, 1999, Wang et al., 2005). A phenomenon of *retroperistalsis* is present pathologically preceding vomiting in the stomach (Thompson and Malagelada, 1982), and physiologically in the late phase of duodenal MMC (Castedal et al., 1997).

When studied through a pressure transducer, smooth muscle movements in the small intestine can be identified and separated in two classes: a low frequency, high amplitude contraction (Huizinga, 1986, Sarna, 1986); and a high frequency, low amplitude contraction with a very similar frequency to the slow waves from ICC-MP in the small intestine (Huizinga et al., 2009, Hennig et al., 2010). It has been hypothesized that the high amplitude contraction is inducible by distention of the intestinal lumen, while the low amplitude contraction is omnipresent in the gut and paced by the ICC (Slow wave associated contraction, SWAC). Whether these contraction patterns interact to develop the peristaltic movements previously described is still being investigated (Huizinga et al., 2011).

The contractions on the muscle layers of the intestine have two distinct sources: myogenic and neurogenic. The myogenic control of smooth muscle contraction is driven primordially by the interstitial cells of Cajal in close interaction with the smooth muscle cells (Sarna, 1986, Huizinga et al., 1995, Der-Silaphet et al., 1998), while the neurogenic control comes from the vagus (Travagli et al., 2006), the mesenteric nerves (coming from the spinal cord) (Brugère et al., 1991) and the neurons located at the myenteric plexus (Waterman et al., 1994, Kunze and Furness, 1999, Mao et al., 2006). When this is the general arrangement for the entire GI tract, the specific function of cell types varies from small intestine to colon, as previously mentioned with the slow waves in the small intestine (generated by ICC-MP) and colon (generated by the ICC-SMP).

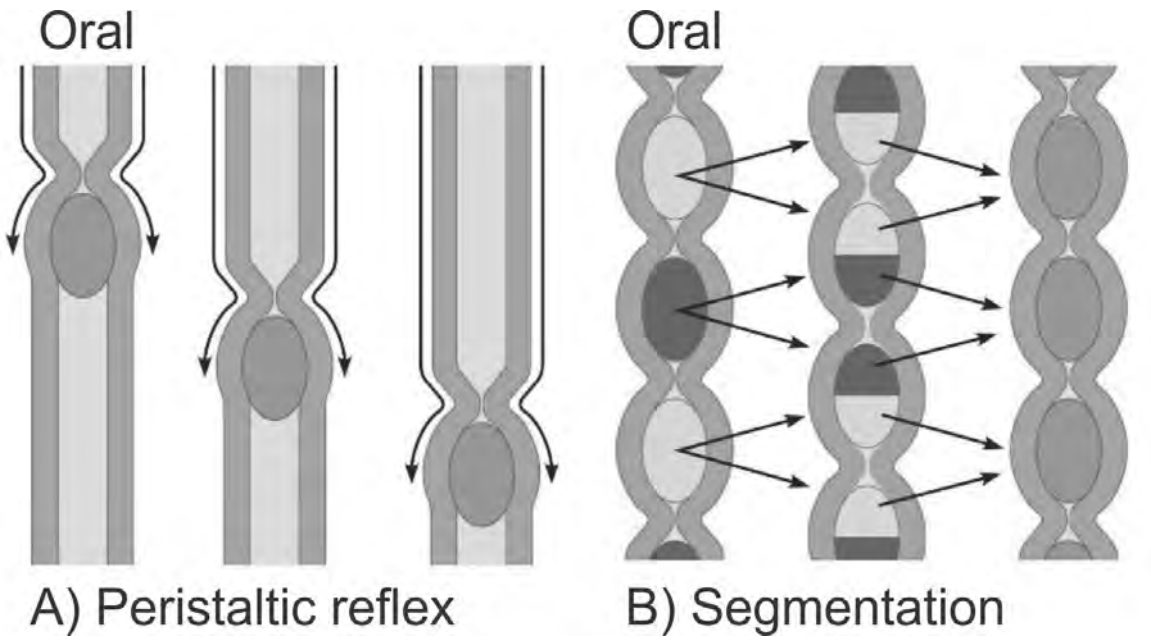

Figure 2. Motility patterns in the gut. A) Peristaltic reflex. The peristaltic reflex is activated when the chime distends the mucosa, then contraction in the circular muscle is followed (oral portion) and relaxation in the anal portion allows content movement in the aboral direction. **B) Segmentation.** Segments of circular muscle alternately contract and relax producing an effect of mixing of the intestinal contents.

1.3. The cellular basis of peristalsis: Importance of ICC and myenteric neurons

Neurons of the myenteric plexus of the intestine are responsible for the initiation of the peristaltic reflex and stimulate contraction and/or relaxation through the action of several neurotransmitters to produce motility patterns (Figure 3A) (Furness and Costa, 1980, Waterman et al., 1994, Kunze and Furness, 1999, Bian et al., 2003). In the myenteric plexus, three types of neurons are recognized based on their basic function and electrical behavior. The first type are the AH neurons, which are

mainly sensory neurons and are thought to be the responsible for the initiation of the peristaltic reflex (Furness and Costa, 1987, Costa et al., 2000, Furness, 2007). The second type is the S neurons, which are mainly motor neurons and are directly responsible for the contraction (excitatory motor neurons) or relaxation (inhibitory motor neurons) of the smooth muscle layers. These neurons can send and receive projections from layers as far as the mucosa. The third type of neurons are located mainly within the ganglia and are the interneurons, which mediate the communication between S and AH neurons (Costa et al., 2000, Furness, 2007). Putting together the knowledge of anatomy, morphology (Dogiel classification), neurotransmitter content and physiological function of myenteric neurons, modern studies often use composed terms to refer to specific neuron subpopulations, however they overpass the purpose of this study and won´t be used.

In experiments done with mechanical stimulation of the intestinal lumen, electrical responses can be identified in the neurons located in the MP, supporting the idea that the initiation of the peristaltic reflex is caused by luminal distention and sensed by myenteric neurons (Kunze and Furness, 1999, Mao et al., 2006). Nevertheless, motor patterns in the gut can be observed in the presence of tetrodotoxin (TTX), a drug that blocks the action of neurons, and these patterns can be modified using drugs targeting smooth muscle cells and ICC (Huizinga et al., 2011). The evidence suggests that the motility in the gut can be regulated by the overlap of neurogenic and myogenic mechanisms of contraction (Huizinga and Lammers, 2009, Zhu et al., 2013).

The contractions of the smooth muscle in the small intestine are driven in part by propagating, oscillatory changes in membrane potential known as slow waves (Barajas-Lopez et al., 1989, Huizinga et al., 1995, Huizinga et al., 1998). The slow waves are paced by a network of interstitial cells of Cajal located close to the myenteric plexus (ICC-MP). These cells generate the slow waves through a mechanism that is still being studied, involving several channels and pumps that produce rhythmic shifts in membrane potential (Koh et al., 2002, Koh et al., 2003, Faville et al., 2009, Zhu et al., 2009, Parsons et al., 2012). Membrane voltage

changes in ICC (pacemaker potentials) stimulate calcium oscillations in the smooth muscle cells through gap junctions, constituting the *pacemaker system* of the myocites of the small intestine (Figure 3B) (Kobilo et al., 2003). There is evidence showing that the slow wave associated contractions generate the low amplitude, high frequency contractions observed in whole intestine preparations *ex vivo* (Hennig et al., 2010). It has been proposed that ICC-MP in the small intestine are responsible for contractions that mix the luminal contents, while ICC-SMP pace this activity in the colon.

While the relationship of ICC with the smooth muscle has been extensively explored, their interaction with myenteric neurons is still being discussed. Experimental evidence shows that ICC helps sustain neurogenic contractions of the small intestine (Ward et al., 2004). Previous works have suggested that ICC may mediate neuron-smooth muscle stimulus, facilitating signalling in conjunction with myenteric neurons (Shuttleworth et al., 1993, Ward, 2000). It also has been suggested that synapse-like junction can be found between ICC and myenteric neurons and they could help their coordinated interaction to produce contraction in the myocites (Figure 5) (Rumessen et al., 1982, Wang et al., 2003, Huizinga et al., 2008). The pacemaker function of ICC is composed by the active interplay between channels, pumps and neurotransmitter receptors; the same accounts for neuronal electric function (Galligan, 2002, Furness, 2007, Furness, 2008b, 2012). To date, the molecular basis of the physiology of myenteric neurons and ICC are not fully understood and represents a very active field of research (Huizinga et al., 2000).

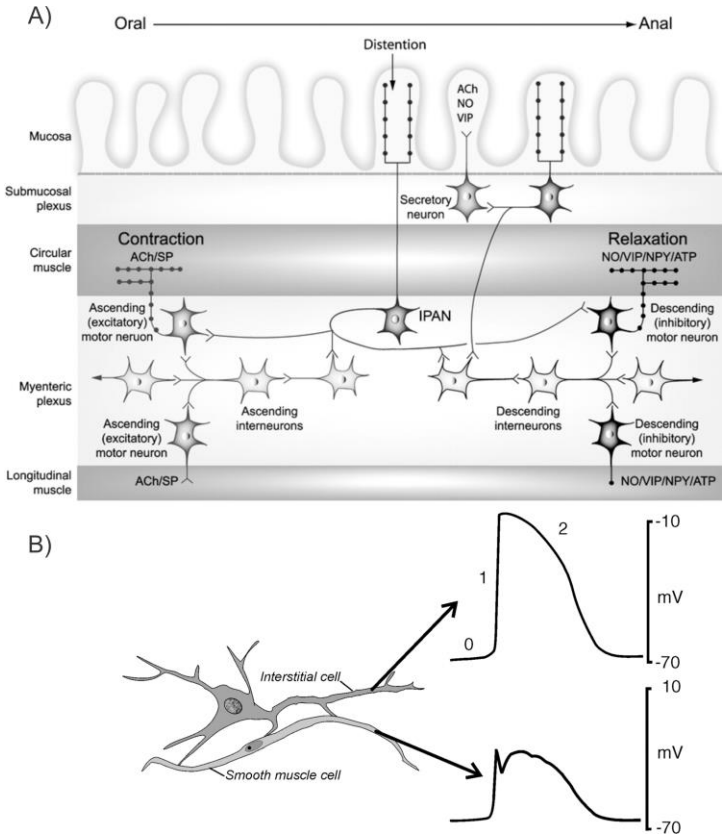

Figure 3. Neurogenic and myogenic control of gut motility. A) Schematic representation of the neurons mediating the peristaltic reflex. Distension of the mucosa by the intestinal contents, as well as some of its chemicals activates intrinsic primary afferent neurons (IPANs). They synapse in both oral and anal directions with interneurons and motor neurons. Contraction in the oral portion of the intestine is mediated by Substance P (SP) and Acetylcholine (ACh), while distention in the anal portion is mediated by nitric oxide (NO), vasoactive inhibitor peptide (VIP), neuropeptide Y (NPY) and ATP. Modified from (Benarroch, 2007). **B) Representation of the interaction of interstitial cells of Cajal and smooth muscle cells.** The upper record shows a pacemaker potential recorded in Interstitial Cells of Cajal at a resting membrane potential of -70 mV. The phases of this potential are the diastolic phase (0), upstroke depolarization (1) and plateau phase. During phase 0 a voltage dependent Ca^{2+} conductance is activated, while potassium conductances may contribute to restore the resting membrane potential. The lower trace shows the simultaneous voltage recording on smooth muscle cell, where the passive conduction of the action potential results in the generation of a slow wave. Modified from (Kito et al., 2005).

1.4. Neurotransmitter receptors in myenteric neurons

In a very general view, myenteric neurons possess two main kinds of receptors: those that increase the probability of generating an action potential (excitatory) and those that decrease that probability (inhibitory) (Costa et al., 2000). Nevertheless, a large diversity of neurotransmitters and their receptors are present in the plasma membrane of myenteric neurons (Furness, 2008a). The most studied inhibitory neurotransmitter in the ENS is the nitric oxide (NO), but its receptor is the guanylyl cyclase (sGC) in the cytoplasm of the innervated cell, which catabolizes the formation of cGMP and reduces the calcium in the cytoplasm, producing relaxation (Costa et al., 2000). Other transmitters exist with inhibitory activity and function through G-proteins coupled receptors such as the vasoactive inhibitor peptide (VIP) (Brookes et al., 1991), enkephalin (Poole et al., 2002), neuropeptide Y (NPY) (Lundberg et al., 1983), pituitary adenylate cyclase-activating polypeptide (PACAP) (Nagahama et al., 1998) and ATP when acting on the muscle cell (Burnstock and Williams, 2000). Enkephalin acts in myenteric neurons through μ and dynorphin receptors (Chavkin and Goldstein, 1981); VIP and PACAP act through VPAC receptors; NPY binds to Y1 receptor in the myenteric neurons, while ATP binds to P2Y receptors in the muscle increasing cAMP and reducing cytosolic calcium, inhibiting contraction. Another neurotransmitter receptor present in myenteric neurons is γ-aminobutiric acid (GABA) receptors type A (termed $GABA_A$) (Baetge and Gershon, 1986). These receptors are localized in AH/sensory neurons and interneurons, however their natural ligand GABA is located in motor neurons supplying the circular muscle (Pompolo and Furness, 1990). In consequence, the synaptic source for $GABA_A$ is still unclear and no synaptic responses to GABA have been reported for the ENS (Galligan, 2002).

The main excitatory neurotransmitter in the enteric nervous system (ENS) is acetylcholine (ACh) which is released by myenteric neurons and acts on nicotinic Ach receptors (nAchR) and muscarinic receptors (mAChR) (North et al., 1985, Steele et al., 1991). nAChRs are ligand-gated ion channels and their action is fast (1 to 100 milliseconds), while muscarinic receptors are coupled to G-proteins and

their action is slower but long lasting (Galligan, 2002). In the post-synaptic neurons, the activation of nAChRs produce a fast excitatory postsynaptic potential (fEPSP), culminating in the release of neurotransmitters on the neuromuscular junction (Galligan et al., 2000). nAchRs and muscarinic receptors can be found in other cell types like myocites, where they drive the contraction of the smooth muscle when activated by ACh (Kunze and Furness, 1999).

Even when ACh is widespread in the ENS, other excitatory neurotransmitters are produced by and elicit their effect on myenteric neurons. Such are the cases of glutamate (acting on AMPA receptors) (Galligan et al., 2000), serotonin (5HT) (Pan et al., 1997), histamine (Starodub and Wood, 2000), tachykinins (Galligan, 1999), neurokinins (Substance P and neurokinin A) (Alex et al., 2001), glycine (Cherubini and North, 1985), ATP and their related hydrolysis products until adenosine (Burnstock, 2007). From these molecules, ATP is prominent because it is co-released with the rest of neurotransmitters in many, if not all, of the synapses in the ENS acting as excitatory neurotransmitter (Burnstock and Williams, 2000, Nurgali et al., 2003, Burnstock and Verkhratsky, 2012). The receptors for ATP in myenteric neurons are metabotropic (P2Y) as well as ionotropic (P2X) in nature. Metabotropic P2Y receptors couple to secondary messengers through G-proteins, while ionotropic P2X receptors contain pores that change their conformation to an open state when activated by ATP, allowing cations to flow into the cytoplasm (Burnstock and Williams, 2000). It is now accepted that P2X receptors participate in the fast excitatory postsynaptic potentials in myenteric neurons (Barajas-Lopez et al., 1996, Galligan et al., 2000, Ren et al., 2003). The existence of seven P2X subunits ($P2X_1$ to $P2X_7$) and their ability to form homo-trimers as well as hetero-trimers may account for the electrophysiological behavior of ATP induced currents, since particular combinations of P2X subunits present characteristic electrical and pharmacological properties (Burnstock and Williams, 2000, North, 2002). $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$ and $P2X_7$ have been reported in the mouse and guinea pig myenteric neurons of the small intestine (Burnstock, 2000, Dunn et al., 2001, Guerrero-Alba et al., 2010, Valdez-Morales et al., 2011, Nieto-Pescador et al., 2013).

There is evidence that P2X receptors mediate to some extent the function of the small intestine. Peristalsis is impaired in mice lacking $P2X_3$ subunit, which presents intestines with altered response to luminal distention. It has been suggested that $P2X_3$ is present in the AH neurons and participates in the sensing of the luminal distention in the mouse (Bian et al., 2003); however in the guinea pig myenteric plexus, $P2X_3$ seems to be located in the motor neurons, as revealed by the co-localization with the marker calretinin and the absence of co-localization with calbindin (Poole et al., 2002, Van Nassauw et al., 2002). When examined with the same mutant mice, neither $P2X_3$ nor $P2X_2$ seem to participate in the peristaltic reflex in the colon (Devries et al., 2010). In the guinea pig MP, the functional characterization of $P2X_3$ has been done using the non-hydrolysable analog α,β-methylene ATP (α,β-meATP), to which $P2X_2$ and $P2X_3$ homomeric receptors as well as $P2X_{2/3}$ heteromeric receptors are sensible (Benko et al., 2005). Given the lack of selective pharmacological tools to discern between P2X subunits, it has been difficult to clarify the role of $P2X_3$ in the physiology of the myenteric neurons of the guinea pig small intestine (Barajas-Lopez et al., 1996), where some authors propose that ATP effects on myenteric neurons are carried out mostly by $P2X_2$ (Zhou and Galligan, 1996).

Another possible role of $P2X_3$ in the ENS is related to development of myenteric neurons. Neurons of sensory ganglia prominently express $P2X_3$ during embryogenesis, which is downregulated in the adult stage (Figure 4) (Ruan et al., 2004). In the myenteric plexus of rat´s stomach, $P2X_3$ appears early in development since immunoreactivity for $P2X_3$ is detected in extrinsic sensory nerves at days E12-14 (Xiang and Burnstock, 2004a); still, in the myenteric plexus it is seen only after birth, when 45% of myenteric neurons express $P2X_3$ receptors. $P2X_3$ positive neurons decreased subsequently and at P30 only about 13% were $P2X_3$ immunoreactive. Nevertheless, similar experiments focusing on development of myenteric neurons in guinea pigs are lacking. Other subunits could also be involved in myenteric plexus development, e. g., in the rat´s brain, $P2X_4$ is regulated during neurogenesis (Burnstock and Ulrich, 2011) and is present in the myenteric neurons of adult guinea pig, but its expression in the early myenteric

plexus is unknown. There is also evidence of strong repression of $P2X_5$ in the development of neural precursors of the peripheral nervous system (Zeng et al., 2013), however, the expression of this subunit during myenteric plexus development is also unknown.

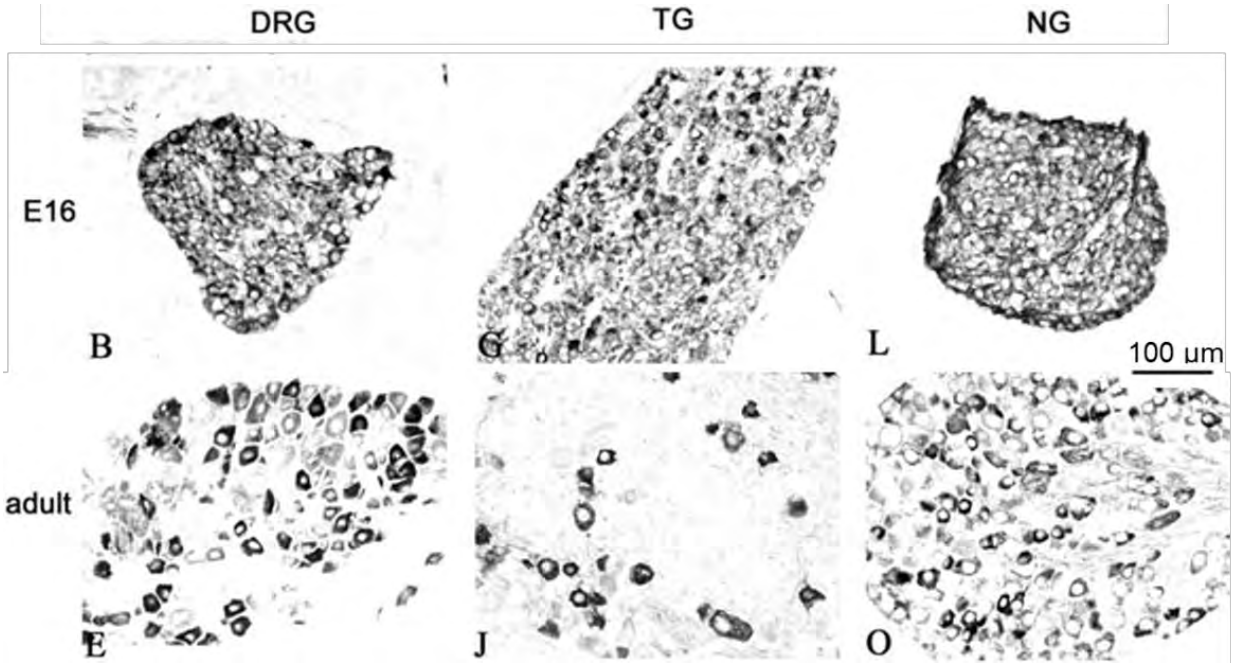

Figure 4. Regulation of $P2X_3$ expression in sensory ganglia. Cross sections of dorsal root ganglia (DRG), trigeminal ganglia (TG) and nodose ganglia (NG) of 16 day embryos (E16) and adult mice were stained to detect $P2X_3$ using immunohistochemistry. While most neurons in E16 ganglia are immunoreactive to $P2X_3$, in the adult only a subpopulation of small diameter sensory neurons are stained. Modified from (Ruan et al., 2004).

1.5. Neurotransmitter receptors in ICC

As well as the myenteric neurons, ICC expresses multiple neurotransmitter receptors. They possess receptors to Substance P and neurokinins called NK receptors (Vannucchi et al., 1999); serotonin can also stimulate ICC via 5HT2B receptors (Wouters et al., 2007), but perhaps the more prominent receptors in ICC are the ones responding to ACh.

There exists seven muscarinic receptor subtypes reported and they are termed M1, M2, M3, M4, M5, M6 and M7 (Caulfield and Birdsall, 1998). ICC of the small intestine expresses M2 and M3 receptors (Chen et al., 2007b). M2 subtype receptors couple to $G_{i/o}$ proteins that reduce adenylyl cyclase activity and lowers levels of the relaxant agent cAMP in the cytoplasm. M3 receptors couple to $G_{q/11}$ proteins that produces inositol 1,4,5-trisphosphate (IP3) and release calcium from the endoplasmic reticulum (Ehlert et al., 2012). In both cases, the activity of muscarinic cholinergic signaling is excitatory and can initiate pacemaker activity in ICC (Kim et al., 2003).

The presence of neurotransmitters in ICC opens the possibility for them to be regulated by neuronal stimulation as previously pointed. In the colon, ICC-MP does not produce the omnipresent slow waves as they do in the small intestine, but they generate rhythmic transient depolarisations that can be related to propulsive patterns in the colonic smooth muscle (Rae et al., 1998, Yoneda et al., 2004). Since the motor complex in the colon is thought to be activated by distention, the neuronal activation of the peristaltic reflex can be helped by the co-activation of ICC through excitatory neurotransmitters (Figure 5) (Costa et al., 2013). One experimental possibility to test this effect is the stimulation of ICC-MP with carbachol, which is an acetylcholine receptor agonist that is not hydrolysed by acetylcholinesterase. Carbachol strongly promotes rhythmic activity in ICC, even when neurotransmission is blocked by TTX (Huizinga et al., 2011); however its effect in the colon has not been thoroughly explored, since most studies in the past have focused in small intestine and stomach (Thuneberg, 1999, Sanders et al.,

2006). The muscarinic cholinergic excitation of ICC can come from a depolarization in the membrane potential through liberation of intracellular calcium, since carbachol stimulation of ICC has shown to activate calcium dependent chloride channels. Another possibility is the inactivation of potassium channels in the plasma membrane, given their roles modulating the upstroke potential of slow waves and the recovery to resting potential of ICC.

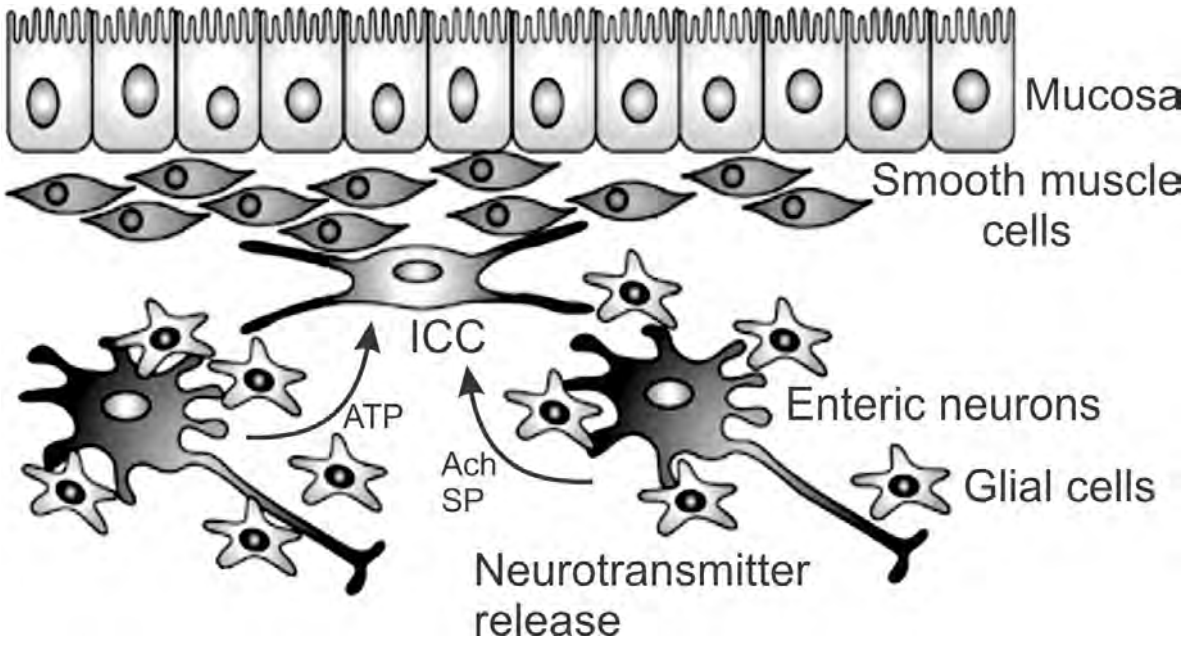

Figure 5. Neurotransmission signaling to interstitial cells of Cajal. Smooth muscle cells are directly innervated by myenteric neurons, but some nerve endings have been found forming synapse-like connections with interstitial cells of Cajal (ICC). When neurotransmitters such as Substance P (SP), Acetylcholine (ACh) or even ATP are released by enteric neurons, receptors in the ICC plasma membrane can change their electrical activity and calcium homeostasis, altering their influence over smooth muscle contractility. Modified from (Bassotti et al., 2007).

THE PREMISE OF THIS STUDY

There exist diverse questions in the research of neurotransmitters present in the cells controlling gut motility. One of them is the composition of the native P2X receptors present in the myenteric neurons and their change during development, given the evidence of $P2X_3$ downregulation in sensory ganglia (related to myenteric ganglia in embryonic origin) (Ruan et al., 2004) and in nerves present in the stomach´s myenteric plexus during development (Xiang and Burnstock, 2004a). There exist only few pharmacological tools to assess the composition of native P2X receptors in myenteric neurons and most of them lack enough selectivity to dissect the participation of a specific subunit in the response to ATP stimulation (Barajas-Lopez et al., 1996). To answer this question we propose the use of single cell PCR protocol, which has been successfully adapted in our laboratory to analyze single myenteric neurons (Valdez-Morales et al., 2011, Linan-Rico et al., 2012, Nieto-Pescador et al., 2013).

Other questions are the neurotransmitter composition and function of colonic ICC. Usually the ICC networks are embedded with the neurons of the different plexus in the gut or within the smooth muscle layers (Rumessen and Thuneberg, 1982, Rumessen et al., 1982, Liu et al., 1993). When performing whole RNA analysis of gut preparations, a mix of genetic material from smooth muscle, pericytes and neurons will be obtained. Given the close embryonic origin of ICC and smooth muscle, sharing of several transcripts is expected; therefore RNA analysis of whole gut portions is not viable. Primary cultures of ICC also have different cell populations that would contribute with their own RNA and potentially cause false results. The solution proposed previously to perform transcript analysis in ICC requires large equipment and enrichment of ICC populations through fluorescence activated cell sorting (Ordog et al., 2004). That is the reason why we decided to adapt single cell PCR, together with other molecular tools, to study neurotransmitter receptor expression in ICC of the colon and help clarify their physiology.

GENERAL OBJECTIVE

To determine the presence of neurotransmitter receptors and channels in cells controlling the motility of the gut through molecular methods and establish possible physiological phenomena related with them.

1.6. Specific aims

- To study the expression of $P2X_3$ and other P2X subunits of myenteric neurons during postnatal development and their possible role in the formation of native P2X channels.

- To identify the effects of cholinergic stimulation of colonic ICC and its mechanism of action.

HYPOTHESES

- Based on what happens in the stomach we expect that P2X3 receptors are downregulated with the postnatal age.

- Since acetylcholine is an excitatory neurotransmitter that induces contraction in most *muscularis externa* it is expected that it would act by inhibiting potassium channels in ICC.

CHAPTER 2. Expression of P2X3 and P2X5 Myenteric Receptors Varies During the Intestinal Postnatal Development in the Guinea Pig

In the rodent brain P2X subunits have been involved in neural differentiation, inhibition of axonal growth and apoptosis during development (Brosenitsch et al., 2005, Huang et al., 2005, Heine et al., 2006, Guo et al., 2013). In ganglionic neurons of the peripheral nervous system, particularly in sensory ganglia, $P2X_3$ expression is abundant in neural precursors during development and decreases over time until adulthood (Dunn et al., 2005). Changes in $P2X_3$ expression occurs between embryonic and postnatal dorsal root ganglion neurons in the mouse, where uniform $P2X_3$ staining was shown by most embryonic cultured neurons, but only 50% of cultured neurons were $P2X_3$ positive at 14 days after birth (P14) (Ruan et al., 2004). Electrophysiological recordings also showed that rat superior cervical ganglion (SCG) neurons responded to alpha-beta methylene ATP (α,β-meATP) at embryonic day 18 (E18), birth and early postnatal stages and not at all in mature rat neurons (P17), where inward currents are still evoked by ATP, suggesting a prevalence of $P2X_2$ over $P2X_3$ in adulthood (Dunn et al., 2005). In rat sensory ganglia, $P2X_3$ expression appears early (E12) in the development and it decreases prior to birth (Xiang and Burnstock, 2004b). The embryonic origin of sensory ganglia and myenteric ganglia arise from the neural crest cells (Bhatt et al., 2013). However, important differences exist regarding the expression of $P2X_3$ receptor in these two sets of ganglia (Xiang and Burnstock, 2004a). Thus, in the rat, immunoreactivity for $P2X_3$ is detected in extrinsic sensory nerves of the stomach very early during development (E12-14) but in the myenteric plexus of this organ it is seen only after birth, when 45% of myenteric neurons express $P2X_3$ receptors. $P2X_3$ positive neurons decreased subsequently and at P30 only about 13% were $P2X_3$ immunoreactive (Xiang and Burnstock, 2004a).

Differential expression of other P2X subunits is observed in the neurogenesis of the rat brain, where $P2X_4$ and $P2X_5$ are detectable from P1 to adulthood but not in the embryo stage (Burnstock and Ulrich, 2011). In the myenteric plexus $P2X_4$ is present in adult ganglion neurons of the guinea pig and

there is evidence of strong repression of $P2X_5$ in the development of neural precursors of the peripheral nervous system (Zeng et al., 2013); however, the expression of these subunits has not been determined in the early myenteric plexus. A previous work reported significant changes in morphology and electrophysiological behavior of myenteric neurons of the duodenum from the neonatal to the adult stage (Foong et al., 2012). Thus, Dogiel Type II (DII) neurons from neonatal animals displayed prominent after-depolarizing potentials and shorter projections than adult DII neurons.

While changes in the physiology of the small intestine during postnatal development are evident, the role of the myenteric plexus and the molecular bases of these changes are not clear. Because $P2X_3$ receptor expression in various tissues appears to be modulated during development, in the present study, we used single cell RT-PCR to determine how often the transcript for $P2X_3$ receptor is expressed in myenteric neurons at different stages of the postnatal development and we tested if similar changes also occur for other myenteric P2X receptors. We found that $P2X_3$ transcripts are expressed by a larger number of neurons in the newborn than in adulthood and this correlates well with the protein expression in the myenteric plexus preparations. Whole-cell recordings of ATP-induced membrane currents, however, indicate that variations of $P2X_3$ receptors do not correlate with higher sensitivity of P2X myenteric channels to ATP and α,β-meATP, a known $P2X_3$ receptor agonist, which would imply that other subunits are more important in determining the properties of these native channels. In agreement with such an interpretation, we found that the number of myenteric neurons expressing $P2X_2$ and $P2X_4$ transcripts remain unchanged between newborn and adult stages, but neurons expressing $P2X_5$ transcripts increased in the adulthood. $P2X_7$ receptor was not studied here because it is known not to form heteromeric channels (North, 2002, Khakh and North, 2006). We discuss how $P2X_3$ subunits could be forming heteromeric channels without altering their sensitivity to ATP and α,β-meATP.

2.1. MATERIALS AND METHODS

2.1.1. Primary cultures of neurons from myenteric plexus of guinea pigs

Dunkin-Hartley guinea pigs (P0-P3, 80 g-100 g for newborns; P7-P15, 150-200 g for young adults and P35 and over ≥250 g for adults) of either sex were killed by decapitation and a segment of 10-15 cm of the proximal jejunum was removed, placed in modified Krebs solution (126 mM NaCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5 mM KCl, 25 mM $NaHCO_3$, 11 mM glucose, gassed with 95% O_2 and 5% CO_2) and opened longitudinally. The mucosa and submucosal layers of the intestinal segment were surgically removed. All the animals protocols used were approved by the Animal Care Committee of the IPICYT and are in agreement with the Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training, approved by the American Physiological Society.

At this point, the whole *muscularis externa* with the myenteric plexus (myenteric preparation) was dissociated using a sequential treatment with two enzymatic solutions as previously described (Barajas-Lopez et al., 1996). Briefly, the first solution contained papain (0.01 mg/ml activated with 0.4 mg/ml L-cysteine) and the second contained collagenase (1mg/ml) and dispase (4 mg/ml). Enzymes were washed with L15 medium and the neurons were plated on round coverslips coated with sterile rat-tail collagen in culture solution (97.5% v/v MEM, 2.5% v/v fetal bovine serum, 2 mM L-glutamine, 10 U/ml penicillin, 10 µg/ml streptomycin, 15 mM glucose, 10 µM cytosine-β-D-arabinofuranoside, 10 µM fluorodeoxyuridine and 10 µM uridine).

2.1.2. Intestinal RNA or protein extraction

A segment of 10-15 cm of the proximal jejunum was placed in a pre-cooled mortar and triturated in presence of liquid nitrogen, then stored in specialized preserving solution according to the technique to be used (either RT-PCR or Western Blot). The resulting powder was weighted (75-100 mg) and used for RNA isolation.

2.1.3. RT-PCR

Tissues extracted from newborn, juvenile and adult small intestine and brain were triturated in a mortar with liquid nitrogen. Afterwards we weighted 500-700 mg of each tissue and collected it in Eppendorf tubes with 1 ml lysis solution from RNAqueous RNA isolation kit (Ambion). The RNA was obtained from the lysis solution using an affinity column and was collected for cDNA synthesis. For each tissue we used 6 µg of mRNA using superscript reverse transcriptase (Invitrogen) in the presence of oligo (dT) for 1.5 h at 42°C. We performed PCR in 25 µl with 300-500 ng of cDNA, 0.2 µM dNTPs, 2.5 µM $MgCl_2$, one unit of Taq DNA polymerase (Invitrogen) and 0.4 micromoles of 10 µM oligonucleotides (see Table 1 for nucleotide sequence and Tm). We assessed the specificity of the designed nucleotides (shown in Table 1) using the FastPCR software (Primer Digital Ltd.). Reaction conditions were as follows: Initial denaturation for 5 min at 95°C, then 35 amplification rounds composed of denaturation for 30s at 95°C, alignment for 30s at specific oligonucleotide temperature, extension for 1-1.5 min at 72°C; the final extension was 5 minutes at 72°C. We analyzed PCR products by electrophoresis in 0.8% agarose gels stained with 1 µg/ml ethidium bromide. For GAPDH, negative controls included omission of the reverse transcriptase reaction; for *P2X*$_3$ negative controls were performed with no cDNA template. Images were obtained with Gel-Doc 2000 documentation system (Bio-Rad).

2.1.4. Single Cell RT-PCR

We used single myenteric neurons from primary 3 days old cultures, which were harvested under visual control and differentiated from others cells types by the presence of long neurites (Eclipse TE200OU, Nikon). The neurons were sucked in with a glass pipette by applying negative pressure. This pipette had a tip diameter of about 4-6 µm and contained 6 µl of RNase-free RT buffer (with RNase inhibitor, 20 U; oligo (dT)18, 2.3 µM; dNTPs, 150 µM; dTT 1.2 mM; $MgCl_2$ 3.6mM and 10X RT Buffer Superscript III First-Strand Syntesis System; Life Technologies , Texas, USA). The content of the pipette was expelled into a PCR-tube containing 12 µl of RNase-free RT buffer and 0.5 µl of NP40 1% to allow cell membrane disruption. The reaction was incubated at 65°C for 2 min. After adding 0.5 µl reverse transcriptase III, the sample was then placed at 50°C for 60 min. Reverse

transcriptase inactivation was done by heating the sample to 70°C for 10 min and then placed on ice. Negative controls were performed without template; no false amplifications were obtained. As pre-amplification step, a PCR was performed using Platinum Taq DNA Polymerase with a first set of primers (Life Technologies, Texas, USA), then a nested PCR was carried on with internal primers (See Table 1). The PCR protocol were as follows: initial denaturation for 3 min at 94 °C, then 30 amplification rounds with denaturation for 15 s at 94°C, alignment for 15 s at 50°C, and extension for 1 min 45 s at 72°C; the final extension was 5 min at 72°C. A nested PCR reaction was performed with internal combinations of primers using the first PCR reaction as template.

2.1.5. Western blot

We obtained the total protein from brain and small intestine of newborn and adult guinea pigs as previously reported (Barclay et al., 2002). Briefly, we placed the protein samples in 5 ml glass tubes with 2 ml of low salt lysis buffer (50 mM Tris-HCL; 10mM NaCl and 1% Triton X-100), and triturated mechanically with a sterile pistil. Tissues where sonicated during one minute whit cycles of 5 s on and 5 s off, interruption of 1 min and another minute of the same pulses. The homogenates were then centrifuged at 2500 g for 5 min, to eliminate tissue debris.

Finally, 2 to 3 µg of protein from the samples with 80mM DTT were separated on 12% SDS polyacrylamide gels. Gels were run at 120 V for 150 min and 4°C. Separated proteins were transferred to nitrocellulose membrane for 1 h at 400 mA, and 4°C. Then nitrocellulose membranes were blocked with 8% non-fat dry milk in Tris buffered saline (20mM Tris base, 136mM NaCl, pH 7.6), 0.1% Tween 20 (TBST) and were then incubated over night with polyclonal antibodies against P2X3 (rabbit IgG, Alomone Labs) diluted 1:500 in TBS-T. Then washed and incubated for 2.5 hours with a horseradish peroxidase- (HRP) conjugated goat anti-rabbit immunoglobulin G antibody, at a 1:3000 v/v dilution (cat. no. 12-348, Millipore, CA, USA). Immunoreactivity was visualized by using 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma, Missouri, USA). Apparent molecular weights were assigned with the protein ladder (Benchmark Prestained Protein Ladder,

Invitrogen Co, CA, USA). Total protein extracted was quantified by the method of Bradford (Bradford, 1976).

Table 1

Identifier	Oligonucleotide	Annealing Temperature (^{o}C)	Annealing region	Position
Guinea Pig *P2X$_2$*				
5	5'- gttctgggcaccatggctgc-3'	55	5'UTR	-12
6	5'- tcctgtctgcagacctggcgt-3'	56	ORF	+1559
7	5'-ttcacagagctggcacacag-3'	55	ORF	+742
8	5'-atggtgggaatcaggctgaa -3'	55	ORF	+944
Guinea pig *P2X$_3$*				
1G	5'-atgaactgcatctcagacttcttcac-3'	53	ORF	+1
2G	5'-ttagtggccgatggagtagg-3'	53	ORF	+1157
3G	5'-tgggggacgtggtccggtttg-3'	56	ORF	+653
4G	5'-gtccacggactcaggcgcctac-3'	56	ORF	+1161
Guinea pig *P2X$_4$*				
5G	5'-ttcctg ttcgagtacga-3'	55	5'UTR	-60
6G	5'-ctgtagtacttggcaaac-3'	55	ORF	+908
7G	5'-cagctcaggaggaaaact-3'	58	ORF	+15
8G	5'-aatgcacgacttgaggta-3'	58	ORF	+546
Guinea pig *P2X$_5$*				
9G	5'-agcgttcgactacaagacagaaaa-3'	55	ORF	+35
10G	5'-ggcccagacatctttgaatc -3'	55	ORF	+526
11G	5'-ggagcctgttagagctggaaat-3'	58	ORF	+414
12G	5'-gccggaagatgggacaatagtga-3'	58	ORF	+678

Oligonucleotides used in the RT-PCR of guinea pig $P2X_2$ (GenBank ID: NM_001172709.1); $P2X_3$ (ENSEMBL: ENSCPOG00000019432); $P2X_4$ (NM_001281791.1) and $P2X_5$ (ENSEMBL: ENSCPOT00000015675).

2.1.6. Electrophysiology

In order to determine the activity of ATP and α,β-meATP on myenteric neurons from both newborn and adult guinea pigs we used the patch clamp technique in its whole cell configuration. We used the Axopatch 1D or the Axopatch 200B amplifiers (Molecular Devices, Sunnyvale, CA, USA). The patch pipette was manufactured as previously described (Barajas-Lopez et al., 1996) and had resistances between 1 and 4 MΩ. The holding potential of the neurons was -60mV. Whole cell currents were recorded using the AxoScope 9 software (Molecular Devices). The composition of the internal pipette solution was (mM): CsCl, 150; EGTA, 10; HEPES, 5; NaCl, 10; ATPMg, 4.5 and GTPNa, 0.1, and was adjusted to pH 7.3–7.4 with CsOH. The external solution composition was (mM): NaCl, 160; CaCl2, 2; glucose, 10; HEPES, 5 and CsCl, 3; the pH was adjusted to 7.3–7.4 with NaOH. Gigaseals obtained were always at least 1 GΩ. Drugs used in these experiments were applied using a fast perfusion system.

2.1.7. Sequence analysis

The oligonucleotides used in this work were designed with the Primer Premier software (Biosoft) and tested for *in silico* PCR with FastPCR software (Primer Digital). The information obtained from the sequencing of the different P2X PCR products were analyzed using the DNAstar software (Lasergene). We made sequence alignments using nBLAST, pBLAST (both searching mouse genomic and transcript database) and the Align software of the European Bioinformatics Institute in his local (water) and global (needle) modes. The software Mega 5 was also used with the Clustal V and Clustal W algorithms.

2.1.8. Statistical analysis

The data obtained from semi quantitative RT-PCR and electrophysiology was tested using one-way ANOVA with Bonferroni-Haselman *post-hoc* test for paired comparisons. The statistical difference in frequency of single cell gene expression was analyzed using Fisher's exact test, a non-parametric statistical test. Two-tailed P-values of 0.05 or less were considered statistically significant. Data are

expressed as the mean ± standard error of the mean (S.E.M.). The number of cells used is represented by "n".

2.2. RESULTS

2.2.1. The $P2X_3$ subunit is downregulated in the small intestine during development

Using semi quantitative RT-PCR we compared the $P2X_3$ expression in small intestine as function of the normalized expression of GAPDH, at least three different cDNAs from different subjects were used for each stage of development. Three stages of development were selected: newborn (P0-P3), juvenile (P7-P15) and adult (≥P35). We found that the highest $P2X_3$ transcript expression levels were found in the newborn specimens, this level diminished in the juvenile intestine until the lowest expression levels in the adult stage and being significantly different at every stage (Fig. 6A).

To assess if changes in the $P2X_3$ transcript correlate with changes in $P2X_3$ protein, we performed the western blot technique in total protein extracts from small intestine (Figure 6B). The amount of total protein extracts loaded into the gels was 5 µg, with this quantity we were able to detect a band between 82 and 115 KDa (90 KDa approximately) in the newborn sample that was barely detectable in the juvenile and adult samples. This observation correlates well with the downregulation seen at the mRNA level. $P2X_3$ transcript is expected to be in myenteric neurons and it is likely that, at least part of this downregulation is by changes occurring in these cells. In order to test this hypothesis we determined the frequency of $P2X_3$ expression in single myenteric neurons.

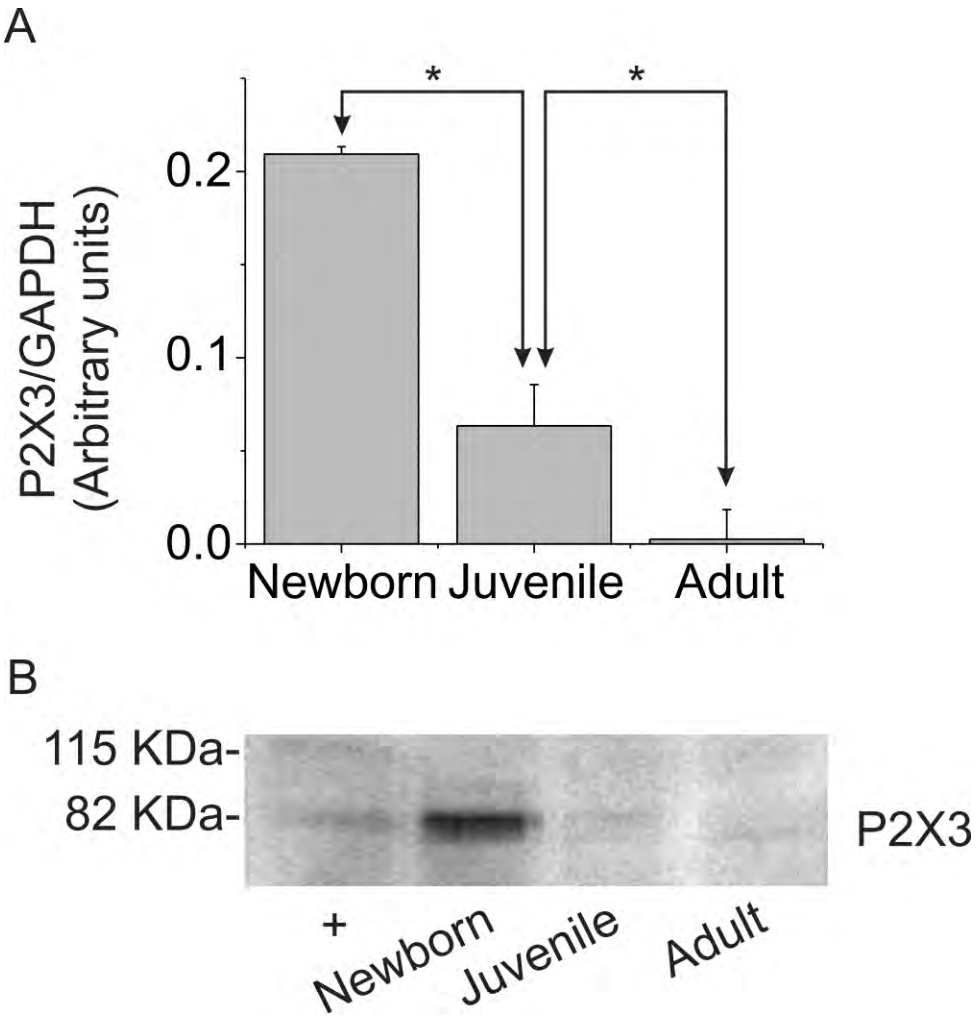

Figure 6. Expression of the $P2X_3$ subunit is decreased with age in the small intestine of guinea pig. **A**) Semiquantitative RT-PCR was performed in total RNA extracts from small intestines of guinea pigs. The expression of $P2X_3$ is shown as a fraction of GAPDH expression. The relative expression of $P2X_3$ is shown for newborn (P0-P3), juvenile (P7-P15) and adult (>P35) guinea pig small intestines. The letters show statistical differences between the three groups ($p<0.05$). **B**) Western blot of whole protein extracts from small intestine of newborn, juvenile and adult guinea pigs. All lines were loaded with 5 micrograms of protein. Primary antibody dilution was 1:1000 and it was revealed with 3,3',5,5'- tetramethylbenzydene substrate.

2.2.2. The $P2X_3$ and $P2X_5$ subunits are differentially regulated in myenteric neurons during development

We examined the presence of the $P2X_3$ subunit in single myenteric neurons from newborn, juvenile and adult guinea pigs (Figure 7), using the single cell RT-PCR technique and the transcripts for $P2X_2$ (known to be expressed by most myenteric neurons; (Linan-Rico et al., 2012)) and GAPDH as controls. We found that for the $P2X_2$ subunit was detected in the great majority neurons and its expression frequency was virtually the same for newborns (87%; 40 out of 46 neurons), juvenile (75%; 6 out of 8 neurons) and adults (89%; 16 out of 18 neurons). The $P2X_3$ subunit, however, is present in 59% of analyzed neurons (14 out of 24) from newborn animals, dropping to a frequency of expression to 38% (3 out of 8 neurons) and 10% (3 out of 29 neurons) in the juvenile and adult animals, respectively (Figure 7A-D). This difference in the frequency of $P2X_3$ expression between newborns and adults was significantly different (Figure 8B). In most analyzed neurons, we tested the expression of GAPDH as control gene to confirm the correct cDNA synthesis, in few neurons of adult animals we used expression of $P2X_2$ as a control gene (Figure 7D).

We also investigated if the downregulation was specific for $P2X_3$ receptor by testing simultaneously for the transcripts of $P2X_4$ and $P2X_5$ in the same myenteric neurons from newborn and adult animals (Figure 8A). We found that the frequency of $P2X_4$ positive neurons in newborn animals (63%, n=24) was not different than that observed in adults (80%. n=10). However, as it shown in Figure 8B, the number of $P2X_5$ positive neurons was significantly lower in newborns (29%, n=24) than in the adults (62%, n=29).

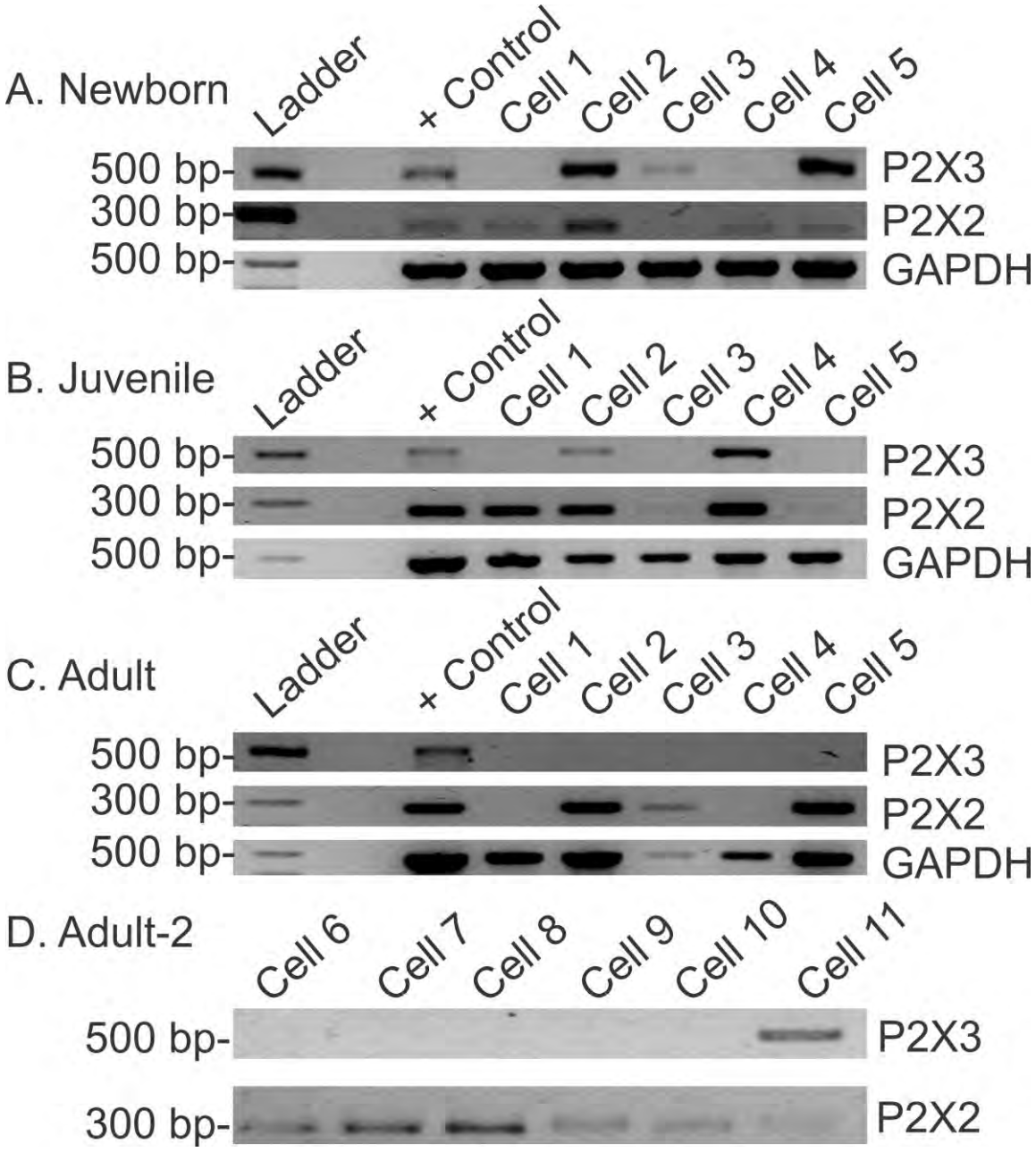

Figure 7. $P2X_3$ transcript is downregulated in myenteric neurons during postnatal development. RT-PCR was performed in single myenteric neurons in culture from newborn (A), juvenile (B), or adult (C-D) guinea pigs to determine $P2X_3$, $P2X_2$, and GAPDH transcripts. Each column corresponds to a cDNA sample synthesized from a single myenteric neuron. Whole brain cDNA was used as positive control. Ladder denotes the molecular weight marker (1Kb plus, Invitrogen).

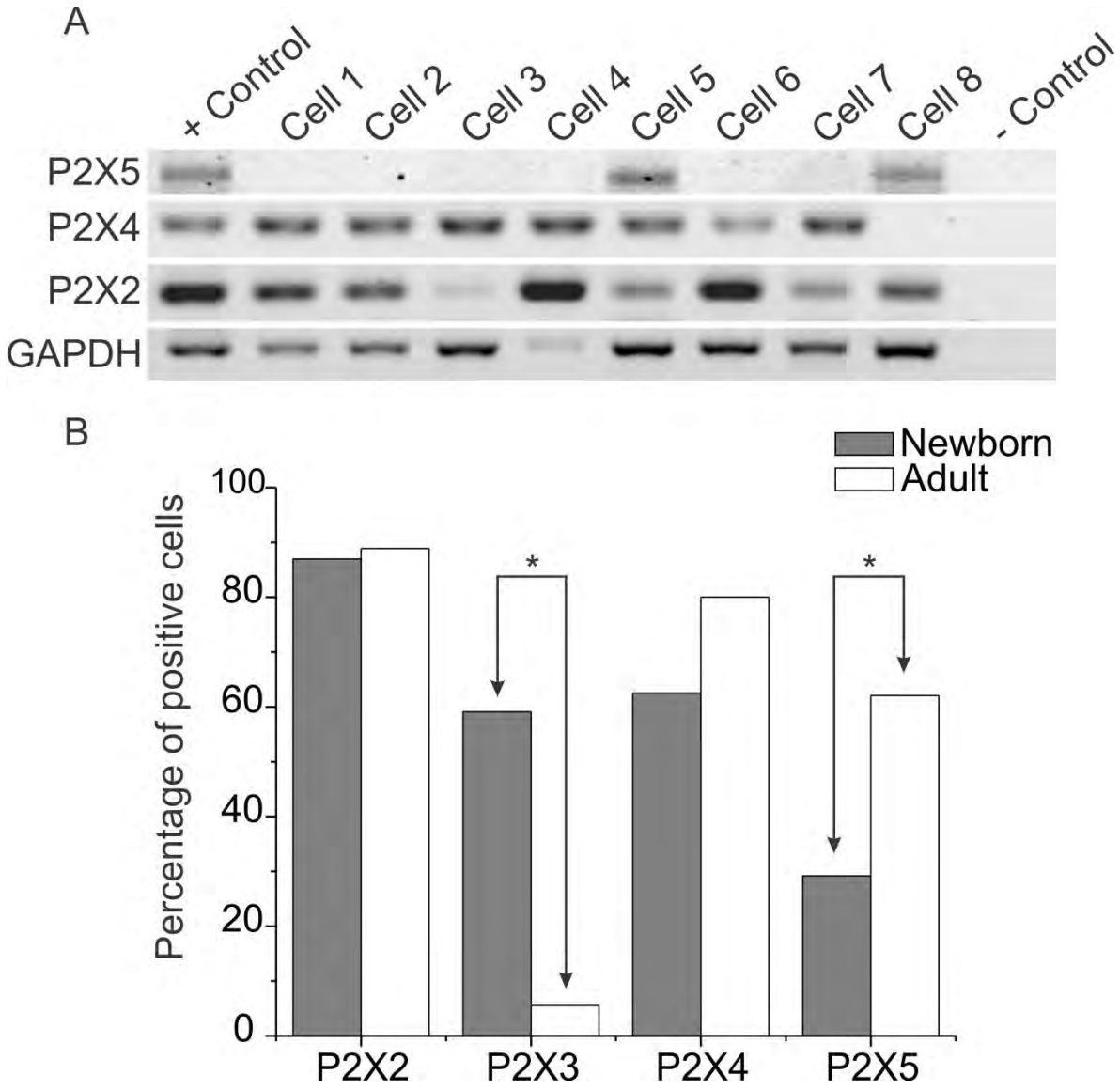

Figure 8. $P2X_3$ and $P2X_5$ transcripts are differentially expressed in myenteric neurons during postnatal development. A) RT-PCR was performed in eight myenteric neurons from a newborn guinea pigs and the expression of $P2X_2$, $P2X_4$ and $P2X_5$ transcripts was determined. GAPDH is used as control. RT-PCR was performed in culture neurons from at least three different guinea pigs. Intestine cDNA was used as positive control. **B**) Frequency histogram for P2X subunits expressed in single neurons. Made with data collected from experiments described in **A,** but in neurons from both newborn ($P2X_2$: 46 cells; $P2X_3$: 22 cells; $P2X_4$: 24 cells; $P2X_5$: 24 cells) and adult guinea pigs ($P2X_2$: 18 cells; $P2X_3$: 18 cells; $P2X_4$: 40 cells; $P2X_5$: 29 cells). The quantity of neurons expressing $P2X_3$ and $P2X_5$ transcripts were found to be significantly different at these two developmental stages, using the Fisher's exact test.

2.2.3. Myenteric P2X receptors from newborn and adult animals have the same ATP sensitivity

Whole cell currents induced by ATP (10, 30, 100, and 300 μM) were normalized (considering as 100% the response to 300 μM ATP) and plotted to generate concentration-response curves of neurons from newborn and adult animals (Fig. 9). For both cases, data were well fitted with a logistic equation, which yield EC_{50}s of 56 μM and 44 μM, respectively.

2.2.4. α,β-meATP is a weak agonist in myenteric P2X channels of newborn animals

We examined the response of newborn guinea pig myenteric neurons to α,β-meATP at a concentration sufficient to activate $P2X_3$ channels (100 μM). ATP (100 μM) induced an average current of -1007±198 pA in 26 out of 27 neurons. In 6 out of these 26 neurons, α,β-meATP induced a current response but in only one neuron this current (175 pA) was similar in amplitude to the one observed with ATP (185 pA). In the other five neurons that responded to α,β-meATP the magnitude of the average response was 6±2.5% of that induced by ATP (100 μM), typical responses to these two agonists are shown in Fig 10A-B.

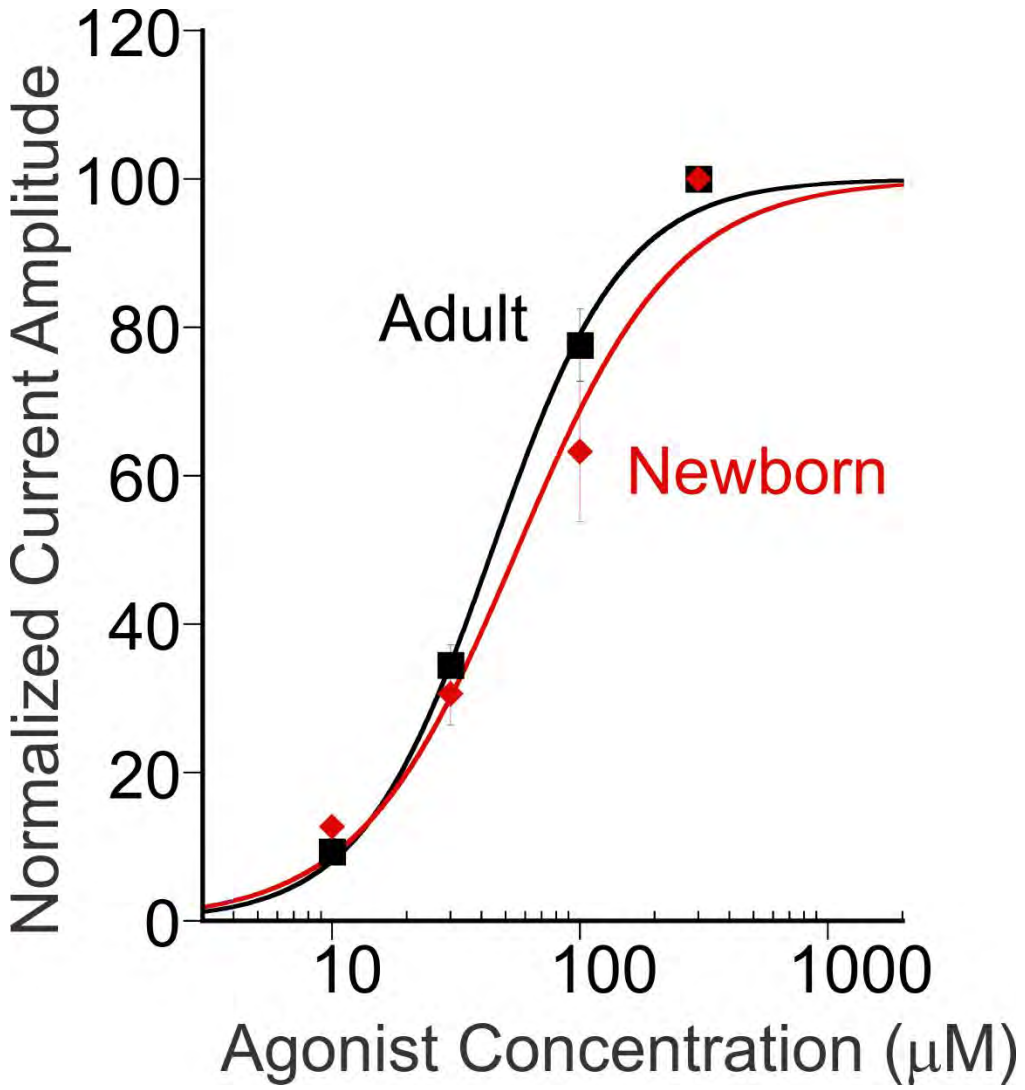

Figure 9. ATP shows, virtually, the same potency to activated P2X myenteric receptors from neurons of newborn or adult animals. For these concentration-response curves data were normalized with respect to the response observed with 300 μM ATP and fitted with a two parameters logistic equation. Each symbol represents the average of 4-10. The calculated EC_{50} for newborn guinea pigs was 56 μM, while for adults was 44 μM. Currents induced at any given ATP concentration, for neurons from animals at both developmental stages, were not significantly different.

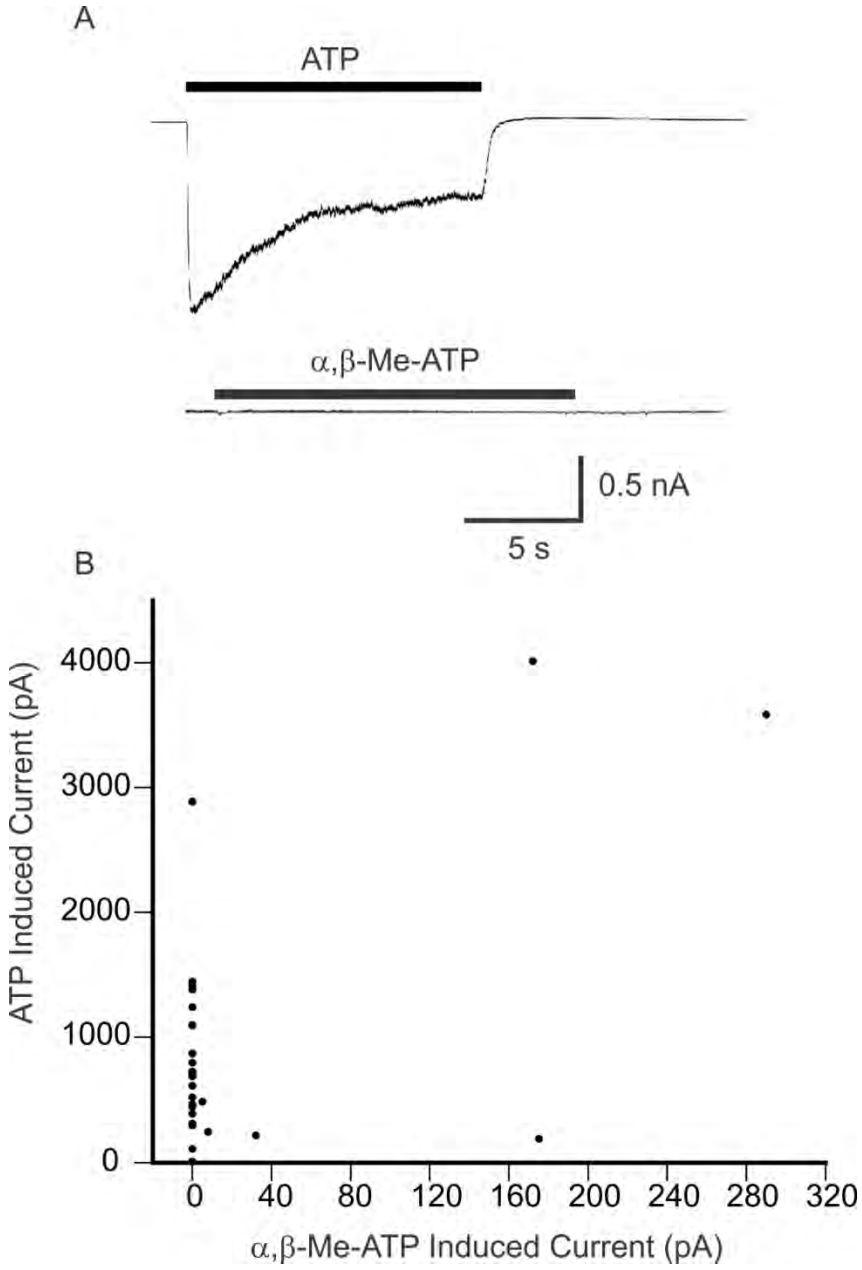

Figure 10. Whole-cell ATP and α,β-Me ATP evoked currents (I_{ATP}) in myenteric neurons from newborn guinea pigs. **A**) Recordings of representative currents from the same neuron induced by ATP (upper panel) or α,β-Me ATP (lower panel). I_{ATP} (100 μM) induced a fast inward current with slow desensitizing kinetics; whereas, α,β-Me ATP evoked only a marginal current. Currents were recorded at -60 mV. **B)** Currents evoked by ATP and α,β-Me ATP in 26 experiments like the one shown in **A**. α,β-Me ATP induced only a small response in in 5 out of 26 neurons as compared to the ATP response and only in one of these 26 neurons responded similarly to ATP. Every circle corresponds to the data from one neuron.

2.3. DISCUSSION

This study presents evidence indicating that in the intestinal myenteric neurons $P2X_3$ receptors are expressed more frequently in the newborn guinea pigs than in adults. Opposite to this, we found that the $P2X_5$ mRNA is upregulated during postnatal development. The great majority of neurons expressing $P2X_3$ also expressed $P2X_2$ transcripts. This is important because these last two subunits are known to form heteromeric channels (Lewis et al., 1995, Jiang et al., 2003). Our electrophysiological experiments indicates, however, that the higher frequency of $P2X_3$ subunits in newborns does not correlate with changes in the sensitivity of P2X myenteric channels to either ATP or α,β-meATP, a known $P2X_3$ receptor agonist. These findings imply that $P2X_3$ subunits are forming heteromeric channels, which are mainly α,β-meATP insensitive. Other subunits expressed in myenteric plexus might be more important as they are more frequently co-expressed. Thus, $P2X_2$ and $P2X_4$ transcripts are more often found in neurons of the myenteric plexus at the different ages tested.

Our data are consistent with previous findings that have shown a higher expression of $P2X_3$ receptors in peripheral neurons at earlier stages of development. Thus, in the rat gastric myenteric neurons (Xiang and Burnstock, 2004a), $P2X_3$-immunoreactivity appears postnatal at P1, and it becomes maximal at P14, when the number of neurons expressing the $P2X_3$ receptor peaked at 45%. This immunoreactivity decreased subsequently, and at P30 is only present in about 13% of gastric myenteric neurons. Similar differences exist regarding the expression of $P2X_3$ receptor in sensory ganglia (Xiang and Burnstock, 2004a). However, in sensory neurons immunoreactivity for $P2X_3$ is detected much early during development (E12-14); whereas, in the myenteric plexus of the rat stomach is seen only after birth (Xiang and Burnstock, 2004a). It has been reported that expression of $P2X_3$ receptors in gut enterochromaffin cells is downregulated during inflammatory conditions (Linan-Rico et al., 2013) and by peripheral nerve injury in sensory ganglia (Bradbury et al., 1998). Therefore, it is possible that the

expression of $P2X_3$ receptor could be downregulated by the dissociation (which removes the neuronal processes) and culturing procedures. Thus, in the jejunum of adult guinea pigs, $P2X_3$ transcript was found in 10% of myenteric neurons, a lower percentage than the calculated (36%) from neurochemical experiments in the guinea pig ileum (Van Nassauw et al., 2002). However, in newborns we detect the transcript of this subunit in about 60% of myenteric neurons meaning that the impact of experimental procedures might decreased the expression to a maximum of 40%; this if the transcript would be expressed by 100% of the neurons, which is unlikely. In agreement with our findings in single neurons we also documented the downregulation of this receptor in acutely dissected myenteric plexus preparations. It is important to underline that the frequency of $P2X_3$ mRNA expression (10%) found here is similar to that reported in mouse (Bian et al., 2003) and rats (Xiang and Burnstock, 2004a).

The fact that in the gastrointestinal tract the upregulation of $P2X_3$ receptor occurs during the weaning period of guinea pigs suggests that it might be related to gut adaptation to new environment and functions like, peristalsis, food and gut colonization by bacteria. Other early adaptations in the gut include the enteric mucosal innervation that appears at birth (Paran et al., 2006), changes in epithelial cell proliferation (Al-Nafussi and Wright, 1982), and in the expression of $P2X_4$ by epithelial cells in the proximal gut (Garcia-Alcocer et al., 2012).

In the present study, we also showed that in myenteric neurons $P2X_5$ is lower and that $P2X_3$ receptor is higher during the early postnatal development than in adults. These findings contrast with previous findings in the rat prostate where ageing associates to higher levels of $P2X_3$ and lower $P2X_5$ receptors (Slater et al., 2000). Suggesting that changes in these receptors might be the result of local modulatory signals and tissue specific.

How the different expression of these receptors affect the physiology of P2X receptors, enteric neurons, and gut physiology needs to be addressed in future experiments. However, the expression pattern of $P2X_3$ in newborn guinea pig myenteric plexus points to a role in the differentiation process of neural

progenitors, acting through a mechanism still unknown. Previous evidence already suggested this phenomenon occurring in the central nervous system (Brosenitsch et al., 2005), in the cochlea (Huang et al., 2005), and peripheral nervous system (Xiang and Burnstock, 2004a, Wang et al., 2008b), particularly in sensory ganglia (Ruan et al., 2004). However, the importance of the regulation of this subunit is still unclear. In spiral neurons of the auditory system it has been shown that $P2X_3$ suppression is needed for the proper onset of hearing in rats, in this system the authors proposed a role in neural identity establishment (Huang et al., 2005). $P2X_3$ receptors are also known to functionally interact with $GABA_A$ receptors (Toulme et al., 2007) and therefore, it is possible that its role is to allow P2X channels to interact with other cytoplasmic or membrane proteins during the maturation of enteric neurons.

At least five different P2X subunits appear to be expressed in the small intestine myenteric neurons of guinea pigs (present study; (Castelucci et al., 2002, Xiang and Burnstock, 2005, Valdez-Morales et al., 2011, Linan-Rico et al., 2012)). Thus, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$ and $P2X_7$ mRNAs are present in these neurons, which is in agreement with the presence of some of these transcripts in enriched cultures of rat (Ohta et al., 2005) and mouse enteric neurons (Maria et al., 2013). The four transcripts are translated into proteins in rodent myenteric neurons as it is indicated by the immunoreactivity for $P2X_2$ (Castelucci et al., 2002, Ren et al., 2003, Ruan et al., 2004), $P2X_3$ (Xiang and Burnstock, 2004b, 2005) $P2X_4$ (Bo et al., 2003, Maria et al., 2013), $P2X_5$ (Xiang and Burnstock, 2004b, Ruan and Burnstock, 2005, Maria et al., 2013) and $P2X_7$ receptors (Hu et al., 2001, Valdez-Morales et al., 2011, Gulbransen et al., 2012).

Our results confirm previous findings where $P2X_2$ is the most prominent P2X subunit in the myenteric plexus of adult guinea pigs (Xiang and Burnstock, 2005, Valdez-Morales et al., 2011, Linan-Rico et al., 2012), and extend the same predominance to myenteric neurons in early stages of postnatal development. In newborn animals, more than half of myenteric neurons expressed $P2X_3$ mRNA and all these neurons also co-expressed the $P2X_2$ transcript. These two subunits have

been shown to form heteromeric channels (Lewis et al., 1995, Jiang et al., 2003). The heteromeric $P2X_{2/3}$ receptors exhibit pharmacological properties similar to those of $P2X_3$ homomeric channels, including sensitivity to α,β-meATP and a similar rank order of agonist potencies, but they can be distinguished from homomeric $P2X_3$ channels by a slow desensitization rate. A more recent study, using heterologous expression of $P2X_2$ and $P2X_3$ homomeric channels and heteromeric $P2X_{2/3}$ channels have provided evidence that the quaternary trimeric structure of the later channels would have the stoichiometry of one $P2X_2$ subunit and two $P2X_3$ subunits (Wilkinson et al., 2006, Hausmann et al., 2012); stoichiometry that described as: $P2X_{2/3}$[1:2]. When non-functional $P2X_2$ mutants ([K69A] or [K308A]) were co-expressed with the wild type $P2X_3$ subunit, a fully functional channel was assembled; whereas, a double $P2X_2$ mutant ([K69A], [K308A]) could not be rescued by the native $P2X_3$ subunit, evidenced by the lack of response to α,β-meATP. Also the same single mutations in $P2X_3$ produced unresponsive channels. This suggests the necessity of two $P2X_3$ subunits to acquire α,β-meATP sensibility.

In the present study, we found that α,β-meATP: i) did not activated most P2X native channels of newborn animals, ii) induced a marginal response (6%) in one fifth of the neurons, and iii) induced a similar current than ATP in only one neuron (1 out of 26). It is unlikely that the marginal response to α,β-meATP could be mediated by either homomeric $P2X_3$ or heteromeric $P2X_{2/3}$[1:2] channels, because, our laboratory has demonstrated that this agonist also marginally activates (~7% of maximal I_{ATP}) the two $P2X_2$ receptor variants ($P2X_{2\text{-}1}$ and $P2X_{2\text{-}2}$) cloned from guinea pig myenteric neurons (Linan-Rico et al., 2012). Furthermore, neurons from adult guinea pigs also show a marginal response to α,β-meATP (Barajas-Lopez et al., 1996). Therefore, the small effect of α,β-meATP seen in one fifth of newborn myenteric neurons is not indicative of the presence of homomeric $P2X_3$ or heteromeric $P2X_{2/3}$[1:2] channels and is more likely an effect of this agonist on $P2X_2$ receptors, which are found more frequently.

We proposed that if $P2X_3$ subunits participate in forming heterologous channels they do it by providing only one subunit to form heterotrimers ($P2X_{n/3}$[2:1]). To date only the stoichiometry $P2X_{2/3}$[1:2] has been reported; however, there is evidence pointing that mRNA expression levels of $P2X_2$ transcripts can alter the subunit composition of the formed channels when co-expressed with $P2X_6$ (Wilkinson et al., 2006, Hausmann et al., 2012). The other two subunits of these heterotrimers could be either $P2X_5$ and/or any of the two $P2X_2$ splicing variants, which could be expected to form heteromeric channels with $P2X_3$ subunits (Burnstock and Verkhratsky, 2012). So far, heteromeric channels formed by some pairs of subunits have been reported (Burnstock and Verkhratsky, 2012) but it is unknown if the co-expression of more than two subunits, as it occurs in myenteric neurons (present study), would facilitate the assembling of heteromeric receptors in native membranes. Thus, at least part of the membrane currents mediated by myenteric native channels could also include homomeric or heteromeric $P2X_4$ receptors (Maria et al., 2013). $P2X_7$ receptor immunoreactivity or mRNA are found in most myenteric neurons (Hu et al., 2001, Valdez-Morales et al., 2011, Gulbransen et al., 2012); however, this subunit is known to only form homomeric channels (Burnstock and Verkhratsky, 2012), which are activated at ATP concentrations higher than 300 μM in the guinea pig (Valdez-Morales et al., 2011), concentrations that we did not use here. Therefore, it is unlikely that this subunit participates in the ATP induced currents recorded in the present study.

In summary, our data indicate that in the intestinal myenteric neurons $P2X_3$ receptors are expressed more frequently in the newborn guinea pigs than in adults; whereas, $P2X_5$ mRNA is found more frequently in adults. Expression of $P2X_2$ and $P2X_4$ genes do not change during postnatal development. In newborn animals, virtually all neurons expressing $P2X_3$ also expressed $P2X_2$ transcripts. This is important because these two subunits are known to form heteromeric channels. α,β-meATP, a known $P2X_3$ receptor agonist, induces only a marginal current despite the fact of the higher presence of $P2X_3$ subunits in newborns. These findings would imply that $P2X_3$ subunits are mainly forming heteromeric channels that are α,β-meATP insensitive perhaps because this subunit contributes with a

maximum of one subunit to the heterotrimers and others (e.g. $P2X_2$, $P2X_5$, or $P2X_4$) complete these channels with the two additional subunits.

CHAPTER 3. Cholinergic signaling-regulated $K_V7.5$ currents are expressed in colonic ICC-IM but not ICC-MP

With respect to the colon, although initial physiological studies were carried out on dog colonic circular muscle, most subsequent studies on ICC pacemaker mechanisms and ion channels have focused on small intestine and stomach (Thuneberg, 1999, Sanders et al., 2006, Huizinga and Chen, 2014). Hence, our knowledge of the colonic pacemaker mechanisms and ion channels involved is still limited. ICC associated with the myenteric plexus (ICC-MP) of the small intestine generate the omnipresent slow waves (Thomsen et al., 1998) that orchestrate rhythmic propulsive muscle contractions in the proximal intestine. In the colon, the omnipresent slow waves are generated by ICC associated with the submuscular plexus (ICC-SMP) in the canine (Smith et al., 1987, Liu and Huizinga, 1993), rat (Pluja et al., 2001), mouse (Yoneda et al., 2004) and human (Rae et al., 1998). ICC-MP from the rat (Pluja et al., 2001) and mouse (Yoneda et al., 2004) colon do not generate omnipresent slow waves, but generate rhythmic transient depolarizations of low and variable frequency, that might depend on activation of L-type calcium channels. Based on these findings, it was proposed that ICC-MP can be induced to generate the pacemaker activity governing a propulsive motor pattern of the colon at that frequency (Huizinga et al., 2011, Costa et al., 2013, Chen et al., 2013). Carbachol strongly promotes this rhythmic activity, even in the presence of TTX (Costa et al., 2013, Chen et al., 2013). The objective of the present study was to find mechanisms of excitation of the ICC-MP. Our specific hypothesis was that cholinergic inhibition of K^+ channels is involved.

3.1. MATERIALS AND METHODS

3.1.1. Cultured Cells

The preparation and electrophysiology for the cultured cells were the same as those described previously, except that colons from 8-15 day old CD-1 mice were used instead of small intestine. All procedures were approved by the Animal Research Ethics Board (AREB) of McMaster University.

3.1.2. *Solutions, Drugs and Electrophysiology*

Carbachol and XE991 were purchased from Sigma Aldrich, Oakville, ON, Canada. Drugs were prepared by dilution in bath solution to concentrations specified in the results from stock solutions in DMSO. Voltage protocols were adjusted according to the junction potential between the pipette and bath solutions (Parsons and Sanders, 2008, Parsons and Huizinga, 2010). The potential that the patch "sees" (E_{patch}) is the summation of the cell membrane potential (E_{cell}) and the potential applied to the patch relative to resting membrane potential (E_{rrmp}).

$$E_{patch} = E_{rrmp} + E_{cell}$$

In the results, potentials are expressed as E_{rrmp} followed in parentheses by E_{patch}, assuming an E_{cell} of -60 mV (Molleman, 2003). See Table 1 for the contents of bath and pipette solutions.

Table 2: Contents of solutions

Solution	Na^+	Cl^-	K^+	$NMDG^+$	Mg^{2+}	Ca^{2+}	HEPES	pH	glucose
Bath	135	146.4	5	-	1.2	2	10	7.35[a]	10
KCl pipette	-	150	150	-	-	-	10	7.0[a]	10
NMDG pipette	-	140	-	140	-	-	10	7.35[a]	10

[a]pH adjusted with Tris base. $NMDG^+$ = N-methyl-D-glucamine; HEPES = 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid.

3.1.3. *Data Analysis and Statistics*

Current traces for figures were filtered with a 140 dB 8-pole Bessel filter on Clampfit (Molecular Devices, Sunnyvale, CA, USA) and a notch electrical interference filter where necessary. The difference in current (ΔI) for the channel transition between one current level and another at a certain potential was measured, this was repeated for channel transitions at different potentials. The ΔI values plotted versus potential were fitted with linear regression, the slope of which

was used to determine the conductance; the x-intercept was used to determine the reversal potential of the currents. Mean reversal potentials and conductances were calculated from the linear fits of ΔI values from individual experiments; pooled data are shown in the figures fitted with linear regression.

Current voltage plots were created by averaging the currents evoked by the voltage ramps from control sweeps or from sweeps after inhibition by drugs. For control sweeps, the linear slope of the current from -80 (-140) to -20 (-80) mV, where no K^+ channels were active, was subtracted from the current evoked by the -80 (-140) to +80 (20) mV voltage ramp. For sweeps after inhibition by drugs, the linear slope of the current from -80 (-140) to +80 (20) mV was subtracted. A Z-test for proportions was used to determine differences between proportions with a significance level of $\alpha=0.05$.

3.1.4. *ICC RNA Extraction and Reverse Transcription*

RNA was obtained from CD-1 mouse brain tissue or single cultured ICC to perform RT-PCR. For tissue, the RNA extraction was done using the RNAeasy RNA isolation kit (Qiagen, Toronto, ON, Canada), according to the manufacturer's instructions. Brain RNA (1 µg) was used for reverse transcription. As a measure to control for gDNA PCR products, an RT minus control was used. To collect single cells, the patch clamp rig was used with unpolished, low resistance glass pipettes containing 6 µL of the RT-PCR mix (20 U of RNase inhibitor, 2.3 µM oligo (dT), 150 µM dNTPs, 1.2 mM dTT, 3.6 mM $MgCl_2$ and 10X RT Buffer (Life Technologies Inc., Burlington, ON, Canada)). The same parameters used to identify ICC for electrophysiology were used to select single cells from primary cultures. After collection, we used NP-40 detergent (1:100) to lyse the cells and release the genetic material. Tissue samples and single ICC were brought to a final volume of 20 µL with RT-PCR mix and 200 U of Superscript III RT. Then the samples were incubated at 50°C for 50 minutes. After the cDNA synthesis, the RT enzyme was deactivated by freezing overnight and subsequent thawing.

3.1.5. Nested PCR Protocol

PCR was performed using Platinum Taq DNA Polymerase (Life Technologies Inc.). To detect transcripts coding for $K_V7.5$ channels, we used the primers $K_V7.5$ ExtR 5′-TGTGTCGGCGTCTATCACTG. A nested PCR was carried afterwards with internal primers $K_V7.5$ intF 5′-GCCAGAGCATTAAGAGCAGAC and $K_V7.5$ intR 5′-ACTCTTGAGCCGTAGTGAGG. To amplify Ano1 we first used the primers Ano1 ExtF 5′-TGTACTTTGCCTGGCTTGGAGC and Ano1 ExtR 5′-CACCTGGCAATGCAGCCGTA, in the same way as with potassium channels, a nested PCR was performed next using the primers Ano1 intF 5′-CAACTACCGATGGGACCTCAC and Ano1 intR 5′-AATAGGCTGGGAATCGGTCC. To control for gDNA PCR products from the single cells, the external primers were designed to span at least one intron of the analyzed genes. No additional bands of higher size were detected. We omitted the template for negative controls. The PCR protocol was performed as follows on a CFX96 thermal cycler (Bio-Rad Laboratories Canada Ltd., Mississauga, ON, Canada): initial denaturation for 3 min at 94°C, then 30 amplification rounds with denaturation for 15 s at 94 °C, alignment for 15 s at 50°C and extension for 1 min 45 s at 72°C per round. The final extension was 5 min at 72°C. For the nested PCR the same protocol was used with 36 cycles and annealing temperature of 58-60°C per cycle. The resulting products were analyzed by electrophoresis in agarose gels (1.5%, Invitrogen, Burlington, ON, Canada) stained with 1 µg/mL ethidium bromide (Sigma-Aldrich). Images were obtained with Gel-Doc 2000 documentation system (Bio-Rad Laboratories Canada Ltd.). The resulting bands were cut then sequenced by MOBIX Laboratories (McMaster University, Hamilton, ON, Canada).

3.1.6. Immunohistochemical Staining

Cell culture preparations were the same as for electrophysiological study. In addition, the whole (proximal, mid and distal) colon was removed for frozen sections and musculature whole-mount preparations. Whole-mount specimens were prepared by peeling away the mucosa and submucosa. Frozen sections were made by embedding the tissues in Tissue-Tek (Miles Lab., Naperville, IL, USA)

and freezing in liquid nitrogen. Ten micron sections were cut with the cryostat and mounted on the coated slides. Staining for c-Kit was performed since it is a selective marker of ICC (Huizinga et al., 1995). For wholemount tissue and culture staining, specimens were fixed in ice-cold acetone for 10 min and then processed for c-Kit staining. After c-Kit staining, specimens were fixed again with 4% paraformaldehyde for 10 min and then processed for $K_V7.5$ staining or vesicular acetylcholine transporter (VAChT) staining. For frozen section staining, sections were only fixed with 4% paraformaldehyde for 10 min and processed for $K_V7.5$ and then c-Kit staining. The non-specific binding was blocked with 5% normal goat serum in all immunohistochemical staining, except for c-Kit/VAChT staining, in which 2% bovine serum albumin was applied. Tissues were then incubated overnight at room temperature in the following two groups of primary antibodies: rat anti-c-Kit (1:200, Cedarlane, Oakville, ON, Canada) and rabbit anti $K_V7.5$ (1:100, Santa Cruz Biotech., Santa Cruz, CA, USA); rat anti c-Kit and goat anti VAChT (1:100, Santa Cruz Biotech.). For c-Kit/Kv7.5 double labeling, secondary antibodies were Cy3 conjugated goat anti-rat IgG (1:400, Jackson Immuno Research, West Grove, PA, USA) and Alexa 488 conjugated goat anti-rabbit IgG (1:200, Jackson Immuno Research). For c-Kit/VAChT double labeling, secondary antibodies were Alexa 488 conjugated donkey anti-rat IgG (1:200, Jackson Immuno Research) and Cy3 conjugated donkey anti-goat IgG (1:400, Jackson Immuno Research). All antibodies were diluted in 0.05M PBS (pH 7.4) with 0.03% triton-X-100. Negative controls included the omission of primary antibodies from the incubation solution. All of the immunostaining was examined using a confocal microscope (Zeiss LSM 510, Göttingen, Germany) with excitation wavelengths (595 nm and 488 nm) appropriate for Cy3 and Alexa 488. Confocal micrographs shown were digital composites of Z-series of 1-4 optical sections through a depth of 0-2 μm.

3.2. RESULTS

3.2.1. Colonic ICC Display K^+ Channel Activity

The potential that the patch "sees" (E_{patch}) is the summation of the cell membrane potential (E_{cell}) and the potential applied to the patch relative to resting membrane potential (E_{rrmp}), $E_{patch} = E_{rrmp} + E_{cell}$. Potentials are expressed as E_{rrmp} followed in parentheses by E_{patch}, assuming an E_{cell} of -60 mV. For example, 20 (-40) mV. One to four channels opened (Fig. 11a-c) at ~ -20 (-80) mV when a ramp protocol from -80 (-140) to +80 (20) mV was applied to cell-attached patches of cultured colonic ICC, using a 150 mM KCl pipette solution. The inward currents were most active at 10 (-50) mV, as shown by the current voltage (IV) plot (Fig. 11d). The inward currents turned off upon patch excision to the inside-out configuration (without changing the bath solution; data not shown). Based on single channel activity – single channel current amplitude (ΔI) versus voltage (Fig. 11e) – the reversal potential was 56.7 (-3.3) ± 1.0 mV and the single channel conductance was 17.9 ± 0.9 pS (n=6). Assuming symmetrical K^+ between the cell and pipette, K^+ currents would reverse at a patch potential of 0 mV. Therefore, the reversal potential of 56.7 mV rrmp suggests a cell membrane potential of -56.7 mV ($E_{cell} = E_{patch} - E_{rrmp}$ = 0 - 56.7 mV = -56.7 mV), confirming it to be close to -60 mV.

The current was outward when a 140 mM NMDGCl pipette solution was used and activated at approximately the same voltage in ramps and steps, consistent with the current being carried by a K^+ channel (Fig. 12a, d-h). The IV plot shows outward K^+ current at positive potentials (Fig. 12b). Based on ΔI versus voltage, the reversal potential (determined as above) was -112.3 (-172.3) ± 4.7 mV and the single channel conductance was 3.3 ± 0.1 pS (n=8; Fig. 12c). The current turned off when patches were excised into the inside-out configuration (data not shown).

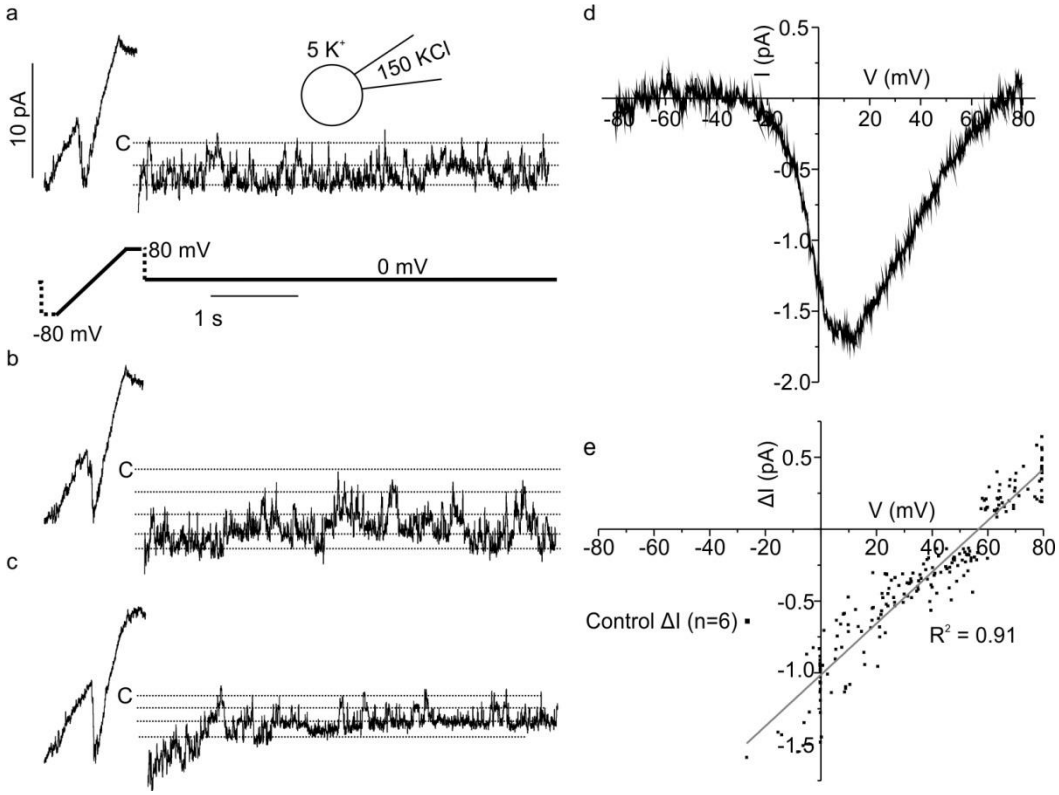

Fig. 11. K^+ currents recorded from cell-attached patches of ICC. Up to four channels were active around 0 (-60) mV. a-c) Control inward K^+ currents evoked by ramps and at 0 (-60) mV. Simultaneous multiple openings were present at 0 (-60) mV. For this and all subsequent figures, dashed lines show current levels and C represents the level where all channels are closed. V is the potential applied to the patch relative to resting membrane potential (E_{rrmp}) and values in parentheses are patch potentials (E_{patch}) assuming a cell membrane potential of -60 mV (see Methods and Results). d) Current voltage plot of average K^+ currents evoked by ramp protocol (n=6). e) Single channel current amplitudes from pooled experiments (n=6); grey line is a linear fit of data with slope = 17.9 pS and x-intercept = 56.7 (-3.3) mV.

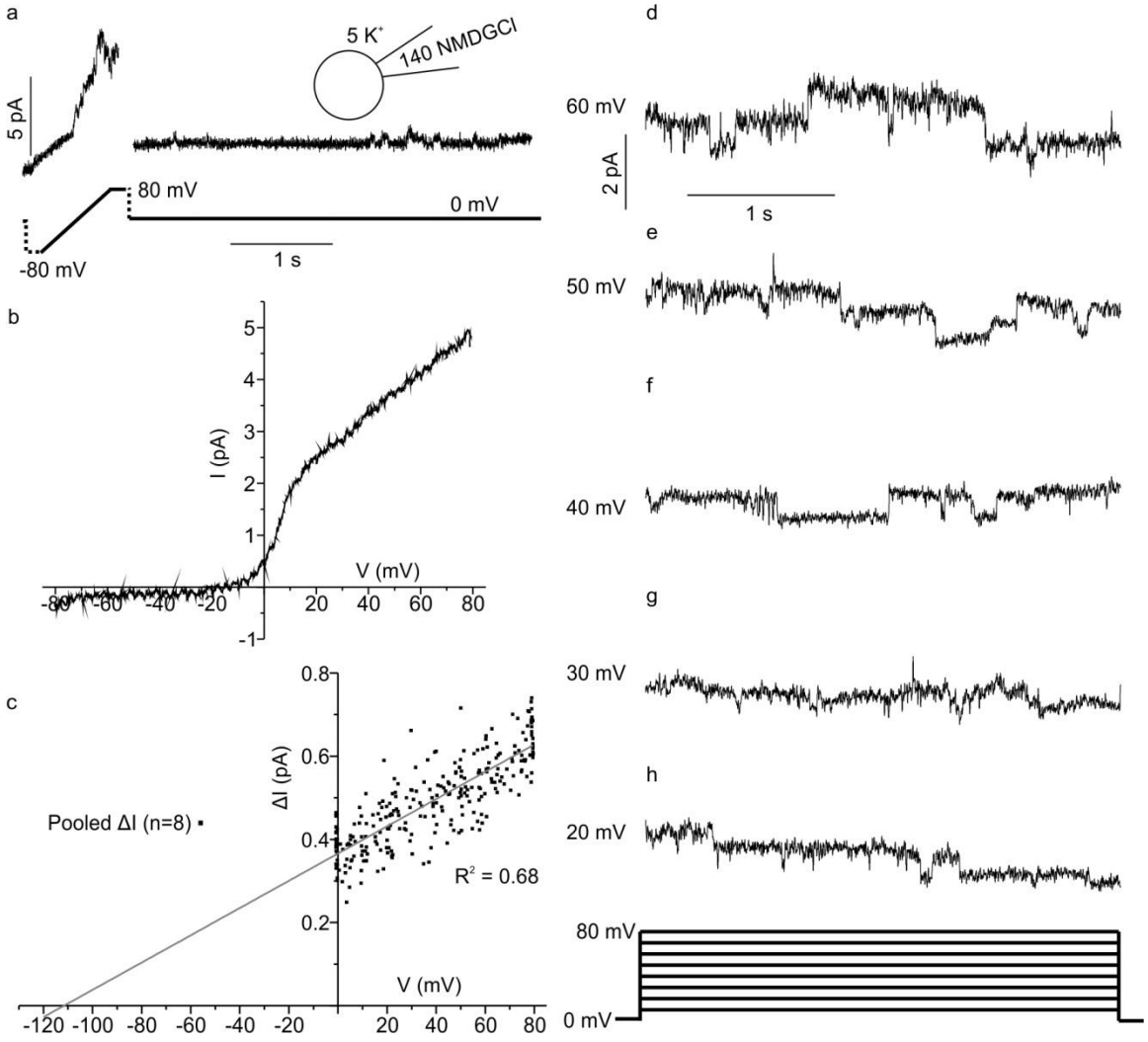

Fig. 12. Outward K^+ currents while cell-attached in 140 mM NMDGCl pipette solution. a) Outward K^+ currents evoked during depolarizing portion of the ramp. b) Current voltage plot of average outward K^+ currents recorded from ramp protocols (n=8). c) Single channel current amplitudes from pooled experiments (n=8); grey line is a linear fit of data with slope = 3.3 pS and x-intercept = -111.6 (-171.6) mV. d-h) Currents recorded from decreasing depolarising voltage steps (inset) and were inactive below 0 (-60) mV (not shown).

3.2.2. Effects of Carbachol on K^+ Currents

Carbachol (CCh; 1 μM) inhibited the K^+ currents completely (n=9; Fig. 13a-c). The current amplitude decreased (Fig. 13b) compared to control (Fig. 13a), before onset of full inhibition (Fig. 13c), which implies that current inhibition began to affect the resting membrane potential, reducing the amplitude of the currents. The IV plot shows that inward currents were most active at 10 (-50) mV, prior to inhibition by CCh (Fig. 13d). Based on ΔI versus voltage, the reversal potential was 59.9 (-0.1) ± 0.6 mV and the single channel conductance was 19.1 ± 0.8 pS (n=8; Fig. 13e). These data imply that the K^+ current is carried by a cholinergic-signaling sensitive channel, hence we tested whether or not the current was carried by Kcnq (K_V7) channels, which are the channels responsible for M-current (Brown et al., 2007).

3.2.3. Effects of XE991 on K^+ Current

XE991 (20 μM), a K_V7 channel blocker (Anderson et al., 2009, Ipavec et al., 2011) completely blocked the currents (n=10; Fig. 14a-c). As with CCh, current amplitude was reduced after addition of XE991 before onset of complete blockade (Fig. 14a & b). Current blockade by XE991 was sustained as is shown by the absence of the K^+ current from ramps recorded at 1 min intervals after XE991 addition (Fig. 14d). The IV plot shows that inward currents were most active at 10 (-50) mV and were blocked by XE991 (n=8). Based on ΔI versus voltage, K_V7 currents had a reversal potential of 55.1 (-4.9) ± 2.3 mV and the conductance was 17.1 ± 1.3 pS (n=8; Fig. 14f). The ΔI versus voltage when all controls were pooled yielded a reversal potential of 57.3 (-2.7) ± 1.0 mV and a conductance of 18.1 ± 0.6 pS (n=22; Fig. 14g). XE991 blocks K_V7 currents from the ICC causing them to depolarize, leading to activation of a large, inwardly rectifying current (n=15; Fig. 15a-c).

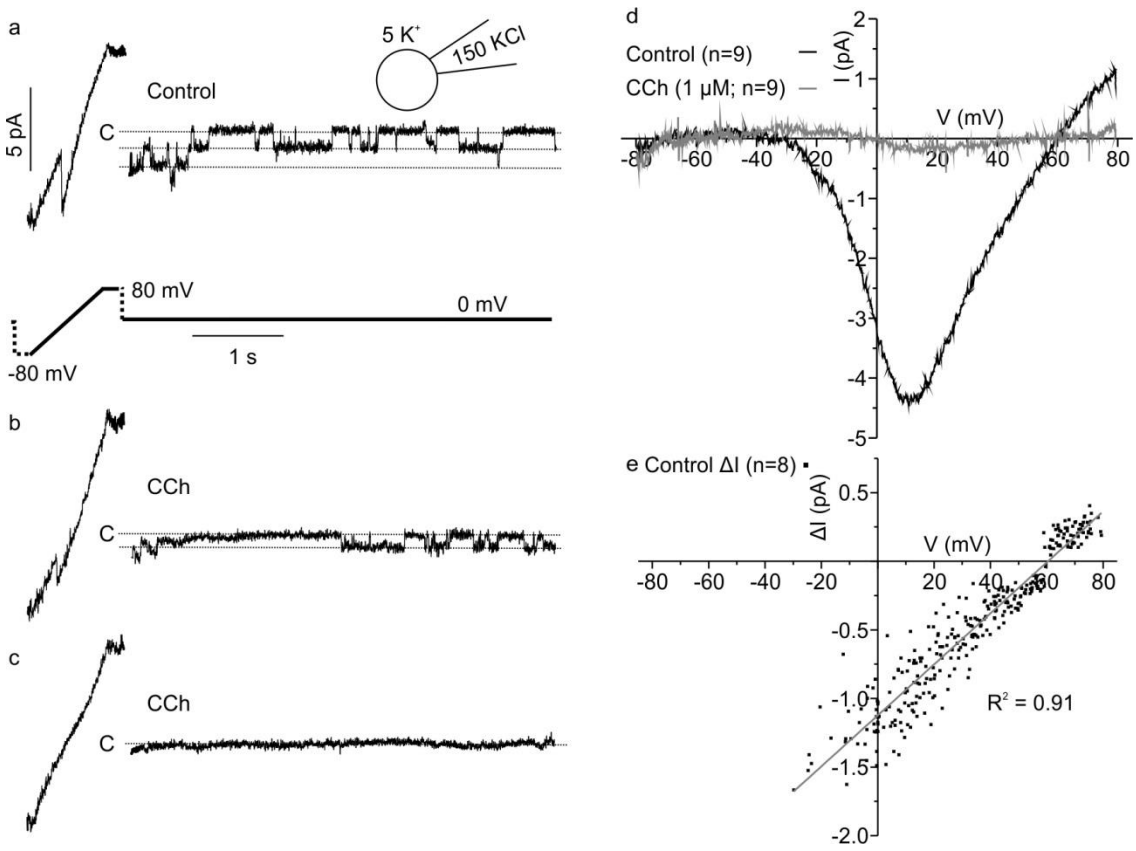

Fig. 13. Effect of carbachol on K^+ current while cell-attached. a) Control K^+ currents evoked by the ramp and at 0 (-60) mV. Note inward current in middle of the ramp. b & c) Carbachol (CCh; 1 µM) inhibited K^+ currents. b was recorded 18 s after CCh addition, c was 114 s after b. d) Current-voltage plots of average currents show inhibition of K^+ current by CCh. e) Single channel current amplitudes from pooled CCh controls (n=8); grey line is a linear fit of data with slope = 18.6 pS and x-intercept = 60.4 (0.4) mV.

In order to confirm that XE991 was blocking the K^+ channels directly, 20 μM XE991 was added to the KCl pipette solution. Time dependent block was shown in Fig. 16a-c. If there were no K^+ currents at the beginning of an experiment, we could not determine whether the channels were blocked rapidly, or whether there were no channels present in the patches. Therefore, many experiments were performed to gain a sample population large enough to accurately determine the effects of XE991 on cultured ICC K^+ currents. Only 30.0% of patches exposed to XE991 exhibited K^+ currents (n=30), in contrast to 57.1% of patches with control 150 mM KCl pipette solution (n=35). There was a significant reduction in the proportion of patches exhibiting K^+ current when exposed to XE991 (9/30) compared to control (20/35), as determined by a Z-test for proportions ($z=2.19$, $\alpha=0.05$, c.v.=1.96). Thus, XE991 blocks the K^+ channels directly when applied to the extracellular side of the patch.

3.2.4. K_V7 Single Cell PCR

Since XE991 blocked the K^+ currents in colonic ICC-IM, we looked for $K_V7.5$ using RT-PCR. We isolated single colonic ICC-IM and found 2/6 Ano1 positive cells were also $K_V7.5$ positive (Fig. 17). The identities of the Ano1 and $K_V7.5$ PCR products were confirmed by sequencing.

3.2.5. c-Kit and $K_V7.5$ Double Immunohistochemical Staining

At the level of myenteric plexus (Fig. 18a), $K_V7.5$ reactivity was found in the enteric neurons of the myenteric ganglia (Fig. 18a2), which were frequently surrounded by c-Kit positive ICC-MP that were negative for $K_V7.5$ (Fig. 18a3). In the circular muscle layer (Fig. 18b-d), $K_V7.5$ was co-expressed with c-Kit in ICC-IM (Fig. 18b3, c3 & d3), both in multipolar and bipolar ICC (arrows in c3). Weak $K_V7.5$ reactivity was also found in the smooth muscle cells (Fig. 18b2 & c2). Transverse sections confirmed that the colonic ICC network expresses $K_V7.5$, not at the level of the

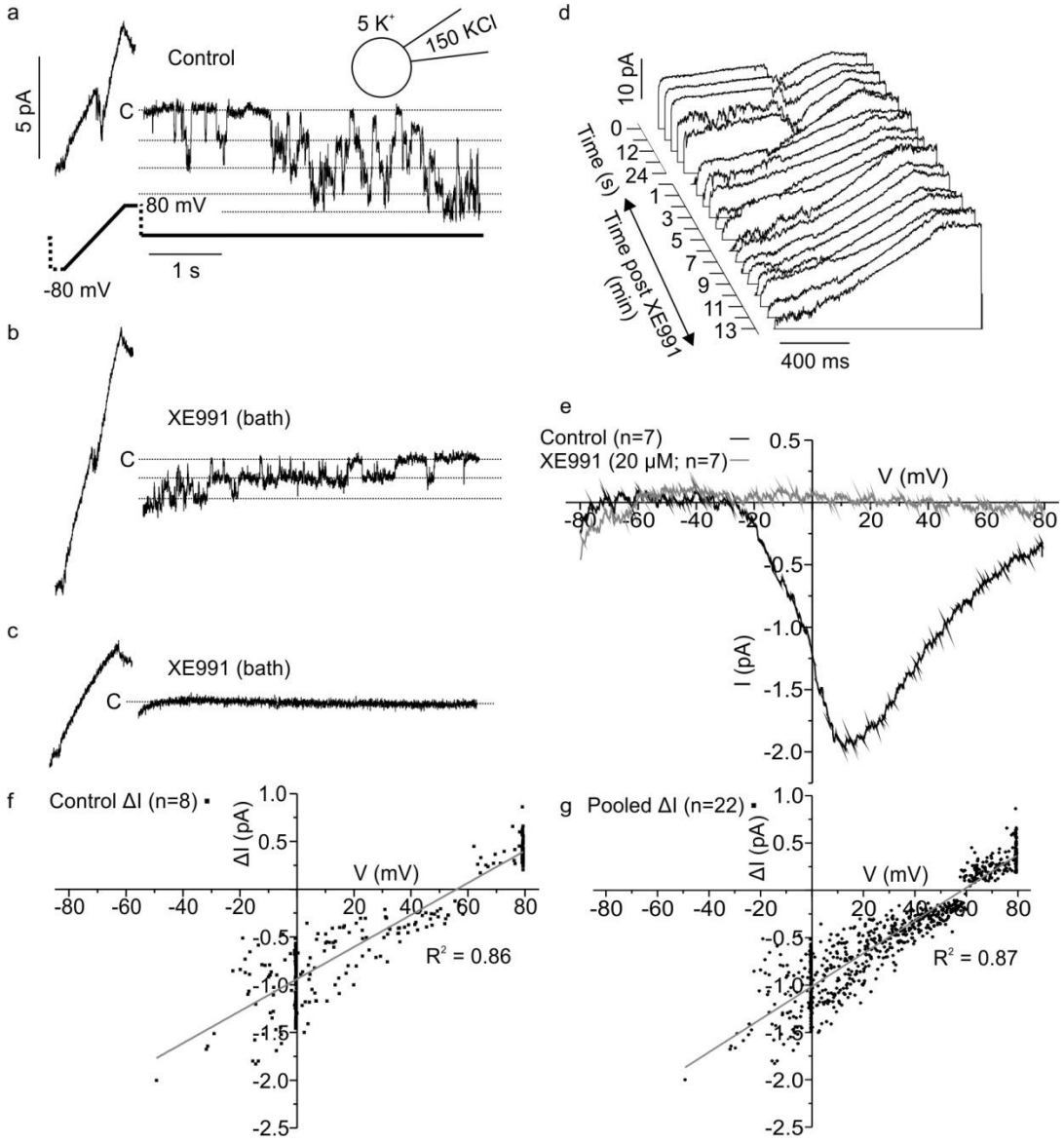

Fig. 14. Effect of bath applied XE991 on K^+ currents while cell-attached. a) Control K^+ currents evoked by the ramp and at 0 (-60) mV. Note inward current in middle of ramp. b & c) XE991 (20 μM) blocked K^+ currents; b was recorded 138 s after XE991 addition, c was 96 s after b. d) Ramp traces where XE991 blocked K^+ currents. The first five traces are sequential from the control with K^+ currents active. The next fifteen traces were recorded after XE991 addition at 1 min intervals; note the absence of K^+ currents. e) Current-voltage plots of average currents show inhibition of K^+ currents by XE991. f) Single channel current amplitudes from pooled XE991 controls (n=8); grey line is a linear fit of data with slope = 16.8 pS and x-intercept = 56.1 (-3.9) mV. g) Single channel amplitudes from pooled control K^+ currents (including ΔI values from Figs. 1e, 3e & 4f; n=22). Grey line is a linear fit of data with slope = 17.4 pS and x-intercept = 58.4 (-1.6) mV.

myenteric plexus, but in the ICC-IM in the circular muscle (Fig. 18d). K_V7.5 positive ICC-IM formed contacts with K_V7.5 negative ICC-MP (Fig. 18d3). We also stained for K_V7.2-4, all of which were negative in the two ICC networks. However, enteric nerves were positive for K_V7.2-4 and the smooth muscle was weakly positive (Fig. 19).

Immediately after cell isolation, most cells were rounded and they gained specific shapes after a few days in culture. Three to four day old c-Kit positive cultured cells were either multipolar (Fig. 18e & f) or bipolar (Fig. 18g & h). Both bipolar and multipolar ICC were found amongst the cells that were positive for K_V7.5 (Fig. 18e3 & f-h; solid arrow in h), which is consistent with the observation of K_V7.5 positive ICC-IM of both morphologies *in situ*. c-Kit positive but K_V7.5 negative cells may be ICC-MP (Fig. 18h, open-headed arrow).

3.2.6. Vesicular Acetylcholine Transporter and c-Kit Double Immunohistochemical Staining

In the mouse colon, anti-vesicular acetylcholine transporter (VAChT) antibodies labelled all the cholinergic nerve varicosities within the myenteric ganglia (Fig. 19a1 & b1) and the nerve fibers in the muscle layer (Fig. 19c1 & d1). c-Kit positive ICC-MP were densely distributed around the myenteric plexus ganglia forming a network (Fig. 19a2, b2, a3 & b3). ICC-IM were observed in both longitudinal and circular muscle layers, parallel to neighbouring smooth muscle cells, connecting each other to form networks (Fig. 19c2 & d2). Intimate apposition between cholinergic varicosities and ICC-IM processes occurred over lengths as long as 250 µm (Fig. 19c3 & d3). Unlike the ICC-MP, which only showed point contact with enteric nerves, ICC-IM (both their cell bodies and processes) contacted nerves with varicosities over long distances.

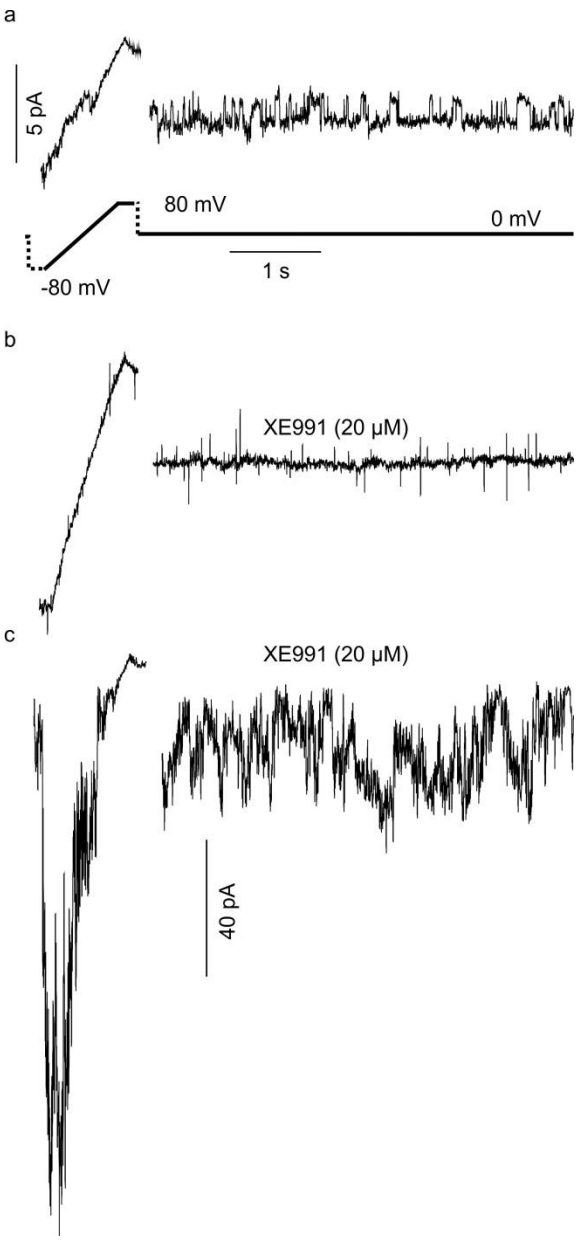

Fig. 15. Activation of an inwardly rectifying current by XE991. a) Inward K^+ currents were active prior to addition of XE991. b) XE991 (20 µM) blocked K^+ currents; b was recorded immediately after switch to XE991 bath solution, which takes 120 s. c) XE991 activated inwardly rectifying currents after 24 s and c was recorded 360 s after addition of XE991.

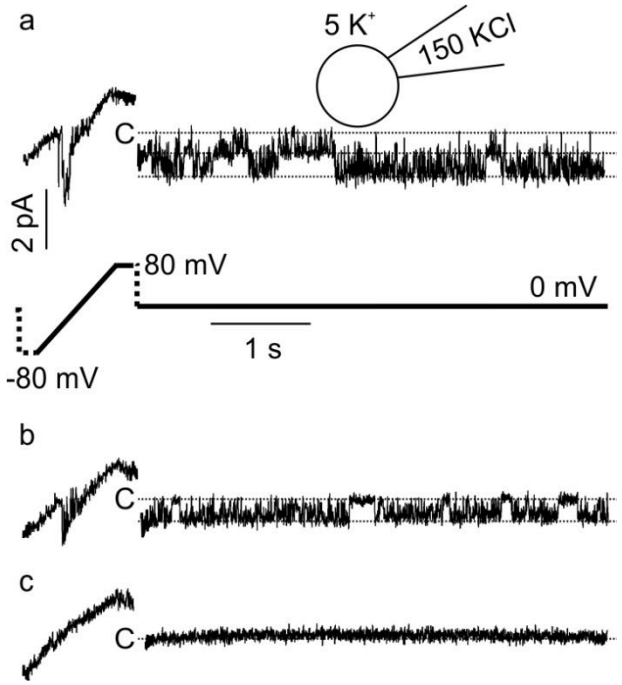

Fig. 16. Effect of XE991 in pipette solution on K^+ currents. a) K^+ currents active prior to onset of XE991 (20 µM) blockade, recorded 144 s from start. b) Time dependent block of current by XE991, 102 s after a. c) Complete current inhibition 18 s after b.

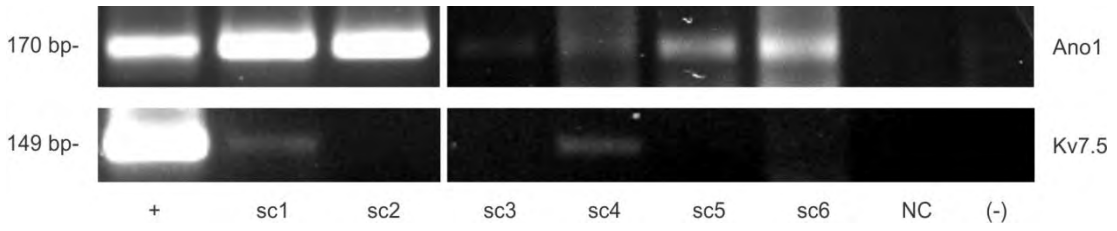

Fig. 17. Expression of K_V7 channels in single ICC-IM. Two of six Ano1 positive cells were positive for $K_V7.5$: single cell 1 (sc1) and single cell (sc4). No cell, positive and negative controls are indicated by NC, + and -

3.2.7. ICC-IM and ICC-MP Networks are Connected

In order to search for structural evidence for communication between ICC-MP and ICC-IM, we conducted wholemount c-Kit immunohistochemistry. ICC-IM, running at a 90^{o} angle from the ICC-MP network parallel to the circular muscle cells, were either bi-polar or multi-polar in shape, with branches occurring from their cell bodies or major processes (Fig. 20). ICC-IM were not independently scattered in the musculature, but were connected to each other to form a network, although the density of their network was not as high as that of ICC-MP. ICC-IM and ICC-MP were connected, providing structural evidence for communication between the two networks.

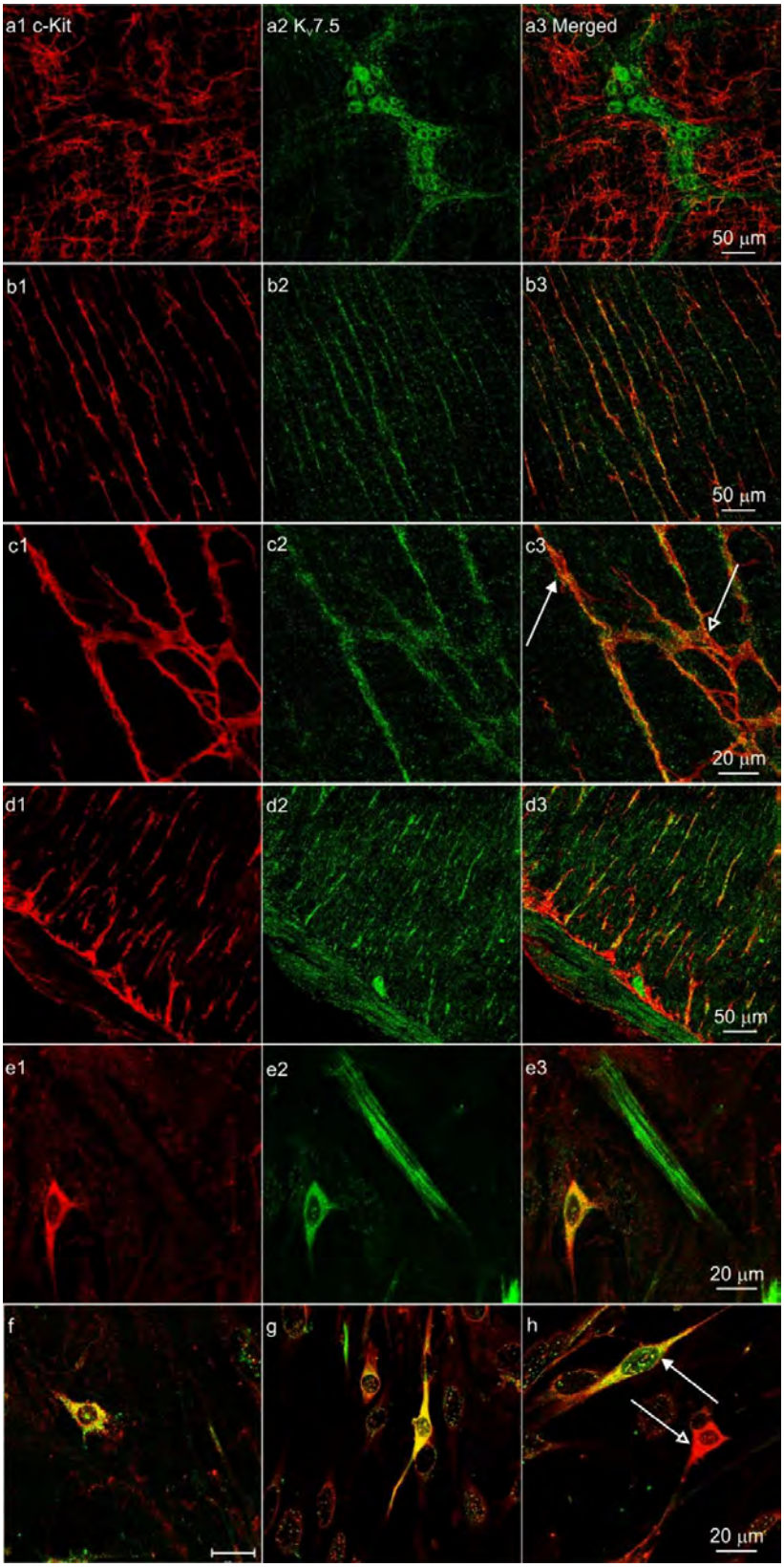

Fig. 18. Double labelling of c-Kit (red) and $K_V7.5$ (green) in mouse colon wholemount (a-c), frozen sections (d) and cultured cells (e-h). a1-3) c-Kit positive ICC-MP network and $K_V7.5$ positive myenteric neurons. No co-localization was found at the level of myenteric plexus. b1-3) c-Kit positive ICC-IM and $K_V7.5$ positive enteric nerves at the level of circular muscle layer. Merged figure shows the co-existence of $K_V7.5$ and c-Kit within ICC-IM. c1-3) Enlarged images to show co-localization of $K_V7.5$ and c-Kit in ICC-IM that were either bi-polar (arrows) or multi-polar (hollow arrow) in shape. d1-3) Transversal sections show the circular muscle, myenteric plexus and longitudinal muscle layers. c-Kit positive ICC-MP surrounded $K_V7.5$ positive myenteric neurons; both c-Kit and $K_V7.5$ immunoreactivities were seen in ICC-IM. Smooth muscle cells also expressed weak immunoreactivity for $K_V7.5$. e-h) Cultured cell staining. e1-3) A triangle-shaped ICC with three processes was positive for $K_V7.5$. f-h) Merged images show a multi-polar shaped ICC that was $K_V7.5$ positive (f), a spindle-shaped bipolar ICC that was $K_V7.5$ positive (g) and a multi-polar shaped ICC that was $K_V7.5$ negative (open-headed arrow), and a spindle-shaped ICC (arrow) that was $K_V7.5$ positive (h).

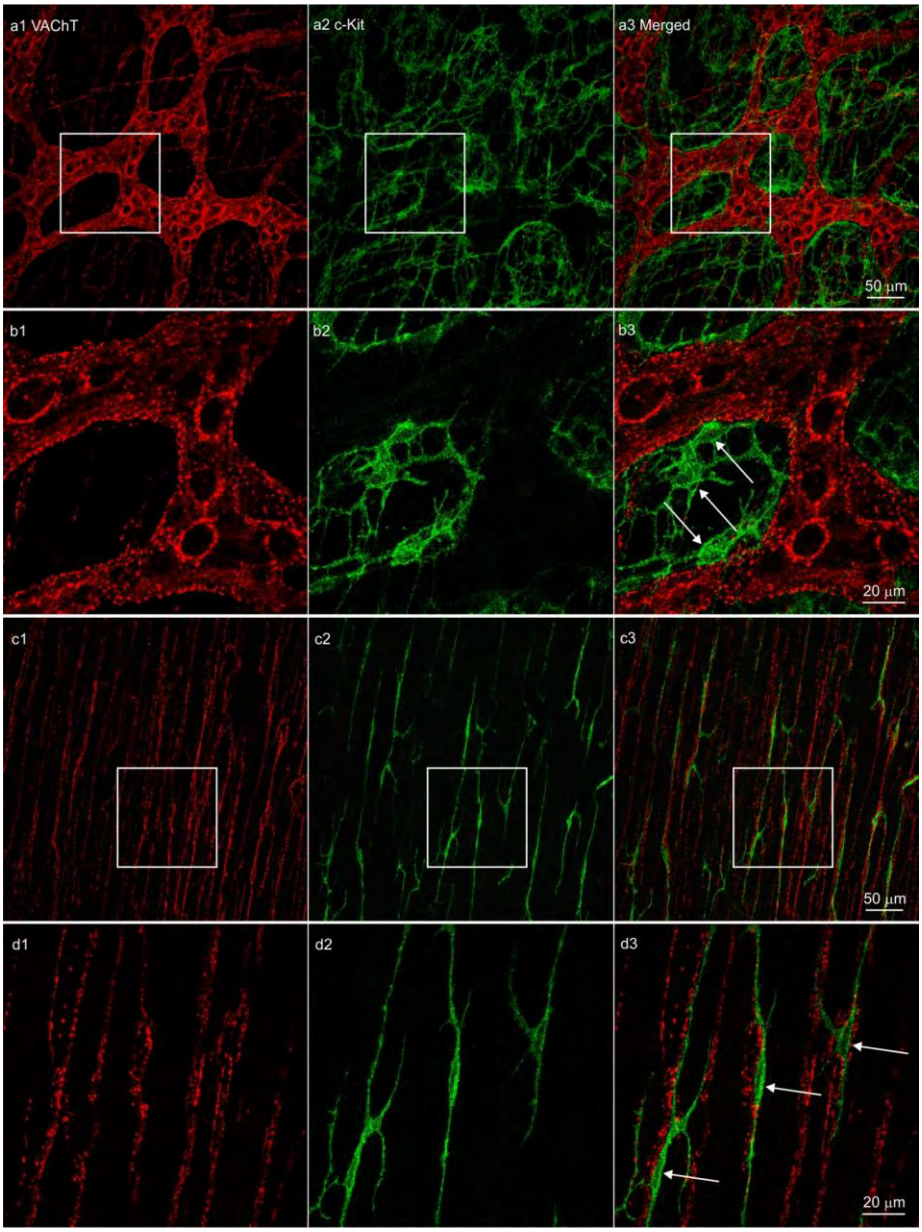

Fig. 19. Double labeling of c-Kit (green) and VAChT (red) in mouse colon wholemount preparation. a1-3) VAChT positive nerve varicosities within the myenteric ganglia and c-Kit positive ICC-MP encompassing the ganglia. b1-3) Enlarged images (single scan confocal micrographs) from the boxes in figures a1-3. Three ICC-MP (arrows) connecting to each other to form network closely aligned the boundaries of the ganglia but never penetrated inside the ganglia. The processes of ICC-MP were not seen lining along the VAChT positive varicosities. c1-3) VAChT positive nerves and c-Kit positive ICC-IM within the circular muscle layer. d1-3) Enlarged images (single scan confocal micrographs) from the boxes in figures c1-3. Three ICC-IM (arrows) with different shapes were all running along cholinergic nerves within the circular muscle layer. Both their cell bodies and processes were surrounded by dotted cholinergic varicosities.

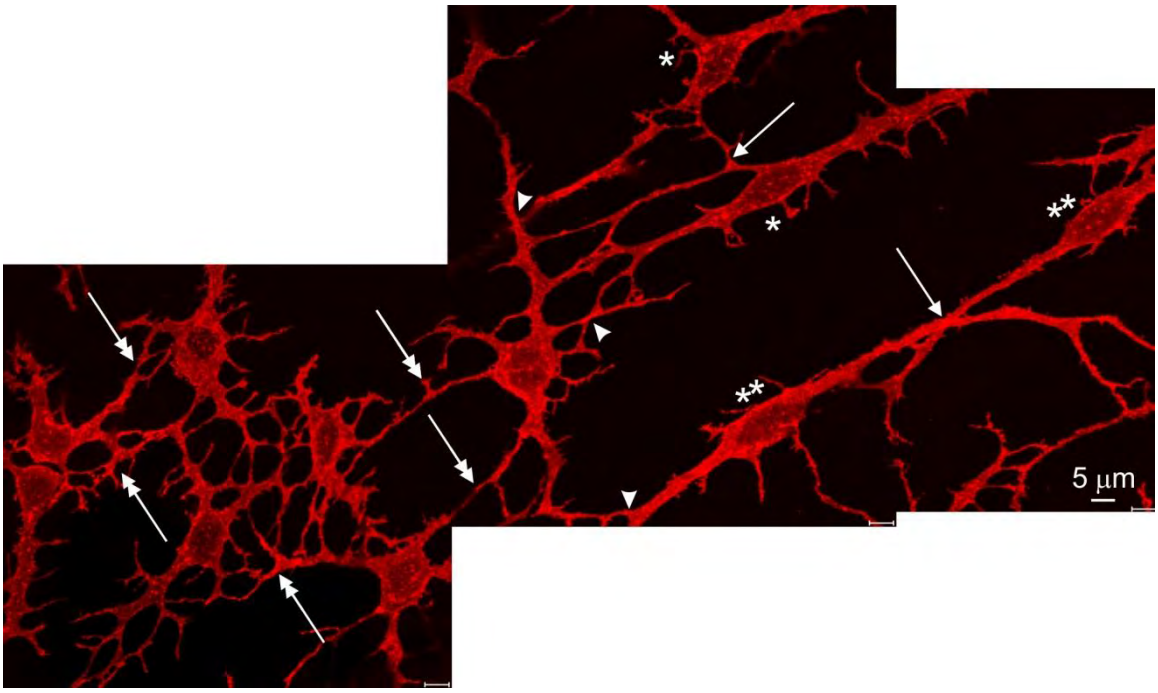

Fig. 20. Combined confocal images with scanning thickness of 1-2 micron (focused on all connections) showing direct connections between ICC-IM, ICC-MP, and between ICC-IM and ICC-MP in mouse colon. On the right side, four ICC-IM in the circular muscle layer close to the level the myenteric plexus were bipolar (**) or multipolar (*) in shape. They were directly connected to each other (arrows) and to nearby ICC-MP (arrowheads). On the left side, several ICC-MP with multipolar shape were closely associated to each other (double arrows) to form a dense network at the myenteric plexus level.

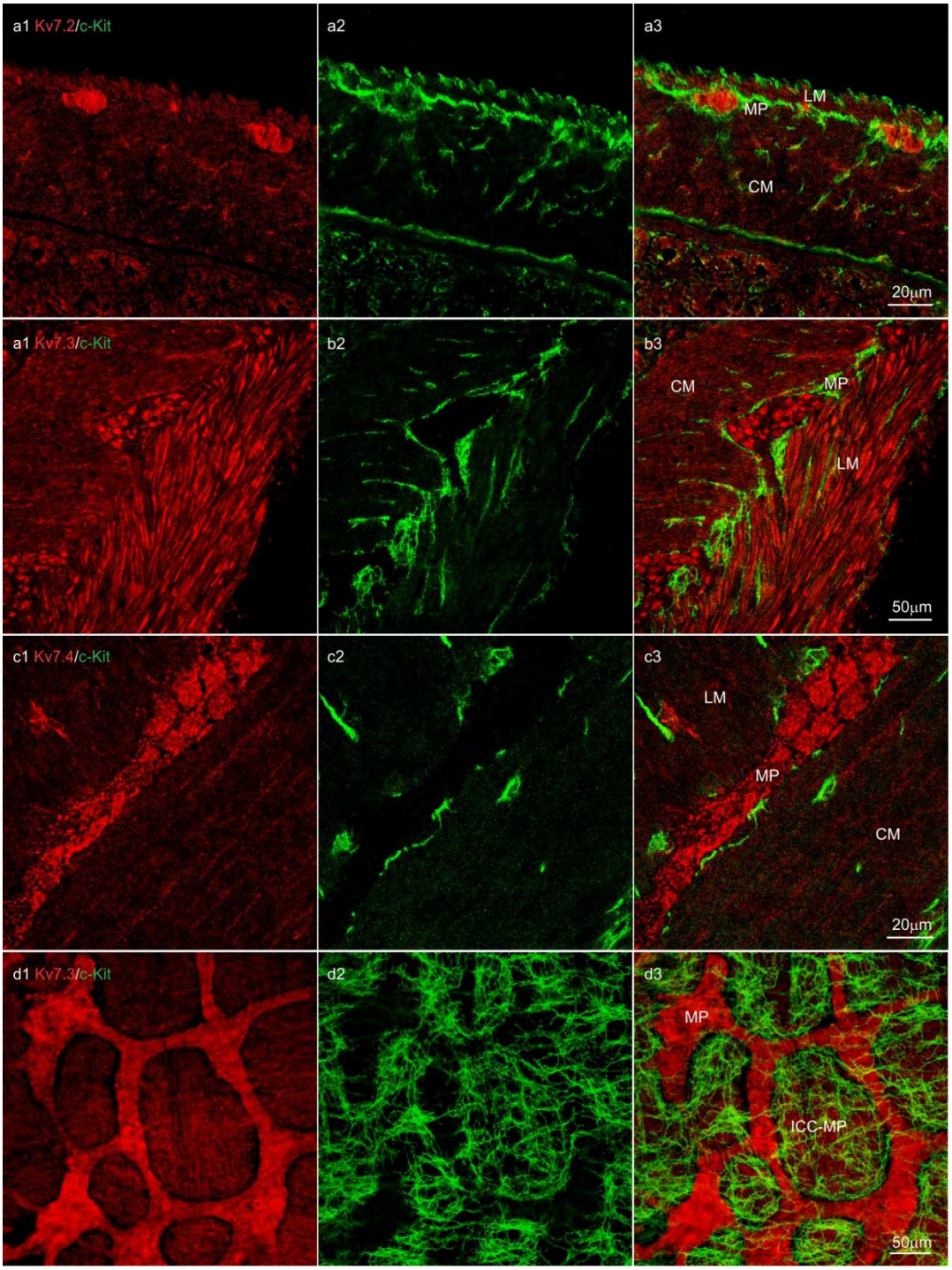

Fig. 21. Double labeling of c-Kit (green) and Kv7.2/7.3/7.4 (red) in mouse colon. Top three groups of figures were from cross sections, bottom group from musculature wholemount preparation. Enteric nerves at the level of myenteric plexus (MP) were positive for Kv7.2 and Kv7.4. Enteric nerves and smooth muscles in the longitudinal muscle (LM) layer were positive for Kv7.3. No co-localization was found between Kv7.2/7.3/7.4 and c-Kit. CM: circular muscle.

3.3. DISCUSSION

Our hypothesis, that ICC-MP excitation was regulated by cholinergic inhibition of K+ channels was not substantiated. Instead, evidence was provided for regulation of ICC-IM excitability by cholinergic inhibition of KV7.5 channels. Therefore, our hypothesis is that ICC-IM have a special role in neurally mediated regulation of pacemaker activity, since we provide evidence that ICC-IM communicate directly with ICC-MP. The notion that ICC-MP and ICC-IM collaborate is well established in the stomach where they cooperate in the generation of pacemaker activity (Hirst et al., 2002a, Hirst et al., 2002b, Edwards and Hirst, 2006, Hirst et al., 2006). In the stomach, the ICC-MP appear to generate the primary pacemaker, and when triggered by the primary pacemaker, the ICC-IM appear to generate a secondary component of the slow wave; this was substantiated by the observation that calcium transients occurred in the ICC-IM following this activity in ICC-MP (Hennig et al., 2004).

3.3.1. Identity of the KV7.5 Positive Cells

Immunohistochemistry showed that in tissue ICC-IM, but not ICC-MP, were positive for KV7.5. ICC-MP are primarily branching cells whereas ICC-IM are a mix of branching and bipolar cells. This was corroborated in the cultured cells where we found branching and bipolar cells, positive both for c-Kit and KV7.5. Although cells in short term culture may differentiate in unpredictable ways, the composition and structural features of c-Kit positive cells in culture was similar to that in tissue: a mix of branching and bipolar cells with some, but not all, branching cells positive for KV7.5. We confirmed using single cell RT-PCR that in our culture, all of the KV7.5 positive ICC were positive for Ano1. Therefore, KV7.5 appears to identify mouse colonic ICC-IM when used in conjunction with Ano1.

3.3.2. Cholinergic Stimulation of ICC: Relationship between ICC-MP and ICC-IM

ICC-IM were shown to be heavily innervated by cholinergic neurons (much more so that ICC-MP) and have the KV7.5 channel; inhibition of KV7 channels by

cholinergic neurotransmitters will enhance cell excitability. When an ICC becomes excited it is possible that pacemaker mechanisms are activated. Cholinergic stimulation causes ICC-IM depolarization since current amplitude decreased before onset of full current inhibition by CCh or blockade by XE991. Cholinergic stimulation leads to depolarization and increased intracellular Ca2+, which activates maxi chloride channels in small intestinal ICC (Wright et al., 2012), which may be involved in generating the ICC pacemaker potential (Huizinga et al., 2002, Wang et al., 2008a, Zhu et al., 2009, Parsons et al., 2012). In the present study we show that high conductance inwardly rectifying currents were activated in colonic ICC-IM by XE991. This strongly supports the hypothesis that cholinergic signalling activates ICC-IM by inhibition of KV7 channels, resulting in increased cell excitability. We hypothesize that depolarization caused by blockade of KV7 channels by XE991 led to activation of the inward currents. The currents resembled inwardly rectifying maxi chloride currents that we have previously recorded from small intestinal ICC-MP (Huizinga et al., 2002, Parsons and Sanders, 2008, Wang et al., 2008a, Zhu et al., 2009, Parsons et al., 2012, Wright et al., 2012). In neurons XE991 enhances cell excitability as evidenced by more frequent action potentials observed during intracellular microelectrode recordings (Brown and Passmore, 2009). ICC-MP and ICC-IM may cooperate in the generation of pacemaker activity; their excitability may be regulated by cholinergic inhibition of KV7.5 in ICC-IM and cholinergic modulation of ICC-MP pacemaker activity may occur through ICC-IM.

3.3.3. Comparison to Other Systems

M-current was first isolated and described in frog sympathetic neurons (Brown and Adams, 1980) and subsequently expression in skeletal muscle (Roura-Ferrer et al., 2009), blood vessels (Zhong et al., 2010) and other smooth muscle cells has been reported. Previous studies have shown expression of KV7.5 in microarray analysis of small intestinal ICC (Chen et al., 2007a). KV7.5 is also expressed in mouse colonic smooth muscle (Jepps et al., 2009). KV7 currents have also been found in ICC from the guinea pig bladder (Anderson et al., 2009). The features of the KV7.5

channel activity described in the present study are similar to those of KV7 channels in Chinese hamster ovary cells transfected with human KCNQ5, where the single channel conductance was 2.2 ± 0.1 pS (Li et al., 2004), which is close to our observation of 3.3 ± 0.1 pS (in NMDGCl pipette solution). The KV7 single-channel amplitude versus voltage relationship we reported was similar to that reported for transfected KV7.5 channels (Li et al., 2004). The activation at ~ -20 (-80) mV of KV7.5 currents recorded from ICC-IM was the same as observed in neurons (Brown and Passmore, 2009). The conductance of the KV7.5 channel currents increased dramatically when high extracellular K+ pipette solution is used. This is because extracellular monovalent cations (such as NMDG+ or Na+ etc.) can block KV channels (Shibasaki, 1987, Block and Jones, 1997, Parsons and Huizinga, 2010), reducing their conductance, which is why studies on KV currents are often performed with high K+ pipette solutions.

3.3.4. KV7 Channel Regulation

There are five members of the KV7 family; channels composed of KV7.2-5 are responsible for M-current (Lerche et al., 2000, Brown et al., 2007, Jepps et al., 2009). M-current is often carried by heteromeric KV7 channels composed of KV7.2 and KV7.3 (Schroeder et al., 2000), KV7.3 and KV7.5 (Lerche et al., 2000) or KV7.4 and KV7.5 (Bal et al., 2008). However, currents we recorded were likely carried exclusively by KV7.5 homomers since we provided negative evidence for the expression of KV7.2-4 proteins in ICC-IM and ICC-MP. It is not known which subtype(s) of muscarinic acetylcholine receptors (mAChRs) are involved in the inhibition of KV7.5 currents in colonic ICC-IM. KV7 channel inhibition can occur via PiP2 degradation (Brown et al., 2007, Brown and Passmore, 2009) or Ca2+-calmodulin (Delmas and Brown, 2005, Linley et al., 2012); the relative contribution of the two KV7 inhibition mechanisms requires investigation. Close proximity of mAChRs to the sarcoplasmic reticulum, as proposed for small intestinal ICC-MP, may favour Ca2+ dependent inhibition in ICC-IM because of the superficial buffer barrier (van Breemen et al., 1995, Wright et al., 2012).

In summary, colonic ICC-IM have a voltage sensitive K+ current, activated around the resting membrane potential of the cell, inhibited by cholinergic stimulation and blocked by a KV7 channel blocker. These data imply inhibition of KV7 channels as a possible mechanism for excitation of colonic ICC-IM by enteric cholinergic nerves. The close association of ICC-IM with ICC-MP and the presence of the maxi chloride channel in ICC-IM suggests the possibility that ICC-IM and ICC-MP cooperate in generating and regulating colonic pacemaker activity associated with propulsive motility (Huizinga et al., 2011, Costa et al., 2013).

4. CONCLUDING REMARKS

In this thesis we used molecular tools of diverse nature (RT-PCR, Immunohistochemistry, western blot, patch clamp), to approach two particular research questions in the physiology of cells controlling motility. In both cases, methodologies like single cell PCR have been used to analyze specific target of diverse nature, from identity markers (Chen et al., 2007a) to oncogenes (Gromova et al., 2011), however their use continues to be highly specialized and limited to a low number of genes and/or to a small number of simultaneously analyzed cells . Good efforts have been done in the analysis of transcriptomes of ICC (Ordog et al., 2004, Chen et al., 2007a), but only smooth muscle-myenteric plexus microarray analysis exist to account for myenteric neurons (Guzman et al., 2006). In any case, they rely on the accumulation of genetic material from large numbers of purified cells of the same nature and not many research groups are able of follow the same methods to perform single cell transcripts analysis. Recent advances in sequencing technologies have opened the possibility of reliably obtaining whole genomes, transcriptomes and proteomes from single cells (Stahlberg et al., 2012, McConnell et al., 2013). It is only integrating all these levels of information that we can exploit the potential of single cells analysis, since correlations between transcript data and protein expression are not 1:1, given the many regulatory mechanisms found among transcription, translation and post-translational processes. Our experience with ICC manifest another important challenge for both molecular biology and physiology that is the incorporation of data from expression

analysis and electrophysiology into the complex systems implicated in the generation and propagation of motility. These tools are already implemented in other branches of physiology (Duan, 2009), however they need to be optimized and validated for the research of excitable cells of the GI tract.

5. REFERENCES

Al-Nafussi AI, Wright NA (1982) Cell kinetics in the mouse small intestine during immediate postnatal life. Virchows Arch B Cell Pathol Incl Mol Pathol 40:51-62.

Alex G, Kunze WA, Furness JB, Clerc N (2001) Comparison of the effects of neurokinin-3 receptor blockade on two forms of slow synaptic transmission in myenteric AH neurons. Neuroscience 104:263-269.

Anderson UA, Carson C, McCloskey KD (2009) KCNQ currents and their contribution to resting membrane potential and the excitability of interstitial cells of Cajal from the guinea pig bladder. The Journal of urology 182:330-336.

Angeli TR, O'Grady G, Paskaranandavadivel N, Erickson JC, Du P, Pullan AJ, Bissett IP, Cheng LK (2013) Experimental and Automated Analysis Techniques for High-resolution Electrical Mapping of Small Intestine Slow Wave Activity. Journal of neurogastroenterology and motility 19:179-191.

Baetge G, Gershon MD (1986) GABA in the PNS: demonstration in enteric neurons. Brain research bulletin 16:421-424.

Bal M, Zhang J, Zaika O, Hernandez CC, Shapiro MS (2008) Homomeric and heteromeric assembly of KCNQ (Kv7) K+ channels assayed by total internal reflection fluorescence/fluorescence resonance energy transfer and patch clamp analysis. The Journal of biological chemistry 283:30668-30676.

Barajas-Lopez C, Berezin I, Daniel EE, Huizinga JD (1989) Pacemaker activity recorded in interstitial cells of Cajal of the gastrointestinal tract. The American journal of physiology 257:C830-835.

Barajas-Lopez C, Huizinga JD, Collins SM, Gerzanich V, Espinosa-Luna R, Peres AL (1996) P2x-purinoceptors of myenteric neurones from the guinea-pig ileum and their unusual pharmacological properties. Br J Pharmacol 119:1541-1548.

Barclay J, Patel S, Dorn G, Wotherspoon G, Moffatt S, Eunson L, Abdel'al S, Natt F, Hall J, Winter J, Bevan S, Wishart W, Fox A, Ganju P (2002) Functional downregulation of P2X3 receptor subunit in rat sensory neurons reveals a significant role in chronic neuropathic and inflammatory pain. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:8139-8147.

Bassotti G, Villanacci V, Antonelli E, Morelli A, Salerni B (2007) Enteric glial cells: new players in gastrointestinal motility? Laboratory investigation; a journal of technical methods and pathology 87:628-632.

Bayliss WM, Starling EH (1899) The movements and innervation of the small intestine. J Physiol 24:99-143.

Benarroch EE (2007) Enteric nervous system: functional organization and neurologic implications. Neurology 69:1953-1957.

Benko R, Undi S, Wolf M, Bartho L (2005) Effects of acute administration of and tachyphylaxis to alpha,beta-methylene ATP in the guinea-pig small intestine. Basic & clinical pharmacology & toxicology 97:369-373.

Bhatt S, Diaz R, Trainor PA (2013) Signals and Switches in Mammalian Neural Crest Cell Differentiation. In: Additional Perspectives on Mammalian Development available: Cold Spring Harbor Laboratory Press.

Bian X, Ren J, DeVries M, Schnegelsberg B, Cockayne DA, Ford AP, Galligan JJ (2003) Peristalsis is impaired in the small intestine of mice lacking the P2X3 subunit. J Physiol 551:309-322.

Block BM, Jones SW (1997) Delayed rectifier current of bullfrog sympathetic neurons: ion-ion competition, asymmetrical block and effects of ions on gating. J Physiol 499 (Pt 2):403-416.

Bo X, Kim M, Nori S, Schoepfer R, Burnstock G, North R (2003) Tissue distribution of P2X4 receptors studied with an ectodomain antibody. Cell and tissue research 313:159-224.

Bradbury EJ, Burnstock G, McMahon SB (1998) The expression of P2X3 purinoreceptors in sensory neurons: effects of axotomy and glial-derived neurotrophic factor. Mol Cell Neurosci 12:256-268.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Brookes SJ, Steele PA, Costa M (1991) Identification and immunohistochemistry of cholinergic and non-cholinergic circular muscle motor neurons in the guinea-pig small intestine. Neuroscience 42:863-878.

Brosenitsch TA, Adachi T, Lipski J, Housley GD, Funk GD (2005) Developmental downregulation of P2X3 receptors in motoneurons of the compact formation of the nucleus ambiguus. The European journal of neuroscience 22:809-824.

Brown DA, Adams PR (1980) Muscarinic suppression of a novel voltage-sensitive K+ current in a vertebrate neurone. Nature 283:673-676.

Brown DA, Hughes SA, Marsh SJ, Tinker A (2007) Regulation of M(Kv7.2/7.3) channels in neurons by PIP(2) and products of PIP(2) hydrolysis: significance for receptor-mediated inhibition. J Physiol 582:917-925.

Brown DA, Passmore GM (2009) Neural KCNQ (Kv7) channels. British journal of pharmacology 156:1185-1195.

Brugère HB, Ferré J-P, Ruckebusch Y (1991) Colonic Motility and Transit After Intermesenteric Nerve Transection and Mesenteric Ganglionectomy in Dogs. Neurogastroenterology & Motility 3:107-116.

Burnstock G (2000) P2X receptors in sensory neurones. British journal of anaesthesia 84:476-488.

Burnstock G (2007) Physiology and pathophysiology of purinergic neurotransmission. Physiological reviews 87:659-797.

Burnstock G, Ulrich H (2011) Purinergic signaling in embryonic and stem cell development. Cellular and molecular life sciences : CMLS 68:1369-1394.

Burnstock G, Verkhratsky A (2012) Purinergic Signalling and the Nervous System: Springer-Verlag Berling Heidelberg.

Burnstock G, Williams M (2000) P2 purinergic receptors: modulation of cell function and therapeutic potential. The Journal of pharmacology and experimental therapeutics 295:862-869.

Castedal M, Bjornsson E, Abrahamsson H (1997) Duodenal juxtapyloric retroperistalsis in the interdigestive state in humans. Scandinavian journal of gastroenterology 32:797-804.

Castelucci P, Robbins HL, Poole DP, Furness JB (2002) The distribution of purine P2X(2) receptors in the guinea-pig enteric nervous system. Histochemistry and cell biology 117:415-422.

Caulfield MP, Birdsall NJ (1998) International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacological reviews 50:279-290.

Costa M, Brookes SJ, Hennig GW (2000) Anatomy and physiology of the enteric nervous system. Gut 47 Suppl 4:iv15-19; discussion iv26.

Costa M, Dodds KN, Wiklendt L, Spencer NJ, Brookes SJ, Dinning PG (2013) Neurogenic and myogenic motor activity in the colon of the guinea pig, mouse, rabbit, and rat. American journal of physiology Gastrointestinal and liver physiology 305:G749-759.

Costa M, Furness JB (1976) The peristaltic reflex: an analysis of the nerve pathways and their pharmacology. Naunyn-Schmiedeberg's archives of pharmacology 294:47-60.

Chavkin C, Goldstein A (1981) Demonstration of a specific dynorphin receptor in guinea pig ileum myenteric plexus. Nature 291:591-593.

Chen H, Ordog T, Chen J, Young DL, Bardsley MR, Redelman D, Ward SM, Sanders KM (2007a) Differential gene expression in functional classes of interstitial cells of Cajal in murine small intestine. Physiological genomics 31:492-509.

Chen H, Redelman D, Ro S, Ward SM, Ordog T, Sanders KM (2007b) Selective labeling and isolation of functional classes of interstitial cells of Cajal of human and murine small intestine. American journal of physiology Cell physiology 292:C497-507.

Chen JH, Zhang Q, Yu Y, Li K, Liao H, Jiang L, Hong L, Du X, Hu X, Chen S, Yin S, Gao Q, Yin X, Luo H, Huizinga JD (2013) Neurogenic and myogenic properties of pan-colonic motor patterns and their spatiotemporal organization in rats. PloS one 8:e60474.

Cherubini E, North RA (1985) Benzodiazepines both enhance gamma-aminobutyrate responses and decrease calcium action potentials in guinea-pig myenteric neurones. Neuroscience 14:309-315.

Delmas P, Brown DA (2005) Pathways modulating neural KCNQ/M (Kv7) potassium channels. Nature reviews Neuroscience 6:850-862.

Der-Silaphet T, Malysz J, Hagel S, Larry Arsenault A, Huizinga JD (1998) Interstitial cells of cajal direct normal propulsive contractile activity in the mouse small intestine. Gastroenterology 114:724-736.

Devries MP, Vessalo M, Galligan JJ (2010) Deletion of P2X2 and P2X3 receptor subunits does not alter motility of the mouse colon. Frontiers in neuroscience 4:22.

Duan D (2009) Phenomics of cardiac chloride channels: the systematic study of chloride channel function in the heart. J Physiol 587:2163-2177.

Dunn PM, Gever J, Ruan HZ, Burnstock G (2005) Developmental changes in heteromeric P2X(2/3) receptor expression in rat sympathetic ganglion neurons. Dev Dyn 234:505-511.

Dunn PM, Zhong Y, Burnstock G (2001) P2X receptors in peripheral neurons. Prog Neurobiol 65:107-134.

Edwards FR, Hirst GD (2006) An electrical analysis of slow wave propagation in the guinea-pig gastric antrum. J Physiol 571:179-189.

Ehlert FJ, Pak KJ, Griffin MT (2012) Muscarinic agonists and antagonists: effects on gastrointestinal function. Handbook of experimental pharmacology 343-374.

Faville RA, Pullan AJ, Sanders KM, Koh SD, Lloyd CM, Smith NP (2009) Biophysically based mathematical modeling of interstitial cells of Cajal slow wave activity generated from a discrete unitary potential basis. Biophysical journal 96:4834-4852.

Foong JP, Nguyen TV, Furness JB, Bornstein JC, Young HM (2012) Myenteric neurons of the mouse small intestine undergo significant electrophysiological and morphological changes during postnatal development. J Physiol.

Furness JB (2007) Enteric nervous system. Massachussets: Blackell.

Furness JB (2008a) The enteric nervous system. Massachusetts, USA: Blackwell Publishing.

Furness JB (2008b) The enteric nervous system: normal functions and enteric neuropathies. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 20 Suppl 1:32-38.

Furness JB (2012) The enteric nervous system and neurogastroenterology. Nature reviews Gastroenterology & hepatology 9:286-294.

Furness JB, Costa M (1980) Types of nerves in the enteric nervous system. Neuroscience 5:1-20.

Furness JB, Costa M (1987) The enteric nervous system: Churchill Livingstone Edinburgh etc.

Galligan JJ (1999) Nerve terminal nicotinic cholinergic receptors on excitatory motoneurons in the myenteric plexus of guinea pig intestine. The Journal of pharmacology and experimental therapeutics 291:92-98.

Galligan JJ (2002) Ligand-gated ion channels in the enteric nervous system. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 14:611-623.

Galligan JJ, LePard KJ, Schneider DA, Zhou X (2000) Multiple mechanisms of fast excitatory synaptic transmission in the enteric nervous system. Journal of the autonomic nervous system 81:97-103.

Garcia-Alcocer G, Padilla K, Rodriguez A, Miledi R, Berumen LC (2012) Distribution of the purinegic receptors P2X(4) and P2X(6) during rat gut development. Neuroscience letters 509:92-95.

Goulet O, Ruemmele F (2006) Causes and management of intestinal failure in children. Gastroenterology 130:S16-28.

Grivel ML, Ruckebusch Y (1972) The propagation of segmental contractions along the small intestine. J Physiol 227:611-625.

Gromova P, Rubin BP, Thys A, Erneux C, Vanderwinden JM (2011) Neurotensin receptor 1 is expressed in gastrointestinal stromal tumors but not in interstitial cells of Cajal. PloS one 6:e14710.

Guerrero-Alba R, Valdez-Morales E, Juarez EH, Miranda-Morales M, Ramirez-Martinez JF, Espinosa-Luna R, Barajas-Lopez C (2010) Two suramin binding sites are present in guinea pig but only one in murine native P2X myenteric receptors. Eur J Pharmacol 626:179-185.

Gulbransen BD, Bashashati M, Hirota SA, Gui X, Roberts JA, MacDonald JA, Muruve DA, McKay DM, Beck PL, Mawe GM, Thompson RJ, Sharkey KA (2012) Activation of neuronal P2X7 receptor-pannexin-1 mediates death of enteric neurons during colitis. Nat Med 18:600-604.

Guo W, Zhang Z, Liu X, Burnstock G, Xiang Z, He C (2013) Developmental expression of P2X5 receptors in the mouse prenatal central and peripheral nervous systems. Purinergic signalling 9:239-248.

Guzman J, Yu JG, Suntres Z, Bozarov A, Cooke H, Javed N, Auer H, Palatini J, Hassanain HH, Cardounel AJ, Javed A, Grants I, Wunderlich JE, Christofi FL (2006) ADOA3R as a therapeutic target in experimental colitis: proof by validated high-density oligonucleotide microarray analysis. Inflammatory bowel diseases 12:766-789.

Hausmann R, Bodnar M, Woltersdorf R, Wang H, Fuchs M, Messemer N, Qin Y, Gunther J, Riedel T, Grohmann M, Nieber K, Schmalzing G, Rubini P, Illes P (2012) ATP binding site mutagenesis reveals different subunit stoichiometry of functional P2X2/3 and P2X2/6 receptors. J Biol Chem 287:13930-13943.

Heanue TA, Pachnis V (2007) Enteric nervous system development and Hirschsprung's disease: advances in genetic and stem cell studies. Nature reviews Neuroscience 8:466-479.

Heine C, Heimrich B, Vogt J, Wegner A, Illes P, Franke H (2006) P2 receptor-stimulation influences axonal outgrowth in the developing hippocampus in vitro. Neuroscience 138:303-311.

Hennig GW, Hirst GD, Park KJ, Smith CB, Sanders KM, Ward SM, Smith TK (2004) Propagation of pacemaker activity in the guinea-pig antrum. J Physiol 556:585-599.

Hennig GW, Spencer NJ, Jokela-Willis S, Bayguinov PO, Lee HT, Ritchie LA, Ward SM, Smith TK, Sanders KM (2010) ICC-MY coordinate smooth muscle electrical and mechanical activity in the murine small intestine. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 22:e138-151.

Hirst GD, Beckett EA, Sanders KM, Ward SM (2002a) Regional variation in contribution of myenteric and intramuscular interstitial cells of Cajal to generation of slow waves in mouse gastric antrum. J Physiol 540:1003-1012.

Hirst GD, Dickens EJ, Edwards FR (2002b) Pacemaker shift in the gastric antrum of guinea-pigs produced by excitatory vagal stimulation involves intramuscular interstitial cells. J Physiol 541:917-928.

Hirst GD, Garcia-Londono AP, Edwards FR (2006) Propagation of slow waves in the guinea-pig gastric antrum. J Physiol 571:165-177.

Hu HZ, Gao N, Lin Z, Gao C, Liu S, Ren J, Xia Y, Wood JD (2001) P2X(7) receptors in the enteric nervous system of guinea-pig small intestine. J Comp Neurol 440:299-310.

Huang LC, Greenwood D, Thorne PR, Housley GD (2005) Developmental regulation of neuron-specific P2X3 receptor expression in the rat cochlea. J Comp Neurol 484:133-143.

Huizinga JD (1986) Myoelectric correlates of colonic motor complexes and contractile activity. The American journal of physiology 251:G724-726.

Huizinga JD (1999) Gastrointestinal peristalsis: joint action of enteric nerves, smooth muscle, and interstitial cells of Cajal. Microscopy research and technique 47:239-247.

Huizinga JD, Berezin I, Chorneyko K, Thuneberg L, Sircar K, Hewlett BR, Riddell RH (1998) Interstitial cells of Cajal: pacemaker cells? The American journal of pathology 153:2008-2011.

Huizinga JD, Chen JH (2014) Interstitial cells of Cajal: update on basic and clinical science. Current gastroenterology reports 16:363.

Huizinga JD, Lammers WJ (2009) Gut peristalsis is governed by a multitude of cooperating mechanisms. American journal of physiology Gastrointestinal and liver physiology 296:G1-8.

Huizinga JD, Martz S, Gil V, Wang XY, Jimenez M, Parsons S (2011) Two independent networks of interstitial cells of cajal work cooperatively with the enteric nervous system to create colonic motor patterns. Frontiers in neuroscience 5:93.

Huizinga JD, Reed DE, Berezin I, Wang XY, Valdez DT, Liu LW, Diamant NE (2008) Survival dependency of intramuscular ICC on vagal afferent nerves in the cat esophagus. American journal of physiology Regulatory, integrative and comparative physiology 294:R302-310.

Huizinga JD, Robinson TL, Thomsen L (2000) The search for the origin of rhythmicity in intestinal contraction; from tissue to single cells. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 12:3-9.

Huizinga JD, Thuneberg L, Kluppel M, Malysz J, Mikkelsen HB, Bernstein A (1995) W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. Nature 373:347-349.

Huizinga JD, Zarate N, Farrugia G (2009) Physiology, injury, and recovery of interstitial cells of Cajal: basic and clinical science. Gastroenterology 137:1548-1556.

Huizinga JD, Zhu Y, Ye J, Molleman A (2002) High-conductance chloride channels generate pacemaker currents in interstitial cells of Cajal. Gastroenterology 123:1627-1636.

Ipavec V, Martire M, Barrese V, Taglialatela M, Curro D (2011) KV7 channels regulate muscle tone and nonadrenergic noncholinergic relaxation of the rat gastric fundus. Pharmacological research : the official journal of the Italian Pharmacological Society 64:397-409.

Janssen PM, Lentle R (2013) Spatiotemporal Mapping Techniques for Quantifying Gut Motility. In: New Advances in Gastrointestinal Motility Research, vol. 10 (Cheng, L. K. et al., eds), pp 219-241: Springer Netherlands.

Jepps TA, Greenwood IA, Moffatt JD, Sanders KM, Ohya S (2009) Molecular and functional characterization of Kv7 K+ channel in murine gastrointestinal smooth muscles. American journal of physiology Gastrointestinal and liver physiology 297:G107-115.

Jiang LH, Kim M, Spelta V, Bo X, Surprenant A, North RA (2003) Subunit arrangement in P2X receptors. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:8903-8910.

Khakh BS, North RA (2006) P2X receptors as cell-surface ATP sensors in health and disease. Nature 442:527-532.

Kim TW, Koh SD, Ordog T, Ward SM, Sanders KM (2003) Muscarinic regulation of pacemaker frequency in murine gastric interstitial cells of Cajal. J Physiol 546:415-425.

Kito Y, Ward SM, Sanders KM (2005) Pacemaker potentials generated by interstitial cells of Cajal in the murine intestine. American journal of physiology Cell physiology 288:C710-720.

Kobilo T, Szurszewski JH, Farrugia G, Hanani M (2003) Coupling and innervation patterns of interstitial cells of Cajal in the deep muscular plexus of the guinea-pig. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 15:635-641.

Koh SD, Jun JY, Kim TW, Sanders KM (2002) A Ca(2+)-inhibited non-selective cation conductance contributes to pacemaker currents in mouse interstitial cell of Cajal. J Physiol 540:803-814.

Koh SD, Ward SM, Ordog T, Sanders KM, Horowitz B (2003) Conductances responsible for slow wave generation and propagation in interstitial cells of Cajal. Current opinion in pharmacology 3:579-582.

Komuro T (2006) Structure and organization of interstitial cells of Cajal in the gastrointestinal tract. J Physiol 576:653-658.

Kunze WA, Furness JB (1999) The enteric nervous system and regulation of intestinal motility. Annual review of physiology 61:117-142.

Langley J (1922) Connexions of the enteric nerve cells. J Physiol (Lond) 56:39.

Langley J, Magnus R (1905) Some observations of the movements of the intestine before and after degenerative section of the mesenteric nerves. J Physiol 33:34-51.

Lentle RG, Janssen PW, Asvarujanon P, Chambers P, Stafford KJ, Hemar Y (2008) High-definition spatiotemporal mapping of contractile activity in the isolated proximal colon of the rabbit. Journal of comparative physiology B, Biochemical, systemic, and environmental physiology 178:257-268.

Lerche C, Scherer CR, Seebohm G, Derst C, Wei AD, Busch AE, Steinmeyer K (2000) Molecular cloning and functional expression of KCNQ5, a potassium channel subunit that may contribute to neuronal M-current diversity. The Journal of biological chemistry 275:22395-22400.

Lewis C, Neidhart S, Holy C, North RA, Buell G, Surprenant A (1995) Coexpression of P2X2 and P2X3 receptor subunits can account for ATP-gated currents in sensory neurons. Nature 377:432-435.

Li Y, Gamper N, Shapiro MS (2004) Single-channel analysis of KCNQ K+ channels reveals the mechanism of augmentation by a cysteine-modifying reagent. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:5079-5090.

Linan-Rico A, Jaramillo-Polanco J, Espinosa-Luna R, Jimenez-Bremont JF, Linan-Rico L, Montano LM, Barajas-Lopez C (2012) Retention of a new-defined intron changes pharmacology and kinetics of the full-length P2X2 receptor found in myenteric neurons of the guinea pig. Neuropharmacology 63:394-404.

Linan-Rico A, Wunderlich JE, Grants IS, Frankel WL, Xue J, Williams KC, Harzman AE, Enneking JT, Cooke HJ, Christofi FL (2013) Purinergic autocrine regulation of mechanosensitivity and serotonin release in a human EC model: ATP-gated P2X3 channels in EC are downregulated in ulcerative colitis. Inflamm Bowel Dis 19:2366-2379.

Linley JE, Pettinger L, Huang D, Gamper N (2012) M channel enhancers and physiological M channel block. J Physiol 590:793-807.

Liu LW, Huizinga JD (1993) Electrical coupling of circular muscle to longitudinal muscle and interstitial cells of Cajal in canine colon. J Physiol 470:445-461.

Liu LW, Thuneberg L, Daniel EE, Huizinga JD (1993) Selective accumulation of methylene blue by interstitial cells of Cajal in canine colon. The American journal of physiology 264:G64-73.

Low PA, Benarroch EE (2008) Clinical autonomic disorders: Lippincott Williams & Wilkins.

Lundberg JM, Terenius L, Hokfelt T, Goldstein M (1983) High levels of neuropeptide Y in peripheral noradrenergic neurons in various mammals including man. Neuroscience letters 42:167-172.

Mao Y, Wang B, Kunze W (2006) Characterization of myenteric sensory neurons in the mouse small intestine. Journal of neurophysiology 96:998-1010.

Maria GN, Raquel GA, Eduardo VM, Rosa EL, Nestor JV, Andromeda LR, Telma LR, Veronica DH, Luis MM, Carlos BL (2013) P2X4 subunits are part of P2X native channels in murine myenteric neurons. Eur J Pharmacol 709:93-102.

McConnell MJ, Lindberg MR, Brennand KJ, Piper JC, Voet T, Cowing-Zitron C, Shumilina S, Lasken RS, Vermeesch JR, Hall IM, Gage FH (2013) Mosaic copy number variation in human neurons. Science 342:632-637.

Molleman A (2003) Patch clamping : an introductory guide to patch clamp electrophysiology. New York: Wiley & Sons.

Nagahama M, Tsuzuki M, Mochizuki T, Iguchi K, Kuwahara A (1998) Light and electron microscopic studies of pituitary adenylate cyclase-activating peptide (PACAP)--immunoreactive neurons in the enteric nervous system of rat small and large intestine. Anatomy and embryology 198:341-352.

Nieto-Pescador MG, Guerrero-Alba R, Valdez-Morales E, Espinosa-Luna R, Jiménez-Vargas N, Liñan-Rico A, Telma L R-L, Verónica D-H, Luis M M, Barajas-Lopez C (2013) P2X4 subunits are part of P2X native channels in murine myenteric neurons. Eur J Pharmacol 709:93-102.

North RA (2002) Molecular physiology of P2X receptors. Physiological reviews 82:1013-1067.

North RA, Slack BE, Surprenant A (1985) Muscarinic M1 and M2 receptors mediate depolarization and presynaptic inhibition in guinea-pig enteric nervous system. J Physiol 368:435-452.

Nurgali K, Furness JB, Stebbing MJ (2003) Analysis of purinergic and cholinergic fast synaptic transmission to identified myenteric neurons. Neuroscience 116:335-347.

Ohta T, Kubota A, Murakami M, Otsuguro K, Ito S (2005) P2X2 receptors are essential for [Ca2+]i increases in response to ATP in cultured rat myenteric neurons. American journal of physiology Gastrointestinal and liver physiology 289:G935-948.

Ordog T, Redelman D, Miller LJ, Horvath VJ, Zhong Q, Almeida-Porada G, Zanjani ED, Horowitz B, Sanders KM (2004) Purification of interstitial cells of Cajal by fluorescence-activated cell sorting. American journal of physiology Cell physiology 286:C448-456.

Pan H, Wang HY, Friedman E, Gershon MD (1997) Mediation by protein kinases C and A of Go-linked slow responses of enteric neurons to 5-HT. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:1011-1024.

Paran TS, Rolle U, Puri P (2006) Postnatal development of the mucosal plexus in the porcine small and large intestine. Pediatr Surg Int 22:997-1001.

Parsons SP, Huizinga JD (2010) Transient outward potassium current in ICC. American journal of physiology Gastrointestinal and liver physiology 298:G456-466.

Parsons SP, Kunze WA, Huizinga JD (2012) Maxi-channels recorded in situ from ICC and pericytes associated with the mouse myenteric plexus. American journal of physiology Cell physiology 302:C1055-1069.

Parsons SP, Sanders KM (2008) An outwardly rectifying and deactivating chloride channel expressed by interstitial cells of cajal from the murine small intestine. JMembrBiol 221:123-132.

Pluja L, Alberti E, Fernandez E, Mikkelsen HB, Thuneberg L, Jimenez M (2001) Evidence supporting presence of two pacemakers in rat colon. American journal of physiology Gastrointestinal and liver physiology 281:G255-266.

Pompolo S, Furness JB (1990) Ultrastructure and synaptology of neurons immunoreactive for gamma-aminobutyric acid in the myenteric plexus of the guinea pig small intestine. Journal of neurocytology 19:539-549.

Poole DP, Castelucci P, Robbins HL, Chiocchetti R, Furness JB (2002) The distribution of P2X3 purine receptor subunits in the guinea pig enteric nervous system. Autonomic neuroscience : basic & clinical 101:39-47.

Preiksaitis HG, Diamant NE (1999) Myogenic mechanism for peristalsis in the cat esophagus. The American journal of physiology 277:G306-313.

Rae MG, Fleming N, McGregor DB, Sanders KM, Keef KD (1998) Control of motility patterns in the human colonic circular muscle layer by pacemaker activity. J Physiol 510 (Pt 1):309-320.

Ren J, Bian X, DeVries M, Schnegelsberg B, Cockayne DA, Ford AP, Galligan JJ (2003) P2X2 subunits contribute to fast synaptic excitation in myenteric neurons of the mouse small intestine. J Physiol 552:809-821.

Roura-Ferrer M, Etxebarria A, Sole L, Oliveras A, Comes N, Villarroel A, Felipe A (2009) Functional implications of KCNE subunit expression for the Kv7.5 (KCNQ5) channel. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology 24:325-334.

Ruan HZ, Burnstock G (2005) The distribution of P2X5 purinergic receptors in the enteric nervous system of mouse. Cell Tissue Res 319:191-200.

Ruan HZ, Moules E, Burnstock G (2004) Changes in P2X3 purinoceptors in sensory ganglia of the mouse during embryonic and postnatal development. Histochem Cell Biol 122:539-551.

Rumessen JJ, Thuneberg L (1982) Plexus muscularis profundus and associated interstitial cells. I. Light microscopical studies of mouse small intestine. The Anatomical record 203:115-127.

Rumessen JJ, Thuneberg L, Mikkelsen HB (1982) Plexus muscularis profundus and associated interstitial cells. II. Ultrastructural studies of mouse small intestine. The Anatomical record 203:129-146.

Sanders KM, Koh SD, Ward SM (2006) Interstitial cells of cajal as pacemakers in the gastrointestinal tract. Annual review of physiology 68:307-343.

Sarna SK (1986) Myoelectric correlates of colonic motor complexes and contractile activity. The American journal of physiology 250:G213-220.

Schroeder BC, Hechenberger M, Weinreich F, Kubisch C, Jentsch TJ (2000) KCNQ5, a novel potassium channel broadly expressed in brain, mediates M-type currents. The Journal of biological chemistry 275:24089-24095.

Shibasaki T (1987) Conductance and kinetics of delayed rectifier potassium channels in nodal cells of the rabbit heart. J Physiol 387:227-250.

Shuttleworth CW, Xue C, Ward SM, de Vente J, Sanders KM (1993) Immunohistochemical localization of 3',5'-cyclic guanosine monophosphate in the canine proximal colon: responses to nitric oxide and electrical stimulation of enteric inhibitory neurons. Neuroscience 56:513-522.

Slater M, Barden JA, Murphy CR (2000) The purinergic calcium channels P2X1,2,5,7 are down-regulated while P2X3,4,6 are up-regulated during apoptosis in the ageing rat prostate. Histochem J 32:571-580.

Smith TK, Reed JB, Sanders KM (1987) Interaction of two electrical pacemakers in muscularis of canine proximal colon. The American journal of physiology 252:C290-299.

Spencer N, Walsh M, Smith TK (1999) Does the guinea-pig ileum obey the 'law of the intestine'? J Physiol 517:889-898.

Stahlberg A, Thomsen C, Ruff D, Aman P (2012) Quantitative PCR analysis of DNA, RNAs, and proteins in the same single cell. Clinical chemistry 58:1682-1691.

Starodub AM, Wood JD (2000) Histamine H(2) receptor activated chloride conductance in myenteric neurons from guinea pig small intestine. Journal of neurophysiology 83:1809-1816.

Steele PA, Brookes SJ, Costa M (1991) Immunohistochemical identification of cholinergic neurons in the myenteric plexus of guinea-pig small intestine. Neuroscience 45:227-239.

Thompson DG, Malagelada JR (1982) Vomiting and the small intestine. Digestive diseases and sciences 27:1121-1125.

Thomsen L, Robinson TL, Lee JC, Farraway LA, Hughes MJ, Andrews DW, Huizinga JD (1998) Interstitial cells of Cajal generate a rhythmic pacemaker current. Nat Med 4:848-851.

Thuneberg L (1999) One hundred years of interstitial cells of Cajal. Microscopy research and technique 47:223-238.

Toulme E, Blais D, Leger C, Landry M, Garret M, Seguela P, Boue-Grabot E (2007) An intracellular motif of P2X(3) receptors is required for functional cross-talk with GABA(A) receptors in nociceptive DRG neurons. J Neurochem 102:1357-1368.

Travagli RA, Hermann GE, Browning KN, Rogers RC (2006) Brainstem circuits regulating gastric function. Annual review of physiology 68:279-305.

Valdez-Morales E, Guerrero-Alba R, Linan-Rico A, Espinosa-Luna R, Zarazua-Guzman S, Miranda-Morales M, Montano LM, Barajas-Lopez C (2011) P2X7 receptors contribute to the currents induced by ATP in guinea pig intestinal myenteric neurons. Eur J Pharmacol 668:366-372.

van Breemen C, Chen Q, Laher I (1995) Superficial buffer barrier function of smooth muscle sarcoplasmic reticulum. Trends in pharmacological sciences 16:98-105.

Van Nassauw L, Brouns I, Adriaensen D, Burnstock G, Timmermans JP (2002) Neurochemical identification of enteric neurons expressing P2X(3) receptors in the guinea-pig ileum. Histochem Cell Biol 118:193-203.

Vannucchi MG, Corsani L, Faussone-Pellegrini MS (1999) Substance P immunoreactive nerves and interstitial cells of Cajal in the rat and guinea-pig ileum. A histochemical and quantitative study. Neuroscience letters 268:49-52.

Wang B, Kunze WA, Zhu Y, Huizinga JD (2008a) In situ recording from gut pacemaker cells. Pflugers Archiv : European journal of physiology 457:243-251.

Wang H, Zhou M, Cong B, He P, Xu M, Ni X, Ma B (2008b) Transient changes in P2X3 but not TRPV1 mRNA expression in rat after prenatal exposure to glucocorticoids. Autonomic neuroscience : basic & clinical 141:112-116.

Wang XY, Lammers WJ, Bercik P, Huizinga JD (2005) Lack of pyloric interstitial cells of Cajal explains distinct peristaltic motor patterns in stomach and small intestine. American journal of physiology Gastrointestinal and liver physiology 289:G539-549.

Wang XY, Paterson C, Huizinga JD (2003) Cholinergic and nitrergic innervation of ICC-DMP and ICC-IM in the human small intestine. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 15:531-543.

Ward SM (2000) Interstitial cells of Cajal in enteric neurotransmission. Gut 47 Suppl 4:iv40-43; discussion iv52.

Ward SM, Sanders KM, Hirst GD (2004) Role of interstitial cells of Cajal in neural control of gastrointestinal smooth muscles. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 16 Suppl 1:112-117.

Waterman SA, Tonini M, Costa M (1994) The role of ascending excitatory and descending inhibitory pathways in peristalsis in the isolated guinea-pig small intestine. J Physiol 481 (Pt 1):223-232.

Wilkinson WJ, Jiang LH, Surprenant A, North RA (2006) Role of ectodomain lysines in the subunits of the heteromeric P2X2/3 receptor. Mol Pharmacol 70:1159-1163.

Wood JD (1999) Enteric nervous control of motility in the upper gastrointestinal tract in defensive states. Digestive diseases and sciences 44:44S-52S.

Wouters MM, Farrugia G, Schemann M (2007) 5-HT receptors on interstitial cells of Cajal, smooth muscle and enteric nerves. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 19 Suppl 2:5-12.

Wright GW, Parsons SP, Huizinga JD (2012) Ca2+ sensitivity of the maxi chloride channel in interstitial cells of Cajal. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 24:e221-234.

Xiang Z, Burnstock G (2004a) Development of nerves expressing P2X3 receptors in the myenteric plexus of rat stomach. Histochem Cell Biol 122:111-119.

Xiang Z, Burnstock G (2004b) P2X2 and P2X3 purinoceptors in the rat enteric nervous system. Histochem Cell Biol 121:169-179.

Xiang Z, Burnstock G (2005) Distribution of P2Y2 receptors in the guinea pig enteric nervous system and its coexistence with P2X2 and P2X3 receptors, neuropeptide Y, nitric oxide synthase and calretinin. Histochem Cell Biol 124:379-390.

Yoneda S, Fukui H, Takaki M (2004) Pacemaker activity from submucosal interstitial cells of Cajal drives high-frequency and low-amplitude circular muscle contractions in the mouse proximal colon. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 16:621-627.

Zeng JW, Cheng SY, Liu XH, Zhao YD, Xiao Z, Burnstock G, Ruan HZ (2013) Expression of P2X5 receptors in the rat, cat, mouse and guinea pig dorsal root ganglion. Histochem Cell Biol 139:549-557.

Zhong XZ, Harhun MI, Olesen SP, Ohya S, Moffatt JD, Cole WC, Greenwood IA (2010) Participation of KCNQ (Kv7) potassium channels in myogenic control of cerebral arterial diameter. J Physiol 588:3277-3293.

Zhou X, Galligan JJ (1996) P2X purinoceptors in cultured myenteric neurons of guinea-pig small intestine. J Physiol 496 (Pt 3):719-729.

Zhu MH, Kim TW, Ro S, Yan W, Ward SM, Koh SD, Sanders KM (2009) A Ca(2+)-activated Cl(-) conductance in interstitial cells of Cajal linked to slow wave currents and pacemaker activity. J Physiol 587:4905-4918.

Zhu YF, Wang XY, Lowie BJ, Parsons S, White L, Kunze W, Pawelka A, Huizinga JD (2013) Enteric sensory neurons communicate with interstitial cells of Cajal to affect pacemaker activity in the small intestine. Pflugers Archiv : European journal of physiology.

APPENDIX A

George W.J. Wright, Sean P. Parsons, Raúl Loera-Valencia, Xuan-Yu Wang, Carlos Barajas-López and Jan D. Huizinga. (2013). **Cholinergic signalling-regulated K(V)7.5 currents are expressed in colonic ICC-IM but not ICC-MP.** Pflugers Arch. Available online December 28. http://dx.doi.org/10.1007/s00424-013-1425-7. ISSN: 1432-2013 (Electronic). 0031-6768 (Print).

Pflugers Arch - Eur J Physiol
DOI 10.1007/s00424-013-1425-7

Cholinergic signalling-regulated $K_V7.5$ currents are expressed in colonic ICC-IM but not ICC-MP

George W. J. Wright · Sean P. Parsons · Raúl Loera-Valencia · Xuan-Yu Wang · Carlos Barajas-López · Jan D. Huizinga

Received: 8 November 2013 / Revised: 2 December 2013 / Accepted: 10 December 2013

Abstract Interstitial cells of Cajal (ICC) and the enteric nervous system orchestrate the various rhythmic motor patterns of the colon. Excitation of ICC may evoke stimulus-dependent pacemaker activity and will therefore have a profound effect on colonic motility. The objective of the present study was to evaluate the potential role of K^+ channels in the regulation of ICC excitability. We employed the cell-attached patch clamp technique to assess single channel activity from mouse colon ICC, immunohistochemistry to determine ICC K^+ channel expression and single cell RT-PCR to identify K^+ channel RNA. Single channel activity revealed voltage-sensitive K^+ channels, which were blocked by the K_V7 blocker XE991 (n=8), which also evoked inward maxi channel activity. Muscarinic acetylcholine receptor stimulation with carbachol inhibited K^+ channel activity (n=8). The single channel conductance was 3.4±0.1 pS (n=8), but with high extracellular K^+, it was 18.1±0.6 pS (n=22). Single cell RT-PCR revealed Ano1-positive ICC that were positive for $K_V7.5$. Double immunohistochemical staining of colons for c-Kit and $K_V7.5$ in situ revealed that intramuscular ICC (ICC-IM), but not ICC associated with the myenteric plexus (ICC-MP), were positive for $K_V7.5$. It also revealed dense cholinergic innervation of ICC-IM. ICC-IM and ICC-MP networks were found to be connected. We propose that the pacemaker network in the colon consists of both ICC-MP and ICC-IM and that one way of exciting this network is via cholinergic $K_V7.5$ channel inhibition in ICC-IM.

Keywords Interstitial cells of Cajal · Pacemaking · Colonic motility · K_V7 channels · Intramuscular ICC · XE991

Abbreviations

GI	Gastrointestinal
ICC	Interstitial cells of Cajal
ICC-MP	Interstitial cells of Cajal associated with the myenteric plexus
ICC-IM	Intramuscular interstitial cells of Cajal
ICC-SMP	Interstitial cells of Cajal associated with the submuscular plexus
$K_V7.5$	Voltage-gated K^+ channel 7.5
mAChRs	Muscarinic acetylcholine receptors
rmp	Relative to resting membrane potential
VAChT	Vesicular acetylcholine transporter

Introduction

Interstitial cells of Cajal (ICC) were proposed as gastrointestinal (GI) pacemaker cells in the late 1970s and early 1980s based on correlations between electrophysiological studies and ultrastructural observations by electron microscopy [12, 18, 35, 41]. There are several populations of ICC in every organ of the GI tract that differ in location, morphology, role and protein expression [18, 23]. With respect to the colon, although initial physiological studies were carried out on dog colonic circular muscle, most subsequent studies on ICC pacemaker mechanisms and ion channels have focused on

Electronic supplementary material The online version of this article (doi:10.1007/s00424-013-1425-7) contains supplementary material, which is available to authorized users.

G. W. J. Wright (✉) · S. P. Parsons · R. Loera-Valencia · X.-Y. Wang · J. D. Huizinga (✉)
Farncombe Family Digestive Health Research Institute, Department of Medicine, McMaster University, HSC-3N8, 1200 Main Street West, Hamilton, ON L8N 3Z5, Canada
e-mail: wrightgw@mcmaster.ca
e-mail: huizinga@mcmaster.ca

R. Loera-Valencia · C. Barajas-López
IPICYT (Instituto Potosino de Investigación Científica y Tecnológica), San Luis Potosi, SLP, Mexico

Published online: 28 December 2013

APPENDIX B

Raúl Loera-Valencia, Egina C. Villalobos, Néstor N. Jiménez-Vargas, Esri H. Juárez, Rosa Espinosa-Luna, Luis M. Montaño, Jan D. Huizinga, Carlos Barajas-López. **Expression of $P2X_3$ and $P2X_5$ Myenteric Receptors Varies During the Intestinal Postnatal Development in the Guinea Pig.** Sent to Cellular and Molecular Neurobiology. Electronic ISSN: 1573-6830.