Role of Interstitial Cells of Cajal in the Pacemaker Activity and Neurotransmission in the Rat Colon

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This is to certify that:

The thesis entitled ‘Role of Interstitial Cells of Cajal in the Pacemaker Activity and Neurotransmission in the Rat Colon’ has been done by Elena Albertí i Martínez de Velasco in order to apply for the PhD in Veterinary Sciences under my supervision.

Bellaterra, November 2004

Dr. Marcel Jiménez i Farrerons
Professor in Physiology in the Veterinary Faculty
Universitat Autònoma de Barcelona

The pictures in the cover are:
Interstitial cells of Cajal (ICCs) from colonic whole mount preparations at the Auerbach's plexus from a Sprague-Dawley rat (Obj. x20 and x60, distal colon, *c-kit* antibody).

The picture in the previous page is a Ws/Ws mutant rat.

Acknowledgements

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Elena Albertí i Martínez de Velasco
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“En els moments de crisis, només la imaginació és més important que el coneixement”

Albert Einstein

Abbreviations

4-AP : 4-aminopyridine

Ach : Acetylcholine

AP : Auerbach’s plexus

ATP : Adenosine 5’-triphosphate

cAMP : Adenosine-3':5'-cyclic monophosphate

cGMP : guanosine-3':5'-cyclic monophosphate

CM : Circular smooth muscle cell

CNS : Central nervous system

CO : Carbon monoxide

DMP : Deep muscular plexus

ENS : Enteric nervous system

GI : Gastrointestinal

HF : High frequency

ICC: Interstitial cells of Cajal

ICC-AP: Interstitial cells of Cajal at the Auerbach’s plexus

ICC-DMP: Interstitial cells of Cajal at the deep muscular plexus

ICC-IM: Intramural interstitial cells of Cajal

ICC-SMP: Interstitial cells of Cajal at the submuscular plexus

IJP : Inhibitory junction potential

IP_3: Inositol triphosphate

LF : Low frequency

LM : Longitudinal smooth muscle cell

L-NAME: N-methyl-L-arginine ester

L-NNA : *N*ϖ-nitro-L-arginine

MCs : Myoelectric complexes

MMC : Migrating motor complexes

NANC : Non-adrenergic, non-cholinergic

NaNP : Sodium nitroprusside

nNOS : Neuronal nitric oxide synthase

NO : Nitric oxide

PACAP : Pituitary adenylate cyclase-activating peptide

PC : Personal computer

RMP : Resting membrane potential

SCF : Stem cell factor

SMP : Submuscular plexus

TEM : Transmission electron microscopy

TKs : Tachykinins

TTX : Tetrodotoxin

VIP : Vasoactive intestinal peptide

Contents

Introduction

1. The gastrointestinal tract: main functions of the colon

The gastrointestinal (GI) tract plays a crucial role in the absorption and digestion and develops contractile activity to facilitate the movement and mixing of luminal contents. The GI tract is divided into several regions: the oesophagus, the stomach, the small intestine and large intestine. In this work we have studied the mechanisms involved in the control of colonic motility. The main functions of the colonic motility are:

(i) Extract water and electrolytes from the contents of the intestinal lumen through mixing movements.
(ii) Keep temporally the content before defecation, allowing bacterial growth.
(iii) Move the content to the rectum and let defecation under voluntary control through coordinated contractions.

These three functions have a physiological feature associated. The first function is important in the adaptation to terrestrial life and dry environments; the second is useful in some animals, like the horse, for nutritional efficiency so that bacteria can digest nutrients by using different ways not provided by mammalian enzymes; and the third plays a role in the ability to control the location of faecal deposits, and it is useful for predators to label their hunting territories and also for victims to escape tracking by scent (Christensen J. 1994).

Gastrointestinal motility depends on the synchronization of the mechanical activity between circular and longitudinal muscle layers and on the coordination given by the enteric nervous system (ENS) and extrinsic neural pathways (Herdt 1997). It is interesting to mention that the enteric nervous system has also been called little-brain or brain in the gut because many of its properties resemble those of the central nervous system (CNS). In this sense, the ENS integrates motility, secretions (exocrine and endocrine), blood flow and immune responses. The ENS is located in the walls of the

entire GI tract, from the oesophagus to the anus, in the associated glands (salivary glands, the pancreas) and in the gallbladder.

In the colon, the wall is divided into two muscle layers, two neural plexuses, a serosa and a mucosa, distributed as it is shown in Figure 1.

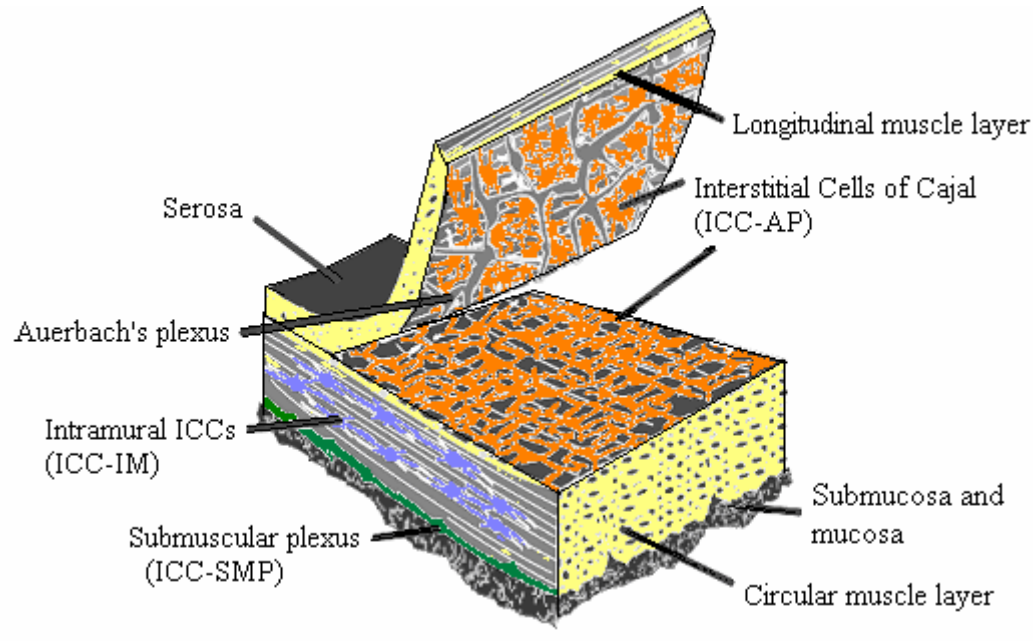

Figure 1. Histological structure of the colon.

The serosa is the most external layer. Smooth muscle layers consist of an outer longitudinal muscle separated from the inner circular muscle by the Auerbach's (AP) or myenteric plexus. The submuscular (SMP) or Meissner's plexus lies between the mucosa and the circular muscle layer. The mucosa consists of an epithelial layer, fibers of the muscularis mucosa and connective tissue (Christensen J. 1994).

2. Pacemaker activity

Intestinal smooth muscle cells show cyclic depolarizations of the membrane potential, called slow waves, which can be recorded *in vivo* as well as *in vitro*. The fact that these slow waves appear in isolated intestinal segments indicates that the pacemaker mechanism is within the intestinal wall itself. When these cyclic depolarizations reach

the calcium channel open threshold, calcium enters inside smooth muscle cells and triggers a muscular contraction. This entrance of calcium is through L-type calcium channels.

Colonic spontaneous contractions were first described in the cat using radiographic techniques (Cannon 1902). The first time to record cyclic depolarizations of the membrane potential (or slow waves) was *in vitro* using extracellular electrodes (Alvarez & Mahoney 1921). In later studies, the correlation between slow waves and muscle contractions was described with experiments using intracellular microelectrode technique.

A typical slow wave consists of a rapid depolarization, a partial repolarization and a sustained plateau followed by a progressive and complete repolarization to the resting membrane potential (RMP) (Figure 2). Slow waves are defined as membrane potential oscillations not blocked by L-type calcium channel blockers (Cayabyab et al. 1996) and are extremely important functionally because they determine the rate and timing of GI smooth muscle contractions. This spontaneous activity is myogenic in origin because the neural blocker tetrodotoxin (TTX) does not abolish them.

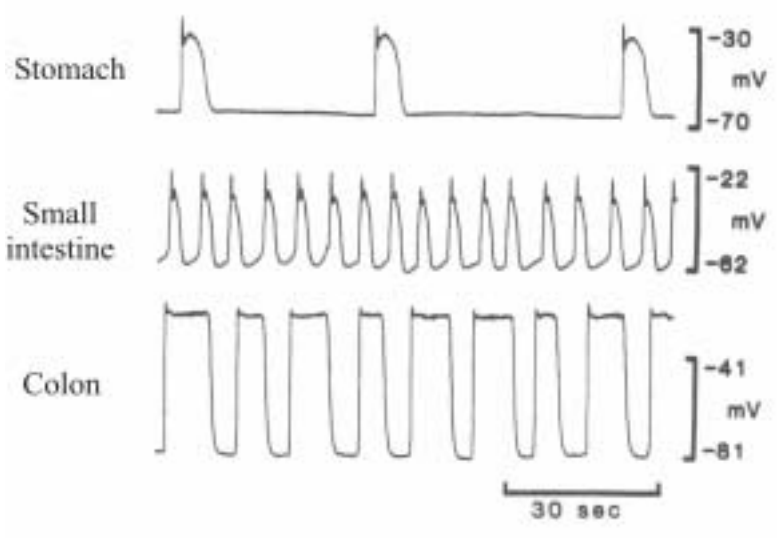

Figure 2. Slow waves recorded with intracellular microelectrode technique in different regions of the gastrointestinal tract of the dog (Sanders 1992).

However, the smooth muscle itself cannot generate slow waves because it lacks the ionic mechanisms responsible for the pacemaker activity. So, where do these cyclic depolarizations come from? Where is the origin of the rhythm of the intestine? Which are the pacemaker cells?

3. History of interstitial cells of Cajal (ICCs)

The origin of the cyclic activity in the gut has been the field of study of many researchers along the history since Santiago Ramón y Cajal, more than one hundred years ago, described a small, stellate or fusiform cells with a prominent nuclei stained with methylene blue (Ramón y Cajal 1892; Ramón y Cajal 1893; Ramón y Cajal 1904).

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> *“Textura del sistema nervioso del hombre y de los vertebrados” (Ramón y Cajal 1904).*

These stellate cells that Cajal drew very accurately were thought to be neural cells because their shape and staining properties were similar to nerve cells. The French neurohistologist Taxi, using the same staining methods as Cajal, showed that these cells were a different cell type (clearly distinguished from neurons, Schwann cells, smooth muscle cells, fibroblasts and macrophages) (Taxi 1952; Taxi 1965). He termed these cells “cellules neuronoïdes” because of their tendency to co-stain with nerves. The work on ICCs by Imaizumi and Hama (Imaizumi & Hama 1969) in the gizzard of the

love-bird and by Yamamoto (Yamamoto 1977) in the mouse and bat small intestine concluded that these “stellate cells” may play a role in the transmission between neurons and smooth muscle cells. The German neurohistologist Stach described at the submuscular plexus of the rat colon close relations between nerve endings, interstitial cells and circular smooth muscle cells (Stach 1972). In that period, Daniel focused his studies on the relations of interstitial cells, smooth muscle cells and nerves (Daniel 1977; Daniel & Posey-Daniel 1984). Later studies revealed that interstitial cells were of mesodermal origin and not derived from neural crest cells, showing that these cells had the same origin as smooth muscle cells.

Faussone Pellegrini (Faussone Pellegrini et al. 1977) and Thuneberg (Thuneberg 1982) suggested that the intestinal pacemaker could be the population of the cells described by Cajal and termed nowadays interstitial cells of Cajal (ICCs). This was the point that started the race to find the physiological role of these special cells that Cajal had seen many years ago with a monocular microscope.

4. Distribution of interstitial cells of Cajal

Interstitial cells of Cajal are differently distributed along the GI tract. During the eighties, identification of ICCs was done by light microscopy with different staining methods (methylene blue, toluidine blue, zinc iodide/osmic acid) (Thuneberg 1982). Transmission electron microscopy (TEM) allowed to characterize the ICC ultrastructure and consequently, identify ICCs (Faussone-Pellegrini & Thuneberg 1999). ICCs have a basal lamina and a cytoplasm rich in caveolae, thin filaments and organelles, including smooth and granular endoplasmic reticulum, Golgi apparatus and a large number of mitochondria, mainly in the branching processes.

However, TEM technique is not useful to visualize the whole network of ICCs at the Auerbach’s and submuscular plexuses and indeed it is not a useful tool to study the distribution of these cells inside each region. The discovery that ICCs express the receptor Kit (see section 6.4) and the use of *c-kit* antibodies has improved the study of

the distribution of ICCs (see Table 1) (Maeda et al. 1992; Ward et al. 1994; Huizinga et al. 1995; Torihashi et al. 1995).

The distribution in Table 1 is a summary of what it has been found along the GI tract in different animal species: dog, rat, mouse, rabbit, cat, pig, guinea-pig (Smith et al. 1987a; Smith et al. 1987b; Du & Conklin 1989; Christensen et al. 1992; Sanders 1996; Ward et al. 1997; Jimenez et al. 1999; Vanderwinden et al. 2000; Pluja et al. 2001) and humans (Rumessen et al. 1993; Rae et al. 1998; Torihashi et al. 1999). Figure 1 shows the distribution of ICCs in the colon.

Gastrointestinal region		ICC-AP	ICC-DMP	ICC-SMP	ICC-IM
Stomach	Antrum	Yes	___	___	Yes
	Corpus	Yes	___	___	Yes
	Fundus	___	___	___	Yes
Small intestine		Yes	Yes	___	___
Colon		Yes	___	Yes	Yes

Table 1. Distribution of ICCs in different regions of the gastrointestinal tract.
ICC-AP: Interstitial cells of Cajal at the Auerbach's plexus; ICC-DMP: Interstitial cells of Cajal at the deep muscular plexus; ICC-SMP: Interstitial cells of Cajal at the submuscular plexus; ICC-IM: Intramuscular interstitial cells of Cajal.

5. Physiological role of ICCs

The two main and established functions of ICCs are:

(i) ICCs generate spontaneous rhythmic electrical oscillations, or **slow waves**, which cause smooth muscle contractions (Torihashi et al. 1995; Sanders et al. 2000; Daniel 2001; Huizinga 2001).

(ii) ICCs serve as a **connection** between enteric nerves and smooth muscle cells, participating in **neurotransmission** and regulating GI motility (Sanders 1996; Huizinga 1999; Ward 2000; Daniel 2001; Huizinga 2001).

6. Experimental procedures to prove that ICCs are pacemaker cells

Experiments to prove the physiological role of ICCs as pacemaker cells of the gut are based on dissection experiments, use of chemicals to lesion ICCs, studies on isolated and/or cultured ICCs, functional immunohistochemistry studies and developmental studies (Sanders 1996).

6.1. Dissection experiments

The procedure of these experiments is to remove surgically one layer of ICCs from one plexus by sharp dissection and leave the other plexus of ICCs intact in order to study the remaining spontaneous activity. In the colon, dissection of ICC-SMP blocks the generation of slow waves in the dog (Smith et al. 1987a; Smith et al. 1987b), cat (Du & Conklin 1989) and rat (Pluja et al. 2001). All these experiments suggest that ICC-SMP are important cells involved in slow wave generation in the colon. In contrast, using the same experimental procedure, the network of ICC-AP is the main pacemaker of the stomach and small intestine.

6.2. Use of chemicals to lesion ICCs

These experiments are performed using chemicals that concentrate “specifically” on ICCs in order to damage them. Two main chemicals have been used: methylene blue and rhodamine 123. Methylene blue stains ICCs and blocks slow waves (after light exposition), as it was shown in the mouse small intestine (Thuneberg 1990) and canine proximal colon (Sanders et al. 1989; Liu et al. 1994). Rhodamine 123 has been shown to abolish slow waves as well, as it was reported in studies from canine proximal colon (Ward & Sanders 1990). Unfortunately, these chemicals are not specific for ICCs and can also damage other cell types such as smooth muscle cells and enteric neurons.

6.3. Studies on isolated and/or cultured ICCs

The aim of these experiments is to obtain single ICCs, using digestion methods, in order to study the ionic mechanisms responsible for the pacemaker activity. Single ICCs are spontaneously active, generating depolarizations similar to slow waves recorded in intact smooth muscle cells, as it was shown in studies from canine colon (Langton et al. 1989). These cells reveal the presence of rhythmic inward currents insensitive to L-type calcium channel blockers, crucial for the generation of slow waves in smooth muscle cells (Koh et al. 1998; Thomsen et al. 1998; Huizinga 1999; Sanders et al. 2000; Zhu et al. 2003). ICCs also show response to messenger molecules known to be released from enteric nerves (Publicover et al. 1992; Young et al. 1993). It is interesting to note that on isolated smooth muscle cells, an ionic mechanism able to generate slow waves has not been found (Lee et al. 1999; Huizinga 1999). These findings are consistent with the hypothesis that ICCs initiate rhythmicity.

6.4. Use of mutant animals

The *c-kit* proto-oncogene encodes Kit, the receptor tyrosine kinase for stem cell factor (SCF). *c-kit* is located in the *Ws* (*white spotting*) locus in the rat and in the *W* locus in the mouse. The Kit receptor plays a crucial role in the development of the following cell lineages: melanocytes, germ cells, mast cells and hematopoietic cells. The extracellular domain contains the receptor for stem cell factor (SCF), which is the natural ligand for Kit receptors. The cytoplasmic domain conveys tyrosine kinase activity. SCF is encoded by the mouse *Sl* locus. The SCF-Kit signalling pathway is crucial for the differentiation and proliferation of ICCs. SCF can be provided by neurons, muscle cells and other cell types (Torihashi et al. 1995).

The use of mutant animals that have genetic deficiencies on the SCF-Kit pathway has been recently studied and they are very useful tools in the knowledge of the role of ICCs in the pacemaker activity. Several animal models have been used, including W/Wv mutant mice, Ws/Ws mutant rats and Sl/Sld mutant mice. *W* mutant animals have spontaneous mutations in the *W* locus and have abnormal Kit protein that

results in underdevelopment of certain classes of ICCs. Sl/Sld mice have a mutation in the *steel* locus that affects the gene encoding stem cell factor (SCF) and consequently these animals lack the ability to make membrane-bound stem cell factor.

In W/Wv mice, ICC-AP fail to develop in the small intestine. These animals lack intestinal pacemaker activity, suggesting that ICC-AP are crucial for the generation of the slow wave activity (Ward et al. 1994; Huizinga et al. 1995; Malysz et al. 1996). W/Wv mice present ICCs only at the DMP in the small intestine, suggesting, at least in the mouse, that ICC-DMP are not dependent on the Kit protein and do not take part in generation of pacemaker activity. In the stomach, interestingly, W/Wv mutant mice lack ICC-IM but have normal ICC-AP (Huizinga 2001). Slow waves are recorded in the stomach of these mutant mice, suggesting that ICC-AP are needed for the generation of slow waves. However, a reduction in NO-dependent postjunctional responses is present, suggesting that ICC-IM play a role in inhibitory innervation (Burns et al. 1996; Ward 2000; Ward & Sanders 2001).

Mutant rats have also been used to study the role of ICCs in the pacemaker activity. In the jejunum of Ws/Ws mutant rats, ICC-AP are greatly reduced (Horiguchi & Komuro 1998) or are absent (Takeda et al. 2001), whereas no changes are found in the number of ICCs at the DMP. The abnormal contractile activity found in these animals is due to the absence of ICC-AP. In the stomach, in situ hybridization shows that *c-kit* expressing cells are completely absent in Ws/Ws rats (Isozaki et al. 1995). Similarly, in the ileum of Ws/Ws rats, the number of *c-kit* positive cells is decreased and the contractile activity is apparently impaired (Isozaki et al. 1995).

Several studies have been done to test whether mutations in stem cell factor (SCF), the ligand for Kit, affect the development of ICC networks. In mice with stell mutations (i.e., Sl/Sld), ICC-AP are not observed in the small intestine and electrical slow waves are absent in these animals. This shows that ICC-AP are involved in the generation of electrical rhythmicity in the small intestine (Ward et al. 1995). In contrast, ICC-DMP are normal and neural imputs are intact, suggesting that the mutation affects certain classes of ICCs but not others. Studies on the gastric fundus of Sl/Sld mice show that ICC-IM are absent, suggesting that membrane-bound SCF is also essential for the development of ICC-IM (Beckett et al. 2002).

6.5. Developmental studies

These studies try to correlate the development of the ICC network with functional changes in electrical and mechanical activity (Faussone-Pellegrini 1992). These experiments use new born animals whose ICC network is not fully developed and arrest ICCs development producing tissues in which the ICC network is impaired. Newborn mice treated with monoclonal antibodies (i.e., ACK2) against Kit receptor for a few days postnatally result in a severe impairment of slow waves and motility (Maeda et al. 1992; Torihashi et al. 1995). These studies suggest that *c-kit* plays a crucial role in the development of the pacemaker system required for the generation of spontaneous rhythmicity.

7. ICCs metabolism

Pacemaker activity in GI muscles has often been considered analogous to cardiac rhythmicity; in fact, slow waves are generated at lower frequencies (between 3-15 cycles/min), have lower amplitudes (maximum of 10-50 mV) and have long action potentials (up to many seconds).

The role of ICCs is to depolarize the smooth muscle syncytium increasing the open probability of voltage-dependent ion channels, like L-type Ca^{2+} channels, which are abundantly expressed in smooth muscle cells (Horowitz et al. 1999). The cyclic electric oscillations that reach the calcium channels open threshold produce calcium inward into smooth muscle cells and, consequently, a muscular contraction. However, the ionic mechanisms whether ICCs generate slow waves are still not clear and therefore, are under the scope of many researchers.

Isolated ICCs show cyclic currents, which are voltage independent. These currents might be through a non-selective cation current, probably Na^{+} and Ca^{2+} (Sanders et al. 2000; Strege et al. 2003), or alternatively a chloride current (Huizinga et al. 2002; Zhu et al. 2004) and have been reported to generate electrical rhythmicity. These currents are located at the ICCs' plasmatic membrane and are activated

rhythmically by intracellular calcium fluctuations. These changes in the intracellular calcium concentration are considered to play a key role in generating slow waves and are due to the release of calcium from the endoplasmic reticulum through inositol triphosphate (IP_3) receptors and the posterior reuptake from mitochondria by active transportation.

8. Pacemaker activity in the colon of different species

In the dog colon, two interacting populations of pacemaker cells have been described. One population at the submucosal edge (ICC-SMP) and the other at the myenteric border of the circular muscle layer (ICC-AP) (Smith et al. 1987a; Smith et al. 1987b; Berezin et al. 1990). Intracellular recordings from circular smooth muscle cells clearly show two pacemaker frequencies in the dog colon: 6 cycles/min slow waves and 20 cycles/min oscillations (Smith et al. 1987a). Slow waves are of higher amplitude near the submuscular plexus (40-45 mV) and decay from this plexus, suggesting that slow waves are generated at the ICC-SMP (Smith et al. 1987b; Liu et al. 1994). The 20 cycles/min events are greatest at the myenteric border. ICCs appear to present gap junctions with other interstitial cells and adjacent smooth muscle cells and are in close contact with nerves (Berezin et al. 1988).

In the rat, two pacemaker populations have been found: one residing at the Auerbach's plexus (AP) and another at the submuscular plexus (SMP). ICC-AP trigger cyclic depolarizations at 0.5-1.5 cycles/min; ICC-SMP trigger slow waves at 13-15 cycles/min (Figure 3) (Pluja et al. 2001). Muscle bath recordings show two types of spontaneous mechanical contractions: high frequency (10-15 contr/min) and low frequency contractions (0.5-2.7 contr/min) (Pluja et al. 2001; Gonzalez & Sarna 2001a; Gonzalez & Sarna 2001b).

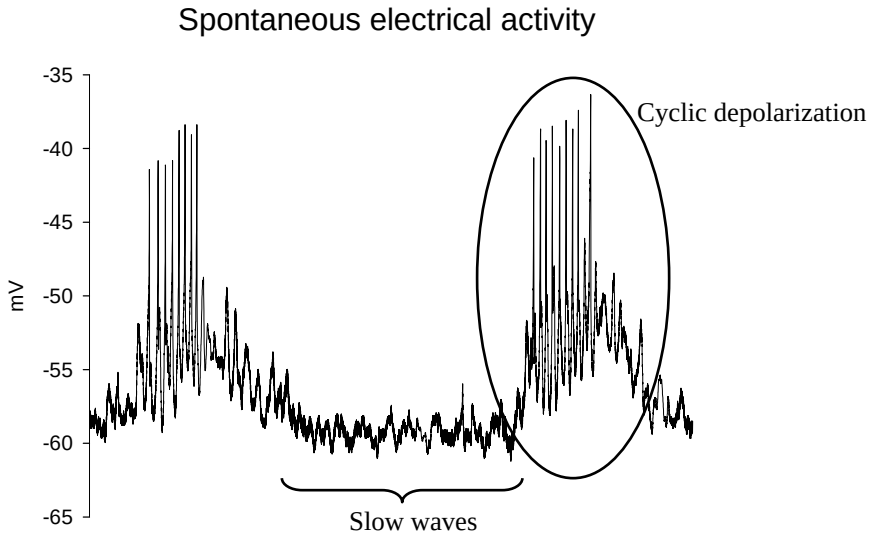

Figure 3. Electrical recording from Sprague-Dawley rat colon showing slow wave activity with superimposed cyclic depolarizations (with action potentials).

In the mouse, a spontaneous electrical and mechanical activity called myoelectric complexes (MCs) or migrating motor complexes (MMCs) respectively, has been reported (Lyster et al. 1995; Spencer et al. 1998c; Spencer et al. 2003). Colonic MCs are abolished by TTX and hexametonium, suggesting that the pacemaker responsible for its origin and propagation is neurogenic (Spencer et al. 1998a; Spencer et al. 1998c). MCs are present in the whole colon of mice (Lyster et al. 1995; Spencer et al. 1998a; Spencer et al. 1998c; Brierley et al. 2001) and alternate with quiescence periods (Lyster et al. 1993; Lyster et al. 1995) and it has been suggested that inhibitory (NO, ATP) and excitatory (Ach and TKs) pathways may be involved in determining whether MCs are formed and may be controlling the quiescent periods (Lyster et al. 1995; Spencer et al. 1998b; Brierley et al. 2001). Moreover, the presence of myogenic slow waves has also been described in mice (Ward et al. 1997; Yoneda et al. 2002). This spontaneous activity is not blocked by tetrodotoxin, suggesting that ICCs are the pacemaker cells.

In human colon, spontaneous electrical and mechanical activities persist in the presence of atropine, phentolamine, propranolol, tetrodotoxin and *N*ϖ-nitro-L-arginine (L-NNA) but are abolished by nifedipine. Human colonic circular muscle cells show

slow waves (12 mV amplitude; 9.4 s duration), which arise at or near the submucosal border at a frequency of 3 cycles/min. Each slow wave is associated with a transient contraction (2-4 contr/min). The myenteric edge exhibits myenteric potential oscillations that are associated with slow-long duration contractions (0.3-0.6 contr/min) with superimposed higher frequency contractions (17-18 contr/min) (Rae et al. 1998). These oscillations are similar to myenteric potential oscillations described in the canine colon.

9. Inhibitory neurotransmission

9.1. Enteric nervous system

The enteric nervous system has specific neural patterns that coordinate motility. These neural patterns are carried out by afferent neurons, interneurons and motor neurons. Both inhibitory and excitatory motor neurons regulate smooth muscle excitability that is paced by the pacemaker cells along the GI tract. The majority of motor neurons that are innervating the circular muscle layer release inhibitory neurotransmitters such as NO, ATP, VIP and PACAP (Okishio et al. 2000). In contrast, the longitudinal muscle layer is mostly innervated by excitatory motor neurons releasing acetylcholine and tachykinins. Enteric neuropathies are complex and might be responsible of gastrointestinal motility disorders, such as achalasia, gastroparesis, intestinal pseudo-obstruction and megacolon (De Giorgio et al. 2004; De Giorgio & Camilleri 2004).

9.2. Mechanisms involved in inhibitory neurotransmission

9.2.1. Action potential in motor neurons

Stimulation of non-adrenergic, non-cholinergic (NANC) motor neurons elicits a transient hyperpolarization of smooth muscle membrane referred to an inhibitory junction potential (IJP), followed by smooth muscle relaxation (Bywater & Taylor

1986). The neural blocker TTX blocks action potentials in neurons and consequently, the IJP is reduced or abolished.

9.2.2. Depolarization of nerve endings

The release of NANC neurotransmitters from the enteric motor neurons is mediated by action potentials that reach the nerve endings and open pre-junctional calcium channels, allowing calcium influx. N-type Ca^{2+} channels (but not P, Q or L-type) are involved in the calcium influx that triggers release of NANC neurotransmitters mediating IJPs, as it is described in the porcine ileum (Borderies et al. 1997). The neuronal N-type Ca^{2+} channel blocker omega-conotoxin GVIA abolishes IJPs, without affecting slow waves, as it is shown in circular muscle cells from canine ileum (Cayabyab et al. 1996). However, in the rat colon, N-type Ca^{2+} channels are not fully responsible for neurotransmitter release from nerve endings (Borderies et al. 1996) and further investigations are needed to characterize the voltage-gated Ca^{2+} channels involved in neurotransmission.

9.2.3. Release of inhibitory neurotransmitters. Candidates: NO, VIP, PACAP, ATP and CO

Non-adrenergic, non-cholinergic (NANC) transmitters are important elements involved in the inhibition of gastrointestinal smooth muscle cells, participating in many physiological reflexes. Several putative neurotransmitters have been proposed during the last 30 years: adenosine 5'-triphosphate (ATP), nitric oxide (NO), vasoactive intestinal peptide (VIP), carbon monoxide (CO) and pituitary adenylate cyclase-activating peptide (PACAP) (Lecci et al. 2002).

9.2.3.1. Evidence supporting VIP and PACAP as NANC inhibitory neurotransmitters

The neuropeptides vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP) are candidates to be considered in playing a role in inhibitory neurotransmission. VIP and PACAP have been found in enteric motor

neurons throughout the gastrointestinal tract and three receptors of the VIP/PACAP family have been characterized: $VPAC_1$, $VPAC_2$ and PAC_1. However, the lack of selective antagonists of these receptors makes the study of the physiological role of these neuropeptides difficult (Jin et al. 1993; McConalogue et al. 1995). All three receptors can be expressed in neurons, ICCs and smooth muscle cells and can mediate inhibitory signals in smooth muscles by activation of cAMP-dependent protein kinase (McConalogue et al. 1995).

9.2.3.2. Evidence supporting ATP as NANC inhibitory neurotransmitter

Adenosine 5'-triphosphate (ATP) is a purine nucleotide released by non-adrenergic, non-cholinergic inhibitory neurons (Burnstock et al. 1970; Burnstock et al. 1978; Giaroni et al. 2002). The quinacrine fluorescence technique evidences the presence of ATP in inhibitory neurons (Belai & Burnstock 1994). The inhibitory response of ATP is mediated by P2Y and/or P2X receptors (Lecci et al. 2002; Giaroni et al. 2002; De Man et al. 2003). ATP induces the fast component of the IJP (see below) through a mechanism that involves the activation of apamin-sensitive small conductance Ca^{2+}-activated K^+ channels, which generate an outward hyperpolarizing current (Pluja et al. 1999; Xue et al. 1999; Serio et al. 2003).

9.2.3.3. Evidence supporting NO as NANC inhibitory neurotransmitter

Nitric oxide (NO) is a molecule that regulates gastrointestinal motility acting as an inhibitory neurotransmitter in the enteric nervous system. The enzymatic reaction that produces NO needs L-arginine and by means of nitric oxide synthase (NOS) results in L-citruline and NO. Three different isoforms of NOS are responsible for NO production: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). L-NNA and L-NAME are widely used inhibitors of the NOS. Immunohistochemical studies have shown that the enzyme necessary for NO synthesis, NOS, is expressed in enteric neurons. Nitric oxide or a NO-releasing substance mediates the NANC relaxation in the canine terminal ileum and ileocolonic junction (Boeckxstaens et al. 1990; Christinck et al. 1991; Boeckxstaens et al. 1991b), the rat gastric fundus (Boeckxstaens et al. 1991a; Boeckxstaens et al. 1992) and human colon (Boeckxstaens et al. 1993). NO receptor is

the guanilate cyclase that is present in ICCs and smooth muscle cells. Activation of guanilate cyclase increases cyclic GMP levels and activates protein kinase G, which in turn acts to open potassium channels and close calcium channels in the smooth muscle cell membrane, leading to hyperpolarization and relaxation of the muscle.

9.2.3.4. Evidence supporting carbon monoxide (CO) as NANC inhibitory neurotransmitter

Heme oxygenase 2 is the enzyme that catalyzes the endogenous production of CO. NADPH-dependent oxidative heme destruction results in the release of biliverdin and CO. Heme oxygenase 2 is present in smooth muscle cells, certain neurons and ICCs (Maines 1988; Miller et al. 1998). Smooth muscle cells express a target site of action for carbon monoxide (Farrugia & Szurszewski 1999). Thus, CO has been proposed as NANC transmitter mediating inhibitory transmission in the gastrointestinal tract and also acting as a messenger molecule between interstitial cells of Cajal and smooth muscle cells. CO increases voltage-dependent potassium currents in smooth muscle cells, leading to membrane hyperpolarization (Farrugia et al. 1993).

9.3. Evidence of co-transmission: Biphasic inhibitory junction potential (IJP)

IJP usually presents two components: a sharp decrease of the membrane potential (fast component) followed by a sustained hyperpolarization and gradual recovery to the resting membrane potential (slow component) (Figure 4).

In the rat colon, the first component of the biphasic IJP is blocked by apamin 1 µM and the second component is blocked by L-NNA 1 mM. This suggests the participation of a purinergic transmitter in the fast component and the participation of a nitrergic mediator in the slow component (Pluja et al. 1999). In the rat proximal colon, almost all neurons contain both ATP (evidenced by quinacrine fluorescence) and NOS, giving a structural support for a co-transmission (Belai & Burnstock 1994). Thus, different inhibitory neurotransmitters are implicated in the IJPs elicited by EFS. The biphasic IJP in the rat colon is similar to those described in mouse colon (Shuttleworth et al. 1997), guinea-pig and human jejunum (Stark et al. 1993), whereas a monophasic

IJP has been recorded from the pig (Borderies et al. 1997) and dog (Stark et al. 1991; Stark et al. 1993). In the dog intestine, IJPs are mediated by nitric oxide (Christinck et al. 1991; Dalziel et al. 1991) and in the pig they are mediated by ATP (Borderies et al. 1997).

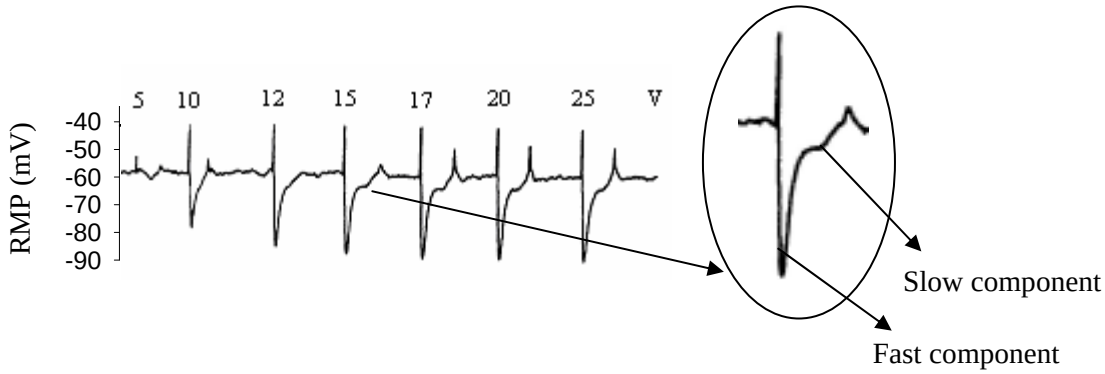

Figure 4. Inhibitory junction potential (IJP) elicited by electrical field stimulation (EFS) at increasing voltages (5, 10, 12, 15, 17, 20 and 25 V). This recording was obtained by intracellular microelectrode technique in the presence of nifedipine 1 μM. From (Pluja et al. 1999).

9.4. A new hypothesis: Interstitial cells of Cajal involved in neurotransmission

The structural relationships between ICCs, varicose nerve fibers, and smooth muscle cells in the gastrointestinal tract have led to the suggestion that ICCs may mediate enteric neurotransmission. Different subclasses of ICCs might play a role in neurotransmission depending on the area of the gastrointestinal tract. In the small intestine, ICC-DMP might act as mediators of the nitrergic neurotransmission to smooth muscle cells (Toma et al. 1999). In other areas, such as the stomach and the colon, ICC-IM are probably involved in neuromuscular transmission.

The study of mutant animals, with genetic deficiencies in ICC subclasses, has been a crucial tool to evaluate the role of ICCs in neurotransmission. In the gastric fundus of wild-type mice, ICC-IM are intimately associated with motor nerve terminals and nerve varicosities, forming synaptic structures with these cells. ICC-IM are also connected with neighbouring smooth muscle cells via gap junctions. In Sl/Sld mutant

mice, ICC-IM are absent in the fundus. These mutants show a reduction in the amplitude of IJPs and they also lack relaxation responses, despite the density of excitatory and inhibitory nerves is not significantly different than in wild-type muscles. This suggests the role of ICC-IM in neurotransmission and that these cells may be the primary site of innervation by enteric motor neurons in gastric muscles (Beckett et al. 2002).

In the stomach, lower oesophageal sphincter and pyloric sphincter of W/Wv mutant mice, ICC-IM are absent. In these animals that lack intramuscular ICCs, the enteric inhibitory responses are extremely reduced, including responses to nitrergic nerve stimulation, supporting a role for ICCs in neurotransmission (Burns et al. 1996; Ward et al. 1998; Ward et al. 2000).

10. Objectives

According to the introduction, the aim of this work is to study the origin of spontaneous motility and neurotransmission in the rat colon. In particular, we have studied the role of ICCs as pacemaker cells and as an interface between nerve endings and smooth muscle cells. To accomplish this objective, we have characterized the spontaneous activity, ICCs distribution and the influence of neural tone in the colon of Sprague-Dawley rats. Moreover, we have performed a similar study on Ws/Ws mutant rats. Finally, we have tested the effect of 4-aminopyridine on the spontaneous activity and neurotransmission. The concrete objectives of each study are described below.

Objective 1: Characterization of the pacemaker activity and neural modulation in the colon of Sprague-Dawley rats.

1.1. Characterize the patterns of spontaneous motility in the proximal, mid and distal colon of Sprague-Dawley rats, in both circular and longitudinal muscle strips.

1.2. Study the neural influence involved in the inhibitory modulation of spontaneous motility.

1.3. Characterize the distribution of ICCs and nitrergic neurons in the colon.

Objective 2: Characterization of the pacemaker activity and inhibitory neurotransmission in the colon of Ws/Ws mutant rats. (For comparison, we have used +/+ rats as controls.)

2.1. Study the distribution of nitrergic neurons and ICCs.

2.2. Characterize the spontaneous electrical and mechanical activities.

2.3. Characterize the inhibitory neurotransmission.

2.4. Study the neural influence involved in the inhibitory modulation of spontaneous motility.

Objective 3: Study the effect of the K^+ channel blocker 4-aminopyridine (4-AP) in the colon of Sprague-Dawley rats.

3.1. Study the effect of 4-AP on the spontaneous electrical and mechanical activities displayed by circular colonic muscle strips.

3.2. Evaluate the effect of 4-AP on NANC inhibitory neurotransmission.

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Chapter 1

Motility patterns and distribution of interstitial cells of Cajal and nitrergic neurons in the proximal, mid and distal colon of the rat

1.1. Summary

The aim of this work was to study the patterns of spontaneous motility in the circular and longitudinal muscle strips and to characterize the distribution of *c-kit* positive interstitial cells of Cajal (ICCs) and nitrergic neurons (nNOS) in the proximal, mid and distal colon of Sprague-Dawley rats. We described two types of spontaneous contractions: high frequency (HF) and low frequency (LF) contractions, which were recorded in the presence of tetrodotoxin, suggesting a non-neurogenic origin. Regional differences were found in the motility patterns depending on the muscle layer and on the part of the colon studied. Muscle strips without submuscular plexus (SMP) showed only LF contractions. The density of ICCs was of the same magnitude along the extent of the colon: about 90 to 120 cells/mm^2 at Auerbach's plexus (AP) and 50 to 60 cells/mm^2 at the SMP. nNOS positive cells were found at the level of the AP and the major density was found in the mid colon. Electrical field stimulation abolished LF but did not affect HF contractions. Our results indicate that HF contractions are due to the ICC network found associated with the submuscular plexus (ICC-SMP). The origin of LF contractions is still unknown.

1.2. Introduction

The control of the mechanical activity of the colon is complex and several mechanisms are involved in its regulation. Elements that contribute to the regulation of colonic motor activity include interstitial cells of Cajal (ICCs) that generate slow wave activity, the smooth muscle layers themselves (circular and longitudinal) and intrinsic neural pathways. Moreover, different regions of the colon might have different electrical and mechanical properties.

Since Santiago Ramón y Cajal reported for the first time the existence of ICCs in the gut (Ramón y Cajal 1892; Ramón y Cajal 1893; Ramón y Cajal 1904), many investigators have studied the mechanisms involved in the control of the gut function (Sanders 1996; Huizinga 1999; Ward 2000; Sanders et al. 2000; Huizinga 2001; Daniel 2001). About 20 years ago it was suggested that ICCs were pacemaker cells responsible for the slow wave activity (Faussone Pellegrini et al. 1977; Thuneberg 1982).

In the colon, a number of studies have demonstrated that ICCs are associated with Auerbach's (ICC-AP) and submuscular (ICC-SMP) plexuses (Berezin et al. 1988; Berezin et al. 1990; Rumessen et al. 1993). In the canine colon, slow waves are originated by the ICC-SMP network, whereas ICC-AP generate myenteric potential oscillations (Smith et al. 1987a; Smith et al. 1987b). In human colon, ICCs located at the submucosal border have also been suggested as responsible for the slow wave activity (Rae et al. 1998). Intrinsic neural patterns such as the peristaltic reflex are also crucial in gastrointestinal motility. A second example of a motor pattern are giant migrating contractions which are not related to slow wave activity but its propagation is controlled by the enteric nervous system (Otterson & Sarna 1994). In rats, electromyographic recordings show the presence of cyclic spike bursts during the fed and fasted state (Ferre & Ruckebusch 1985). These regular spike bursts are muscular action potentials that cause cyclic contractions that can be recorded with strain gauges (Li et al. 2002). The frequency of spike bursts and subsequent contractions is higher in the proximal compared to distal areas of the colon (Ferre & Ruckebusch 1985; Li et al. 2002). *In vivo*, the regular cyclic activity might have a neurogenic origin because the cyclic activity is blocked by hexamethonium and consequently a parallelism between

giant migrating contractions (GMCs) and the colonic motor pattern has been established (Li et al. 2002). In contrast, *in vitro*, GMCs can be recorded in the presence of neural blockade suggesting a myogenic origin (Pluja et al. 1999; Pluja et al. 2001; Gonzalez & Sarna 2001a; Gonzalez & Sarna 2001b).

In mice, a neurogenic pacemaker generates *in vitro* a well characterized colonic motor complex (Lyster et al. 1995; Spencer et al. 1998). This neurogenic mechanism causes cyclic depolarizations and repolarizations of smooth muscle cells which cause cyclic contractions.

Finally, as it has been reported in other species, in the colon of rodents (rats and mice) *c-kit* positive cells at the submucosal border generate slow waves (or plateau potentials) and in rats generate rhythmic contractions of low amplitude and high frequency (HF) (10-15 cycles/min) (Pluja et al. 2001; Yoneda et al. 2002). The origin of this cyclic activity is controversial because: (i) neurogenic or myogenic mechanisms have been proposed; (ii) there is no uniform agreement regarding the nomenclature of the motility; (iii) discrepancies between *in vivo* and *in vitro* results have been reported and (iv) differences have been found between rats and mice.

The aim of the present study was to characterize the motility patterns of the rat colon *in vitro* and to quantify, by using an unbiased stereological method, the distribution of nitrergic cells and ICCs in the proximal, mid and distal areas of the rat colon. This study might help to characterize the elements that contribute to the control of colonic motility. Preliminary data from this work were presented at the XI European Symposium on Neurogastroenterology and Motility in Tübingen (Germany).

1.3. Methods

1.3.1. Animals

In the present study, male Sprague-Dawley rats (Iffa-Credo, Lyon, France) 8-10 weeks old and 300-350 g were used. Animals were kept at a constant temperature (20-21ºC) and humidity (60%) in groups of three animals and had unlimited access to water and food. Ambient illumination was maintained on a 12 h light/dark cycle. Before the *in vitro* motility studies, rats were kept individually and fasted for 16-18 h with *ad libitum* access to water. They were stunned before being decapitated and bled. The housing and handling of animals were approved by The Ethics Committee of the Universitat Autònoma de Barcelona.

1.3.2. Tissue preparation

The colon was removed from 1 cm below the ileocecal junction to the pelvic brim. The colon was placed in (a) carbogenated Krebs solution, for motility studies or in (b) phosphate buffered saline (PBS) with nifedipine 1 µM for 15 min to ensure relaxation, for morphological studies. The colon was opened along the mesenteric border in both types of experiments and pinned to a Sylgard base with the mucosa facing upwards.
Considering site-specific anatomical parameters we divided the colon into three different parts: proximal, mid and distal colon (Figure 1).

1.3.3. Motility studies

In order to evaluate the spontaneous mechanical activity, muscle bath technique was performed. The following preparations were used: (i) full-thickness strips and (ii) muscle strips without submucosa. The submucosal border was removed with fine forceps by sharp dissection under a magnifying glass. These preparations (~1 cm long and 0.3 cm wide) were made for each colonic part (proximal, mid and distal) and

following both circular and longitudinal smooth muscle layers. Strips were mounted under 1 g tension with a 2/0 silk thread in a 10-ml muscle bath containing carbogenated Krebs solution maintained at 37 ± 1 ºC. One strip edge was tied to the bottom of the muscle bath using suture silk and the other one to an isometric force transducer (Harvard Apparatus Inc., Holliston, MA, USA). Output from the transducer was fed to a PC through an amplifier. Data were digitized (25 samples/s) and displayed with Data 2001 software (Panlab, Barcelona, Spain).

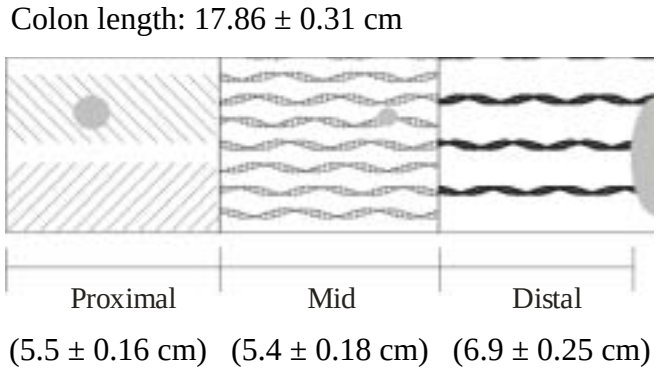

Figure 1. Anatomical criteria to define the different parts of the colon. The proximal colon has a characteristic mucosa with marked folds 45º from the longitudinal axis and one Peyer's patch. The mid colon was considered to extend from the end of the proximal colon to 1 cm below the second Peyer's patch. The mucosa in the mid colon had smaller folds, which were longitudinally oriented. The distal colon was considered to extend from the end of the mid colon to a third Peyer's patch (more developed) where it was cut near the pelvic brim. Peyer's patches are represented in grey.

Strips were allowed to equilibrate for ~1 h before experiments started**.** Preparations did not developed active tension, and a passive tension (baseline) between 0.1 and 0.7 g was obtained. Spontaneous phasic contractions were recorded. The amplitude of a contraction was the difference between the peak of a contraction (mean peak of more than 10 contractions) and the baseline. The frequency, i.e., the number of contractions per minute, was the average in time over more than 10 contractions.

Non-adrenergic, non-cholinergic (NANC) conditions were obtained by adding atropine sulphate, phentolamine and propranolol to the Krebs solution at 1 μM. The

effects of the different pharmacological agents on the amplitude and frequency of the contractions were studied in individual muscle strips before and after drug addition.

To study the presence of a NANC inhibitory neural tone on the spontaneous mechanical activity in the different areas of the colon, muscle strips were studied in the presence of the neural blocker TTX (1 µM), the nitric oxide synthase inhibitor L- NNA (1 mM) and the small conductance calcium activated K^+ channel blocker apamin (1 µM). The release of inhibitory neurotransmitters was studied by placing circular muscle strips in a muscle bath under NANC conditions and stimulating them by electrical field stimulation (EFS: 28 to 40 V, 4 Hz, 0.3 ms during about 3-4 minutes).

1.3.4. Morphological studies

In rats used for morphological experiments (n = 5) each part (proximal, mid and distal) was cut into three pieces respectively. The first piece was frozen, the second and third were prepared for whole mount immunohistochemistry using antibodies towards nitrergic neurons (nNOS) and *c-kit* receptor. The mucosa was removed by sharp dissection in all preparations.

1.3.5. Immunohistochemistry studies

Whole mount preparations were used to study the morphology and areal density (number of cells/mm^2 surface area) of nNOS positive cells and *c-kit* positive cells. When using nNOS antibodies, whole mounts were fixed with 4% paraformaldehyde for 3 h immediately before immunostaining and with Zamboni's fixative when using *c-kit* antibodies. The tissue was preincubated with human serum, and incubated with primary antibodies overnight and with biotinylated secondary antibodies for 4 h. Immunoreactivity was demonstrated with the streptavidin-biotin (ABC-complex, Dako, Glostrup, Denmark) method using 0.5% diaminobenzidine in 0.035% H_2O_2 in PBS as substrate. They were rinsed in distilled water and 1% H_2O_2, dehydrated in dimethoxypropan, benzene and methyl benzoate, and mounted with Eukit. Frozen sections were used to confirm the distribution of *c-kit* and nNOS positive cells

throughout the thickness of the colon. For frozen sections, the unfixed tissue was pinned to a Sylgard base and quick-frozen in isopentane cooled in liquid nitrogen and stored at -80 ºC. The sections where fixed in Zamboni before immunostaining. The rest of the procedure was similar to that used for the whole mounts.

1.3.6. Stereological analysis

Both nNOS and *c-kit* positive cells were counted in the three different colonic segments using unbiased stereological technique. The total number of cells per region was estimated by counting the number of cells in a known fraction of the region and multiplying that result by the reciprocal of the known fraction, the so-called fractionator technique (Larsen J.O. 1998; Gundersen 1986; Mayhew 1988). The actual counting was performed on systematic random fields of vision by moving an unbiased counting frame (Gundersen 1977) through the full-thickness of the whole mount specimen. The number of cells per surface area, i.e., the areal density of cells, is $Q_A = N_{tot}/OA$, where N_{tot} is the total number of cells in the region and OA is the surface area of the region. The surface areas of the whole mounts were estimated by point counting using a grid of systematic uniform test points that was randomly placed on the whole mount profile. The number of points OP that hit the profile, multiplied by the area associated with each point *a(p)* in the grid, are used to estimate $A = a(p) \cdot OP$.

The stereological analysis of nNOS and *c-kit* positive cells was carried out on a computer monitor using computer-assisted interactive stereological test systems (The CAST-grid software, Olympus Denmark). Live video images of the fields of vision in the microscope were transmitted by a video camera to the computer screen. The microscope was equipped with stepping motors that controlled stage movements via the software. The entire region was delineated at a low magnification (x102 using a x2 PlanApo objective). Cells were counted at a final magnification of x1022 using a x20 UPanApo oil immersion objective (NA = 0.8) to which the x2 objective used for delineation was paracentered. At high magnification the computer-controlled stage of the Olympus BX51 microscope was programmed to move the section systematically, random in a raster pattern within the delineated region with interactively defined steps.

At each point in the raster pattern the image of an unbiased counting frame was superimposed on to the microscope image via the video-computer interface and was 'moved' through the entire thickness of the whole mount specimen, and all cells within the unbiased counting frame were counted.

nNOS positive cells were counted transmurally with a counting frame of area 7412 σm^2 using step-lengths, dx and dy, of 500 σm. The counting frames covered ~3% of the area of the whole mount.

All *c-kit* positive cells, present in the whole mounts, were divided into two groups and counted (i) at AP and the connected cells in LM and CM; (ii) at the submucosal border (ICC-SMP) and adjacent cells in CM (ICC-CM), using a counting frame of area 2965 σm^2 and dx and dy of 700 σm, covering approximately 0.6% of the area of the whole mount. The point counting used to make the area estimation of the specimens was performed with a Leica DMLB projection microscope using a X1.6 HCX PL Fluotar objective, that provided a final magnification of X 22.25. For estimating the areas, the a (p) was 1.82 mm^2.

1.3.7. Solutions and drugs

a) *Motility studies*

The composition of Krebs solution was (in mM): glucose 10.10; NaCl 115.48; $NaHCO_3$ 21.90; KCl 4.61; NaH_2PO_4 1.14; $CaCl_2$ 2.50 and Mg SO_4 1.16 (pH 7.3-7.4). The solution was bubbled with carbogen (95% O_2 and 5% CO_2). The following drugs were used: phentolamine and Nϖ-nitro-L-arginine (L-NNA) (Sigma Chemical, St. Louis, MO, USA), atropine sulphate (Merck, Darmstadt, Germany), tetrodotoxin and apamin (Latoxan, Valence, France), propranolol (Tocris, Tocris Cookson Ltd., Bristol, UK). Stock solutions were prepared by dissolving drugs in distilled water, except tetrodotoxin, which was diluted in 1% glacial acetic acid.

b) *Morphological studies*

Nifedipine was used at 1 μM (diluted in PBS), paraformaldehyde 4%, Zamboni's fixative: 4% paraformaldehyde, 0.15% picric acid in 0.1 M PBS (pH 7.4), HCl,

methanol, H_2O_2, human serum albumen, triton X-100, azid, 0.5% diamino benzedine, dimethoxypropan, benzene, methyl benzoate, Eukit and isopentane.

1.3.8. Antibodies

Primary antibodies were rabbit anti-neuronal nitric oxide synthase (nNOS) (Chemicon, Temecula, CA, USA) 1:500 and rabbit anti-*c-kit* receptor 1:500 (Santa Cruz, Sc 168, CA, USA). Secondary antibody was biotin-conjugated donkey anti-rabbit $F(ab)_2$ (Jackson, ME, USA) 1:2000. All the antibodies were diluted in 1% human serum albumen in PBS + 0.3% triton-X-100. Negative controls included the omission of primary or secondary antibodies or preincubation of the primary antibody with the corresponding peptides.

1.3.9. Statistics

Statistics was performed with GraphPad Prism v.3.0 (San Diego, CA, USA) software. An analysis of variance (ANOVA) with a Bonferroni *post-hoc* test was used to compare the proximal, mid and distal colon in the different experimental protocols in both circular and longitudinal muscles. A non-paired Student's *t*-test was used to compare the mechanical activity between circular and longitudinal muscle strips. A paired Student's *t*-test was used to compare mechanical activity in the absence and presence of drugs. Data are expressed as mean values ∂ SE. $p < 0.05$ was considered to be statistically significant. For the morphological studies, a two-way ANOVA was used to test for regional differences of the three parts (proximal, mid and distal) using a 5% significance level. *Post-hoc* comparisons between different segments were performed with a paired *t*-test given that significant difference was found with the ANOVA. In order to avoid "mass-significance" the null hypothesis was rejected when *3p* Ω0.05.

1.4. Results

1.4.1. Patterns of spontaneous mechanical activity in the rat colon

In Krebs solution we recorded two types of spontaneous contractions in preparations with intact SMP (Figure 2). Low frequency (LF) contractions ranged from 0.5 to 3.7 contractions per minute (cpm) and high frequency (HF) contractions from 8 to 12 cpm. LF contractions usually show higher amplitude (between 250 and 2000 mg) than HF contractions (between 50 and 300 mg) (Table 1). In strips without the SMP, HF contractions were not observed and only LF contractions were recorded from both circular and longitudinal muscle strips (Figures 3 A,B). However, the pattern of spontaneous motility defined as the amplitude and frequency of these two types of contractions depend on the orientation of the strip (circular or longitudinal) and depend on the location of the muscle strip along the colon (proximal, mid or distal).

1.4.2. Patterns of motility in circular muscle strips

In circular muscle preparations, both LF and HF contractions were of higher amplitude in the proximal compared to those from the mid and distal colon (see Table 1). LF contractions had higher frequency in proximal segments and decreased distally, whereas HF contractions showed a steady frequency (between 10 and 13 cpm) along the colon (Table 1).

1.4.3. Patterns of motility in longitudinal muscle strips

Muscle strips oriented longitudinally also showed two types of contractions in the proximal, mid and distal areas (Figure 2B). In contrast to the circular muscle strips, the amplitudes of both contraction types (both LF and HF contractions) were higher in the distal part of the colon compared to more proximal areas (Table 1). In the proximal colon, approximately one-third of the strips showed continuous small amplitude HF contractions.

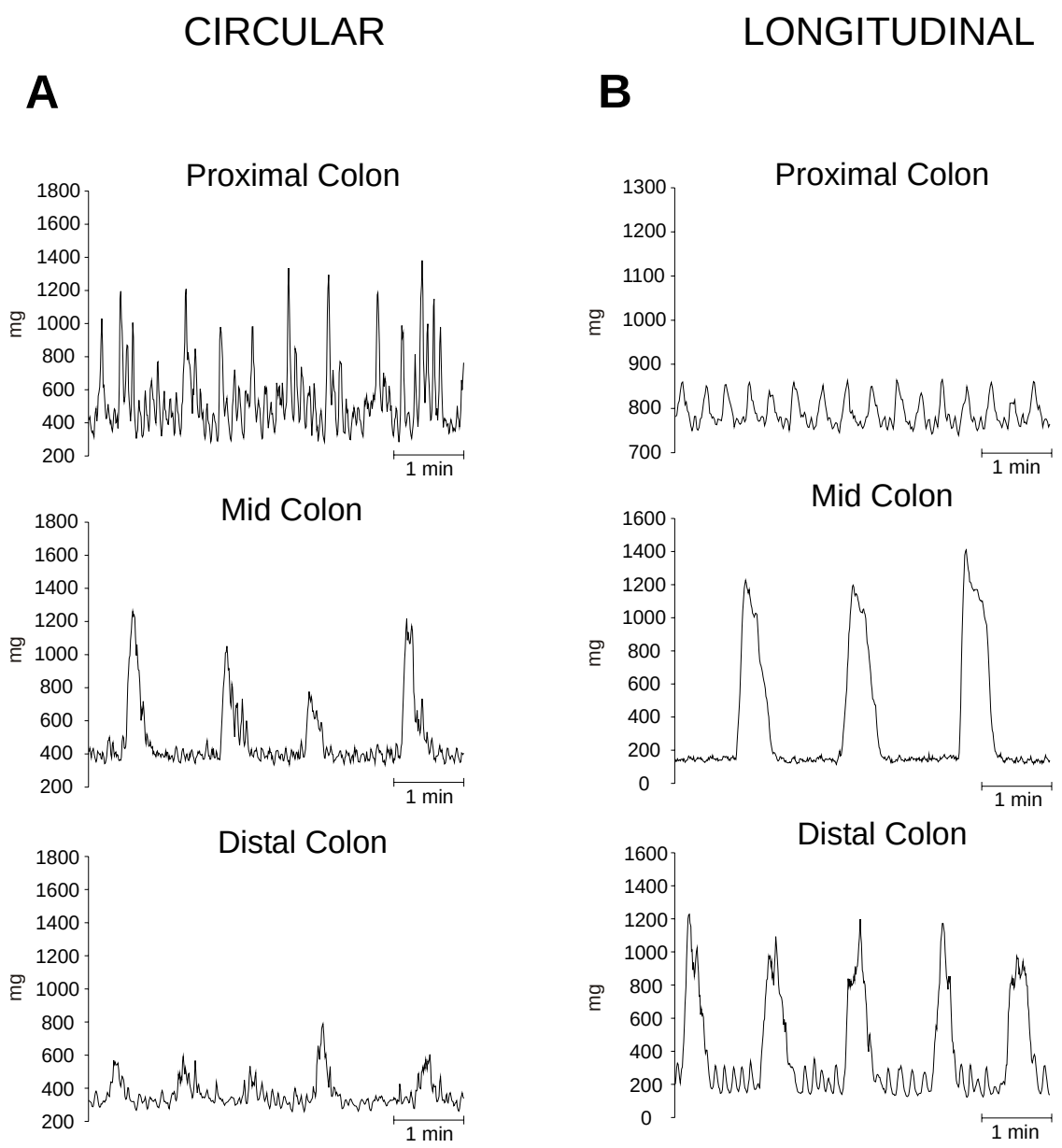

Figure 2. Muscle bath recordings showing the spontaneous cyclic mechanical activity displayed by circular (A) and longitudinal (B) muscle strips in colonic rat preparations with preserved Auerbach's plexus (AP) and submuscular plexus (SMP).

In the rest of proximal colon strips, two contraction types were distinguished although very similar amplitudes were found between LF and HF contraction types. The frequencies of the two contraction types followed the same patterns as those described for circular muscle strips: HF contractions were about 10 cpm and LF contractions were higher in proximal compared to mid and distal areas (Table 1).

CIRCULAR				
	LF		HF	
KREBS	Ampl. (mg)	Freq. (cpm)	Ampl. (mg)	Freq.(cpm)
Proximal	877 ± 182 (n = 7)	2.25 ± 0.49 (n = 7)	240 ± 45 (n = 6)	11.75 ± 0.51 (n = 6)
Mid	630 ± 152 (n = 7)	0.67 ± 0.09 (n = 7)	84 ± 11 (n = 8)	10.74 ± 0.89 (n = 8)
Distal	298 ± 57 (n =7)	0.55 ± 0.09 (n = 7)	70 ± 5 (n = 7)	11.02 ± 0.88 (n = 7)
Anova	$p < 0.05$	$p < 0.01$	$p < 0.001$	*n.s.*

LONGITUDINAL				
	LF		HF	
KREBS	Ampl. (mg)	Freq. (cpm)	Ampl. (mg)	Freq.(cpm)
Proximal	362 ± 87 (n = 9)	2.76 ± 0.23 (n = 9)	57 ± 17 (n = 3)	10.35 ± 0.25 (n = 3)
Mid	1406 ± 347 (n = 6)	0.72 ± 0.05 (n = 6)	126 ± 26 (n = 6)	9.56 ± 0.96 (n = 6)
Distal	1440 ± 305 (n = 7)	0.81 ± 0.09 (n = 7)	185 ± 31 (n = 7)	8.58 ± 0.25 (n = 7)
Anova	$p < 0.01$	$p < 0.001$	*n.s.*	*n.s.*

Table 1. Motility patterns observed in rat colonic muscle strips with both plexuses intact in Krebs solution. LF: low frequency contractions; HF: high frequency contractions; Ampl: amplitude; Freq: frequency; Cpm: contractions per minute; Anova: differences between proximal, mid and distal colon.

1.4.4. Comparison between circular and longitudinal muscle strips

The amplitudes of contractions in the proximal colon were highest in circular strips. Conversely, the amplitude in distal segments was highest in longitudinal muscle strips (Figure 4 top). In strips from the same region we did not find differences in the frequencies of LF and HF contractions when comparing between circular and longitudinal muscle layers (Figure 4 bottom).

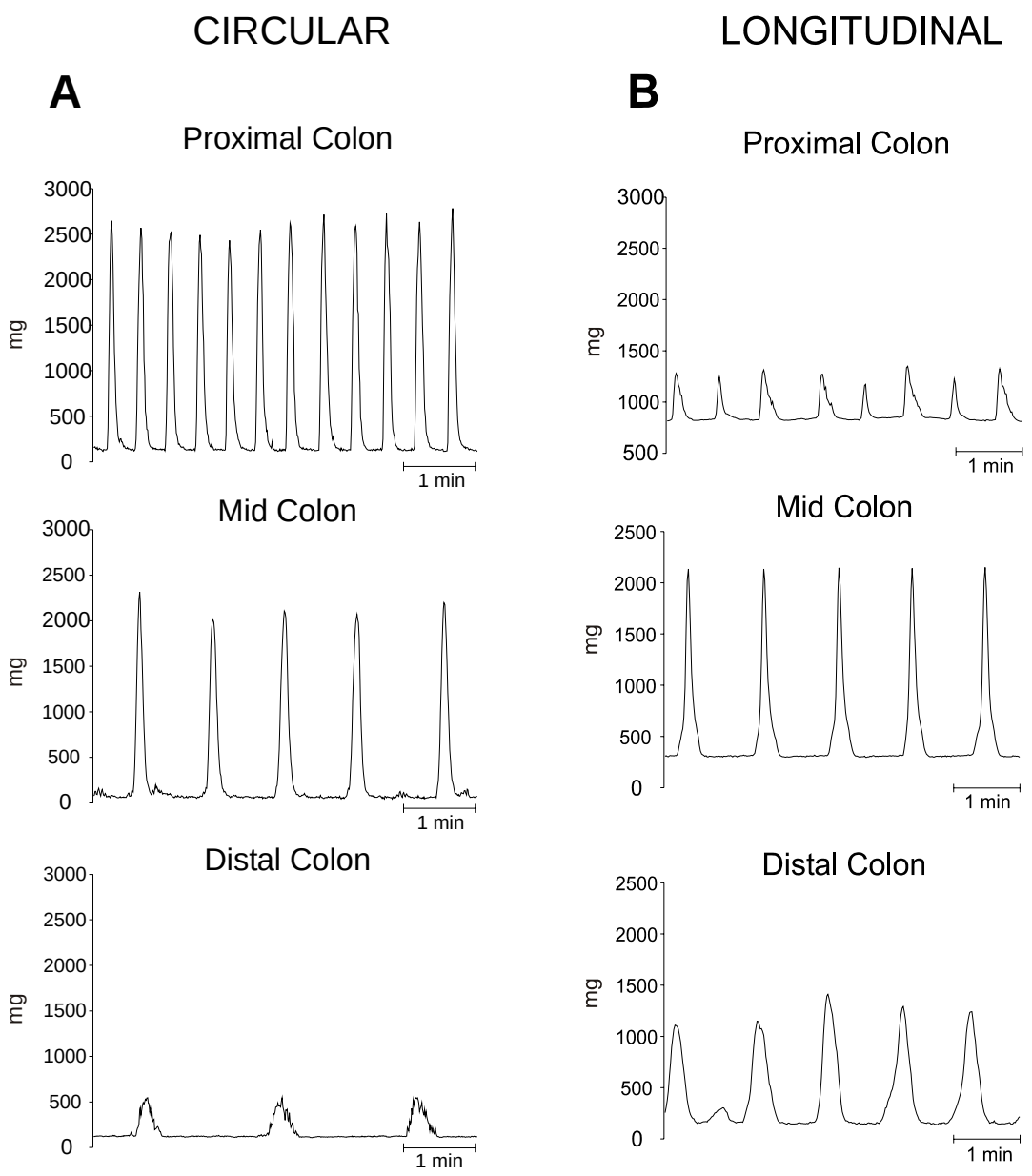

Figure 3. Mechanical recordings showing the spontaneous cyclic activity displayed by the circular (A) and longitudinal (B) muscles in strips devoid of submuscular plexus (notice the absence of high frequency contractions).

1.4.5. Patterns of motility in strips without submucosa

In strips without submucosa only LF contractions were recorded from both circular and longitudinal muscle strips. HF contractions were not observed (Figures 3 A,B). The

pattern (relative amplitude and frequency between areas) of spontaneous LF contractions was preserved in preparations without SMP (Table 2) when strips were placed in Krebs solution and under NANC conditions (not shown).

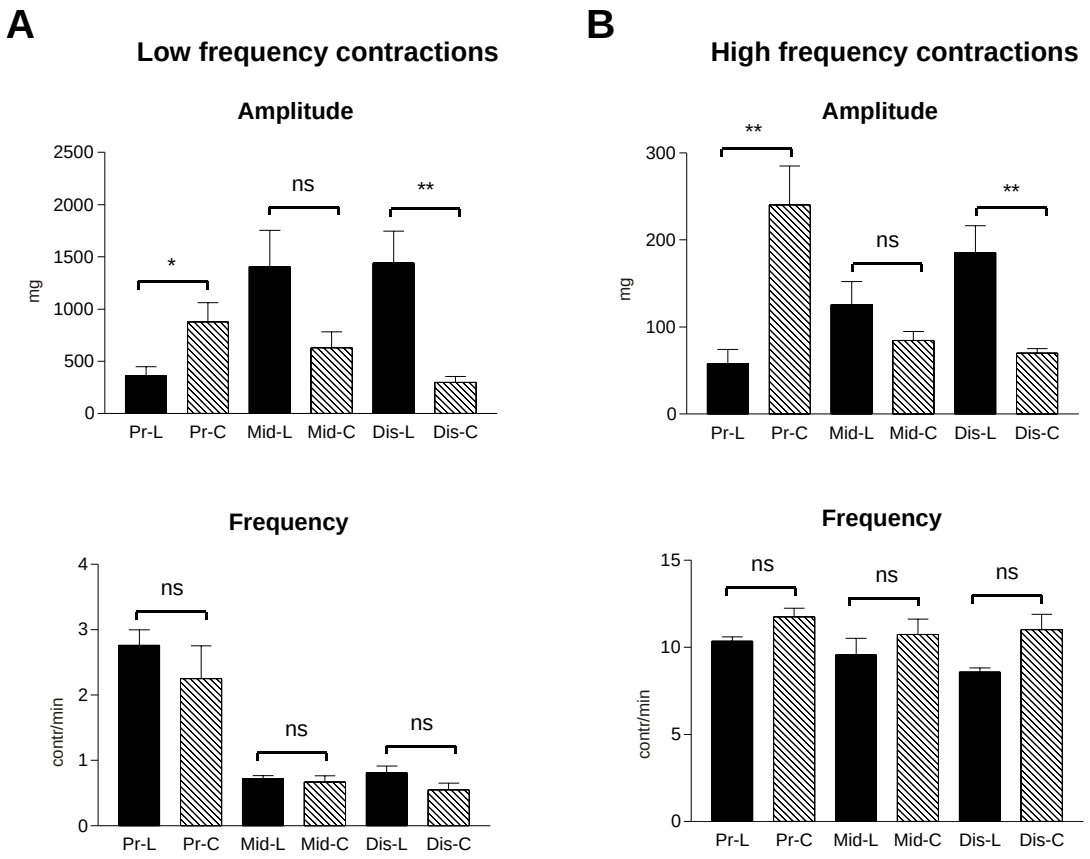

Figure 4. Bar diagrams comparing the amplitude and frequency between circular and longitudinal muscle strips for both low frequency (A) and high frequency (B) contractions. Pr-L and Pr-C: proximal longitudinal and circular muscle strips, respectively. Mid-L and Mid-C: mid longitudinal and circular muscle strips, respectively. Distal-L and Distal-C: distal longitudinal and circular muscle strips, respectively.

1.4.6. Patterns of motility in the presence of the neural blocker TTX

In the presence of the neural blocker TTX (1 μM) the motility pattern was preserved both in preparations with intact (Table 3, Figures 5 A,B) and without SMP (Table 2)

and when the tissue was studied in normal Krebs solution and under NANC conditions (not shown).

KREBS				
	Circular		Longitudinal	
	Ampl. (mg)	Freq. (cpm)	Ampl. (mg)	Freq.(cpm)
Proximal	1777 ± 190 (n = 6)	1.85 ± 0.20 (n = 6)	311.4 ± 49.5 (n = 7)	1.28 ± 0.18 (n = 7)
Mid	1551 ± 206 (n = 6)	0.98 ± 0.09 (n = 6)	856.2 ± 155.6 (n = 6)	0.59 ± 0.08 (n = 6)
Distal	418 ± 120 (n = 6)	0.44 ± 0.05 (n = 6)	512.5 ± 100.9 (n = 6)	0.73 ± 0.05 (n = 6)
Anova	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.05$

TTX				
	Circular		Longitudinal	
	Ampl. (mg)	Freq. (cpm)	Ampl. (mg)	Freq.(cpm)
Proximal	4193 ± 863 (n = 10)	1.86 ± 0.13 (n = 10)	559 ± 92 (n = 6)	1.08 ± 0.17 (n = 6)
Mid	4203 ± 523 (n = 11)	0.83 ± 0.14 (n = 11)	1109 ± 293 (n = 6)	0.52 ± 0.07 (n = 6)
Distal	776 ± 145 (n = 10)	0.32 ± 0.06 (n = 10)	1040 ± 424 (n = 6)	0.59 ± 0.12 (n = 6)
Anova	$p < 0.001$	$p < 0.001$	*n.s.*	$p < 0.05$

Table 2 Characteristics of the contractions (low frequency) observed in rat colonic muscle strips without submuscular plexus in Krebs solution and in the presence of TTX 1 µM. Ampl: amplitude; Freq: frequency; Cpm., contractions per minute; Anova: differences between proximal, mid and distal colon.

1.4.7. Non-adrenergic, non-cholinergic inhibitory neural tone

The amplitude of HF contractions of both longitudinal and circular muscle strips was not modified by any of the drugs tested (Figure 6). In contrast, the amplitude of LF contractions amplitude was usually increased by TTX, L- NNA and apamin (Figure 6).

Comparing data from circular and longitudinal layers we found that TTX increased (almost twice) the amplitude of contractions in circular muscle strips whereas the increase in amplitude was moderate in longitudinal muscle strips, suggesting a poor inhibitory tone in this layer (Figure 6). Nitric oxide (NO) and/or apamin-sensitive mediators, such as ATP, might be involved in the inhibitory tone of the circular muscle layer (Figure 6).

CIRCULAR				
	LF		HF	
TTX	Ampl. (mg)	Freq. (cpm)	Ampl. (mg)	Freq.(cpm)
Proximal	2492 ± 543 (n = 9)	3.77 ± 1.01 (n = 9)	230 ± 56 (n = 6)	12.71 ± 0.71 (n = 6)
Mid	1970 ± 313 (n = 8)	1.02 ± 0.16 (n = 8)	92 ± 16 (n = 6)	12.55 ± 0.60 (n = 6)
Distal	1086 ± 219 (n = 10)	0.80 ± 0.09 (n = 10)	85 ± 13 (n = 7)	9.24 ± 1.10 (n = 7)
Anova	$p < 0.05$	$p < 0.01$	$p < 0.05$	$p < 0.05$

LONGITUDINAL				
	LF		HF	
TTX	Ampl. (mg)	Freq. (cpm)	Ampl. (mg)	Freq.(cpm)
Proximal	822 ± 249 (n = 6)	1.75 ± 0.36 (n = 6)	51.18 ± 12.78 (n = 4)	10.93 ± 2.35 (n = 4)
Mid	1355 ± 276 (n = 6)	0.53 ± 0.08 (n = 6)	91.73 ± 23.38 (n = 4)	11.11 ± 1.59 (n = 4)
Distal	1341 ± 130 (n = 6)	0.73 ± 0.22 (n = 6)	253.60 ± 81.53 (n = 4)	8.12 ± 0.39 (n = 4)
Anova	*n.s.*	$p < 0.01$	$p < 0.05$	$p < 0.05$

Table 3 Motility patterns observed in rat colonic muscle strips with both plexuses intact in the presence of TTX 1µM. LF: low frequency contractions; HF: high frequency contractions; Ampl: amplitude; Freq: frequency; Cpm: contractions per minute. Anova: differences between proximal, mid and distal colon.

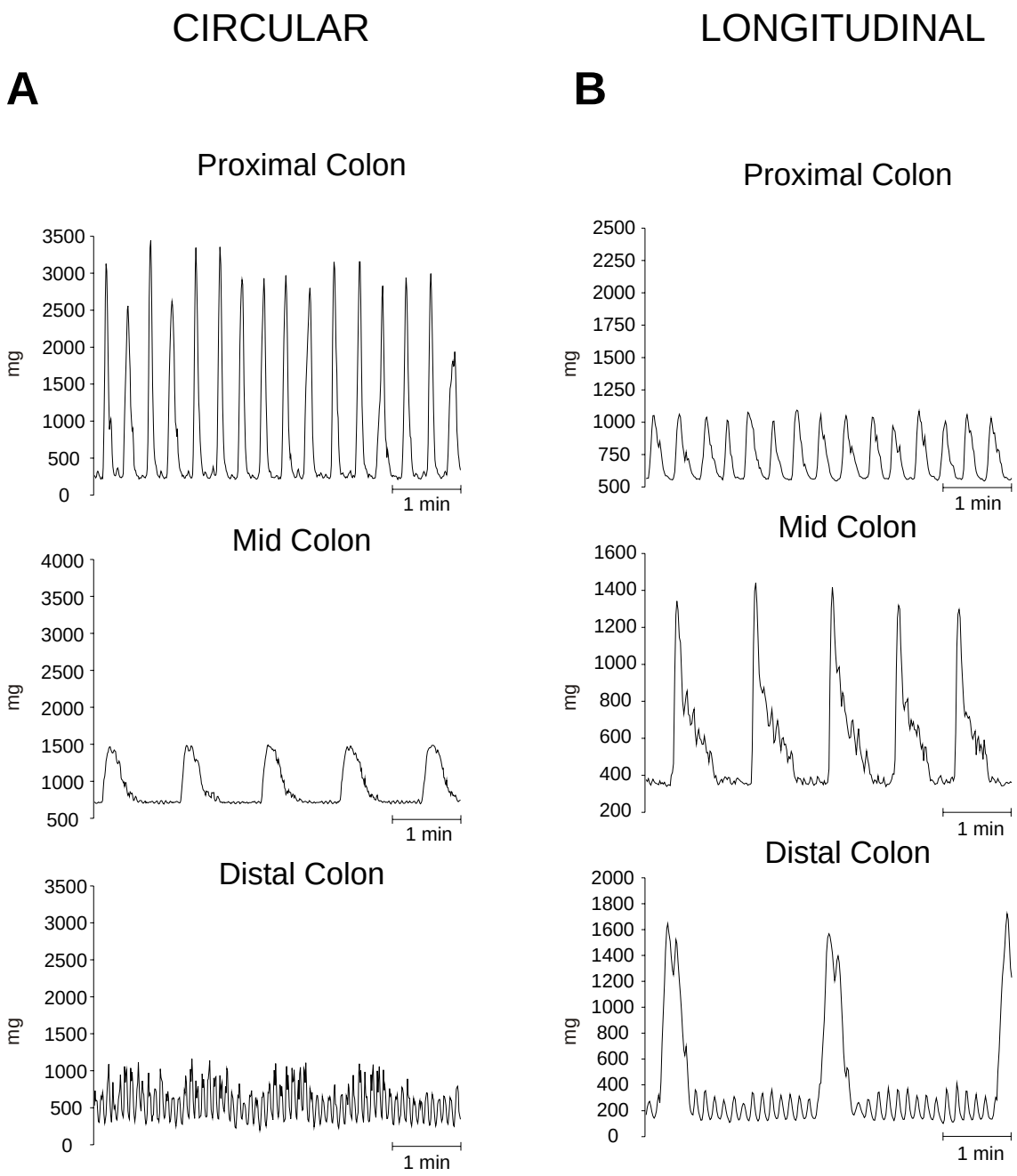

Figure 5. Muscle bath recordings showing the spontaneous cyclic mechanical activity of circular (A) and longitudinal (B) muscle strips with Auerbach's plexus (AP) and submuscular plexus (SMP) kept intact in the presence of TTX 1 μM.

1.4.8. Effect of the release of NANC inhibitory transmitters on the spontaneous contractions

In the circular muscle, LF contractions were abolished by EFS. In contrast, HF contractions were less sensitive to EFS and were only slightly affected using high voltages. After the addition of TTX (1 µM), EFS did not modify the two types of contractions (Figure 7).

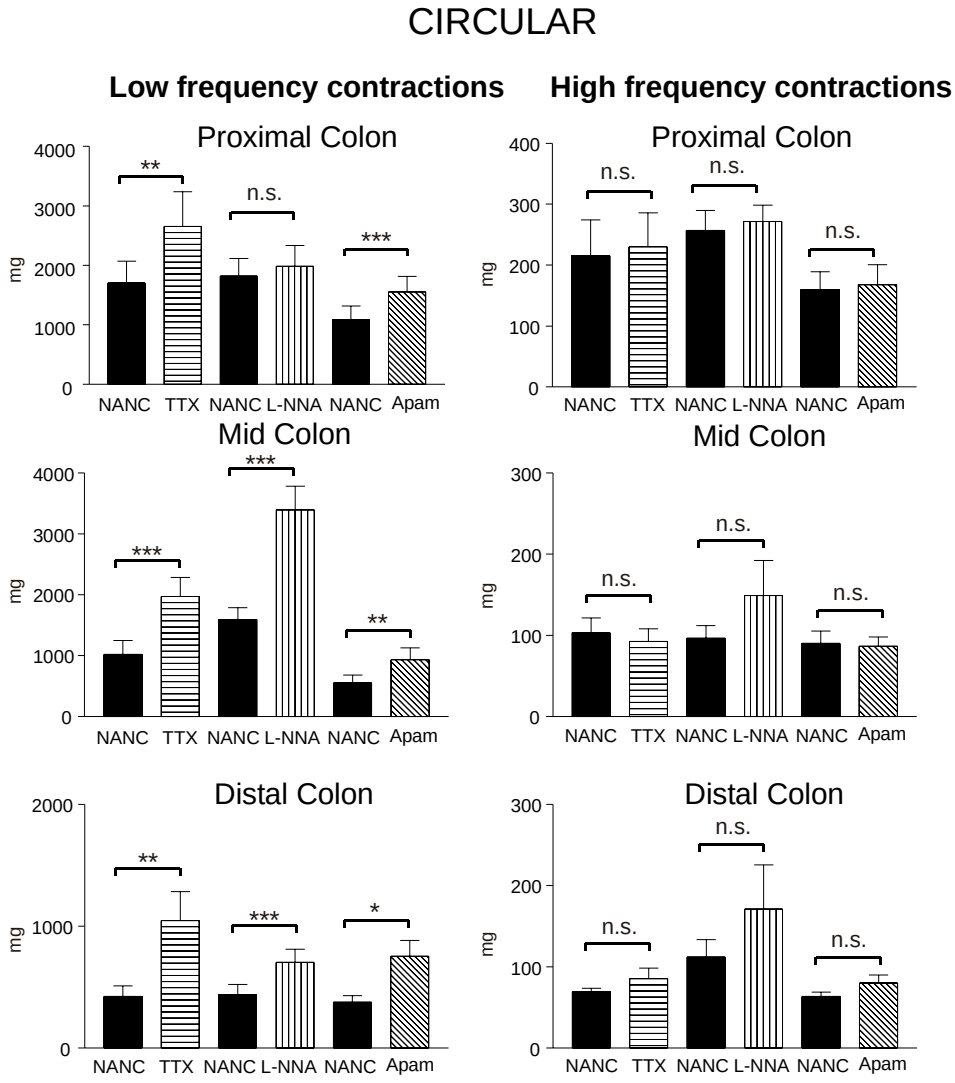

Figure 6A. Effect of TTX (1 µM), L-NNA (1 mM) and apamin (1 µM) on the amplitude of LF and HF contractions in the proximal, mid and distal areas in circular colonic muscle strips. Experiments were performed in non-adrenergic, non-cholinergic (NANC) conditions.

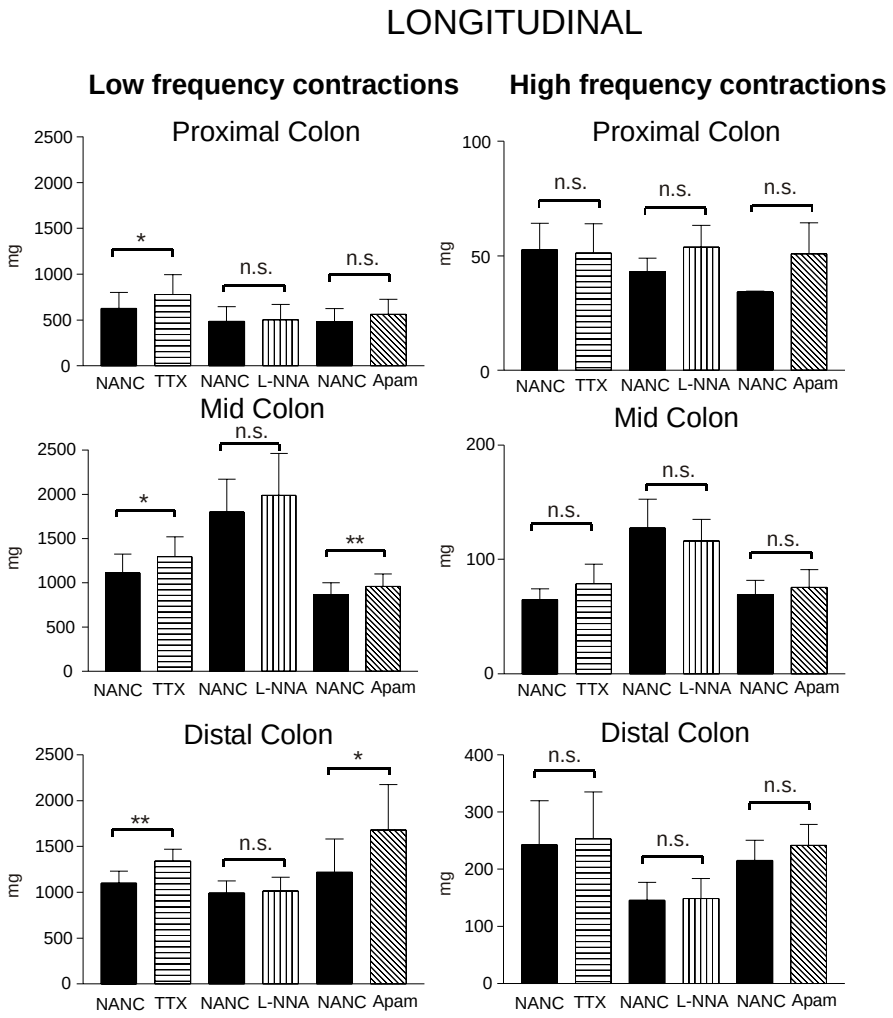

Figure 6B. Effect of TTX (1 µM), L-NNA (1 mM) and apamin (1 µM) on the amplitude of LF and HF contractions in the proximal, mid and distal areas in longitudinal colonic muscle strips. Experiments were performed in non-adrenergic, non-cholinergic (NANC) conditions.

1.4.9. Immunohistochemistry

nNOS positive cells were distributed between the circular and the longitudinal muscle layers at the level of the Auerbach's plexus (AP) forming a network of nerve strands and ganglia (Figures 8 B-D). The cell bodies of the nNOS positive cells displayed dark somata and non-reacting nuclei. Usually, several positive cell bodies were present in each ganglion (Figure 8E). Auerbach's ganglia were interconnected by nerve strands of variable length and thickness. Three components were seen in the AP (Figure 8A):

primary plexus, secondary plexus and a tertiary plexus. Together, the ganglia and internodal strands make up the primary plexus. The fine nerve strands constituted the secondary component of the plexus and the tertiary plexus was made up of fine nerve bundles that zigzagged in the spaces between the meshwork formed by the primary plexus.

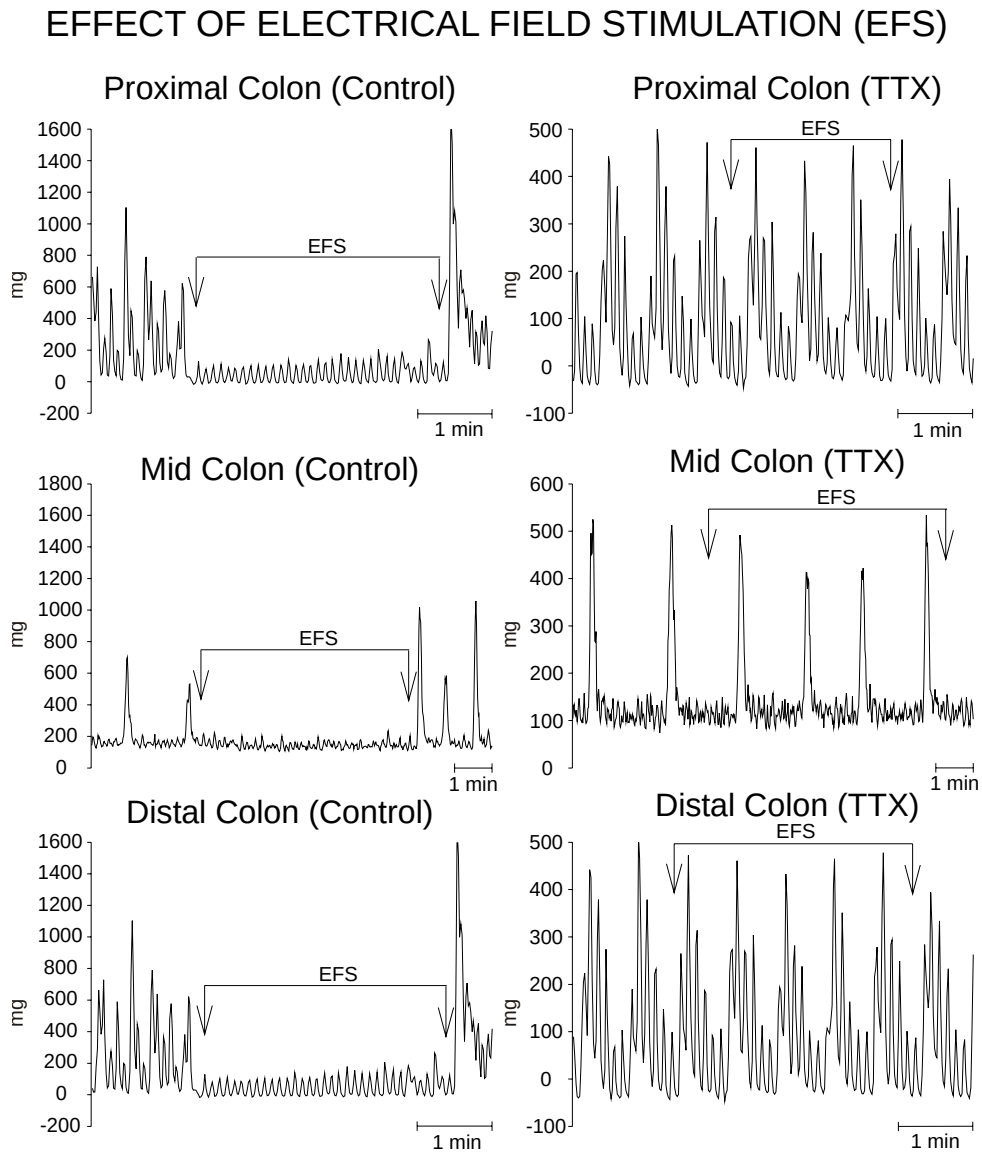

Figure 7. Muscle bath recordings showing the effect of electrical field stimulation (EFS, 28V-40V, 4Hz, 0.3ms) on the spontaneous mechanical activity in intact circular muscle strips. Strips were studied under NANC conditions (left recordings) and in the presence of the neural blocker TTX 1 μM (right recordings).

Occasionally, some of the nNOS positive cell bodies were found in nerve strands that ran along smooth muscle bundles or between the ganglia (Figure 8E). Fine fibers were found running parallel to the long axes of the smooth muscle cells both in the circular and the longitudinal muscle layers. However, the circular muscle layer was more densely innervated than the longitudinal layer. nNOS positive fibers were found throughout the thickness of the circular muscle layer. At the submucosal border, nNOS positive cell bodies were not observed.

At the level of AP (ICC-AP) multipolar *c-kit* positive cells were found (Figure 8F) with long branching processes that form an interconnecting network (Figures 9 A-C). Using blind examination of all preparations, in 9 of the 10 preparations from the proximal colon, the cell bodies had more processes, which also were more ramified than observed in nerve cells in mid and distal areas. The denser network in the proximal colon is probably due to the presence of more processes (not cell bodies: see Stereology). Some of the long branching processes from cells residing at the AP extended into the circular and longitudinal muscle layers and seemed to be in contact with some ICC-IM. These ICC-IM within the circular (ICC-CM) and longitudinal (ICC-LM) muscle layers were spindle-shaped cells that ran parallel to the muscle fibers. A network of *c-kit* positive cells was observed at the submucosal border (ICC-SMP) (Figure 9D).

1.4.10. Stereology

The stereological data are shown in Figure 10. Significant differences in the areal density of nNOS positive cells were found between different regions of the colon (Figure 10). The highest density of nNOS positive cells was found in the mid colon. Significant regional differences were neither found in the areal density of *c-kit* positive cells at the level of AP nor at the submucosal border.

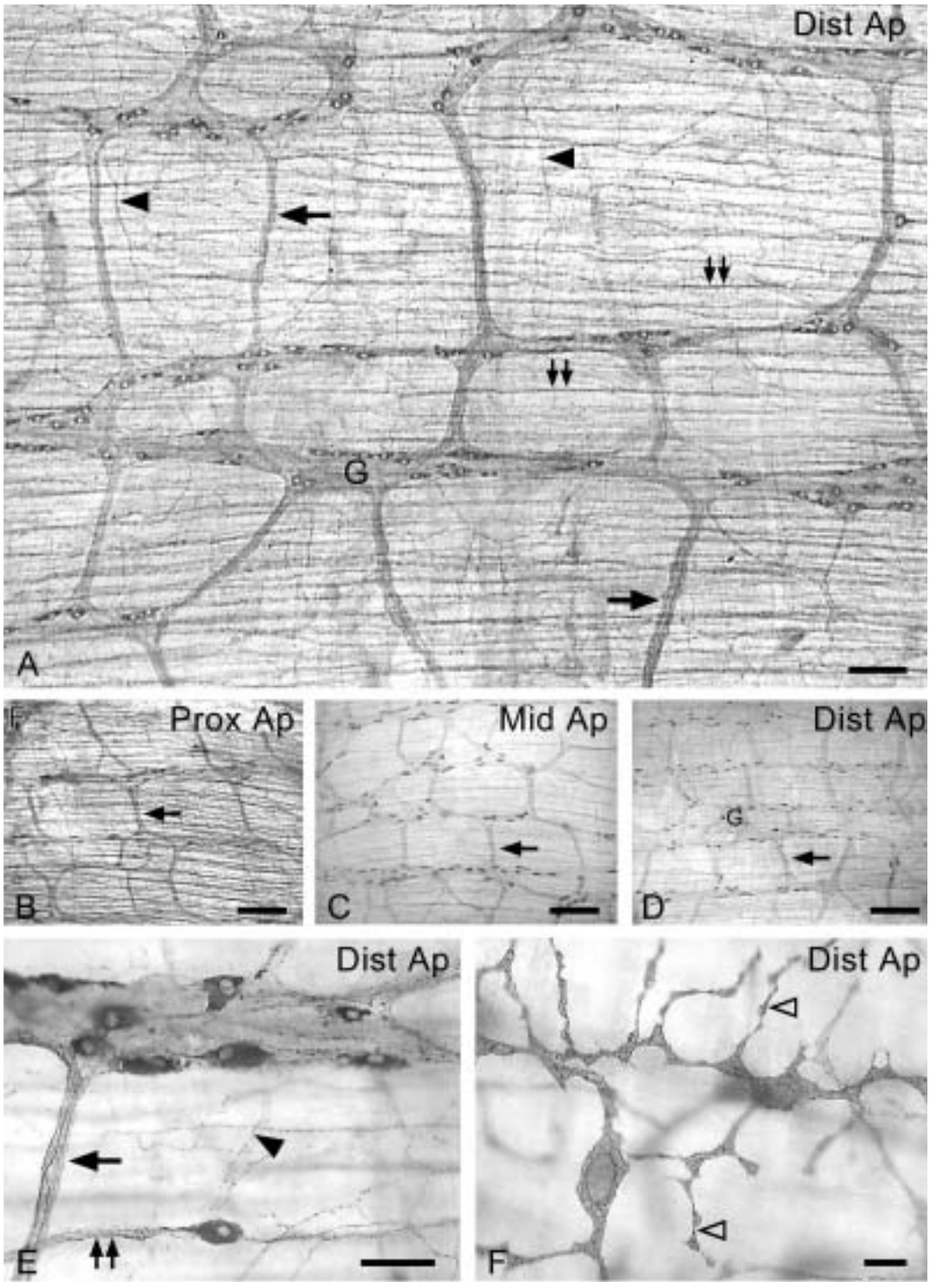

Figure 8. (A) nNOS immunohistochemistry in a whole mount preparation in the distal colon. Proximal (B), mid (C) and distal (D) colon nNOS whole mount preparations. One nitrergic ganglion in detail (E) at the level of Auerbach's plexus in a distal colon whole mount preparation. There is one nNOS cell body in a nerve strand (two small arrows). (A-E) Auerbach's ganglia (G) were interconnected to each other by

nerve strands (big arrows) of variable length and thickness. Fine fibers were found running parallel to the circular (two small arrows) and the longitudinal (arrow heads) muscle layers. (F) ICC cells at Auerbach's plexus region in a distal colon whole mount. The *c-kit* positive cells were stellate with a complex secondary branching pattern; three to four long and several short processes protruded in every direction. Some of the long branching processes (white arrow heads) at the level of Auerbach's plexus extended into the circular and longitudinal muscle layers. Several short processes ran between the muscle fibers. Scale bar applies were: (A) 100 µm, (B-D) 270 µm, (E) 50 µm and (F) 10 µm.

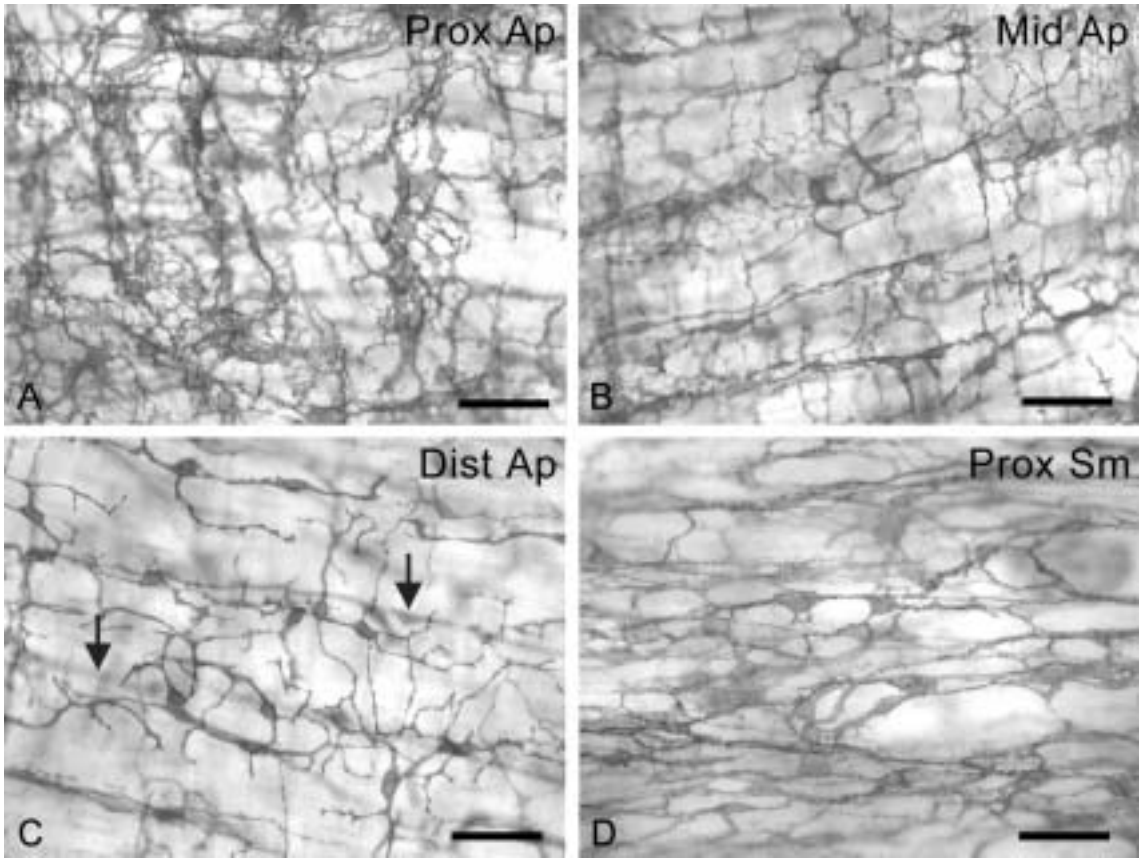

Figure 9. (A-D) *c-kit* immunoreactivity in the rat colon. Whole mount preparations show *c-kit* positive cells at Auerbach's plexus region of the proximal (A), mid (B) and distal colon (C). *c-kit* positive cells showed a multipolar shape with long branching processes that form an interconnecting network. This network was also observed at the level of the submuscular plexus (D). Intramural spindle-shaped cells (ICC-CM) can be seen out of focus running parallel to the muscle fibers (big arrows). Scale bar applies to all figures 50 µm.

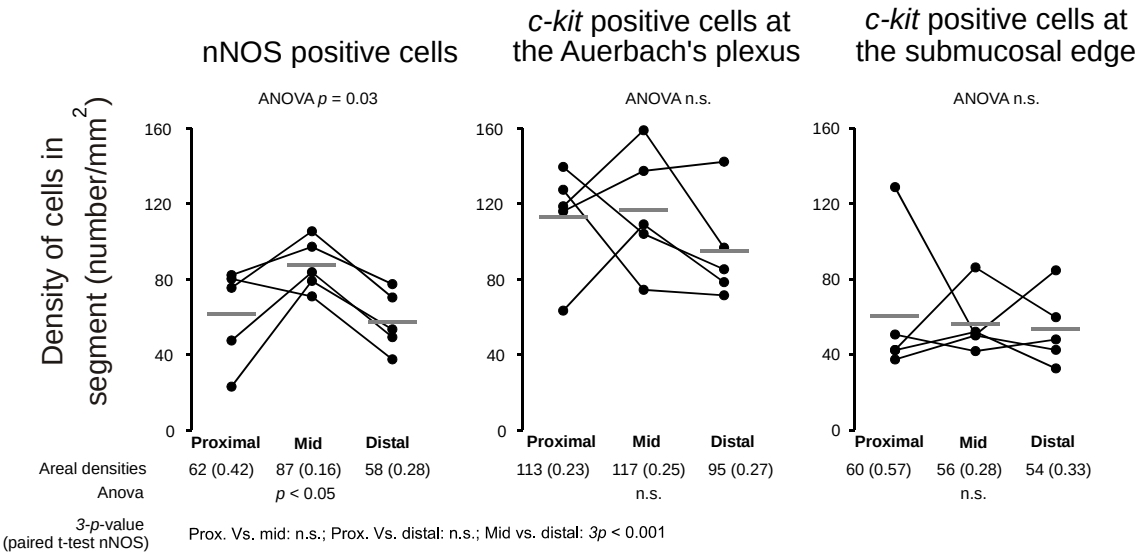

Figure 10. Areal densities of nNOS positive cells and *c-kit* positive cells in the rat colon. The values for the three different regions are connected for each rat by solid lines. The grey horizontal lines show group mean. The values show areal densities (means) of nNOS positive cells and *c-kit* positive cells (number of cells/mm^2 surface area). Coefficients of variation (CV=SD/group mean) are shown in parenthesis.

1.5. Discussion

In the present study, two functional pacemakers are found along the rat colon. These two pacemakers generate two types of contractions that have been termed in this paper as HF and LF contractions because this is probably the most characteristic and stable property of both contraction types along the colon. The presence of two types of contractions in the rat colon has been reported in other works *in vitro* (Pluja et al. 2001; Gonzalez & Sarna 2001a; Gonzalez & Sarna 2001b) and *in vivo* (Li et al. 2002). However, these two types of contractions vary in amplitude and frequency according to the region (proximal, mid and distal) of the colon and orientation of the strip (circular or longitudinal).

The amplitude of both contraction types is different if strips are longitudinally or circularly oriented. In circular strips, contractions are higher in amplitude in the proximal compared to the distal area. In contrast, in longitudinal strips, the amplitude of contractions is higher in the distal compared to proximal areas. The reasons underlying these differences between layers are presently unclear but it might be related to their structural properties such as thickness, number of cells, coupling or contractile proteins but is probably independent of the pacemaker mechanism.

Regarding the frequency, LF contractions (in strips oriented circularly and longitudinally) show a gradient along the colon and they are higher in proximal compared to distal areas. In contrast, HF contractions are constant all along the colon. Similar results have been reported *in vivo* using electromyographic (Ferre & Ruckebusch 1985) or strain gauges recordings (Li et al. 2002). Li and co-workers (Li et al. 2002) recently reported *in vivo* a pattern of motility with two types of contractions: low amplitude contractions of 10-15 per minute and high amplitude contractions of < 1 per minute. In this study performed with strain gauges, the frequency of high amplitude contractions decrease distally whereas the frequency of low amplitude contractions was constant along the colon. These results are very similar to those found *in vitro* in our study.

Our data show that (a), the pacemaker responsible for both HF and LF contractions is present in small strips of tissue and do not depend on the entire colon and that (b), the relative frequency of both contractions (gradient for LF contractions) and (constant for HF contractions) is also an intrinsic property of the strip. In the mouse, in contrast to what is found in the rat, migrating motor complexes are constant all along the colon (Brierley et al. 2001).

When the submucosa and its associated ICC-SMP network was dissected (Pluja et al. 2001) only LF contractions were recorded in both circular and longitudinal muscle layers. This is consistent with the hypothesis that the pacemaker responsible for HF contractions is the ICC network located at the submucosal border. Moreover, it has been recently reported that in the mouse colon near the submucosa, *c-kit* positive cells generate 'plateau potentials' at a frequency of about 15 per minute (Yoneda et al. 2002; Yoneda et al. 2003). These plateau potentials are very similar in frequency to the slow wave activity previously described in the rat colon (Pluja et al. 2001) and are responsible for HF contractions described all along the colon in the present work. Taken together, these results infer that ICC-SMP are the pacemaker cells responsible for the HF contractions in the circular muscle. However, it is difficult to explain the presence of HF contractions in the longitudinal muscle on the basis of physiological studies. One possible hypothesis could be that the electrical activity, which originated in the ICC-SMP, reaches the longitudinal muscle but good coupling through gap junctions between smooth muscle cells from both layers has not been demonstrated. However, in canine colon ICC can couple both muscle layers through close appositions and in this case ICC might mediate communication between the circular and longitudinal muscle layers (Liu et al. 1998). Another possibility is the mechanical interaction between layers that transfers an active event from one layer to another (Wood & Perkins 1970). This might be because of mechanical transmission through peg and socket junctions between layers as it was reported in the deep muscular plexus of the small intestine (Thuneberg & Peters 2001). Recently, the presence of cyclic mechanical activity in the longitudinal muscle of the small intestine in mutant mice that lack ICCs at AP has been documented (Daniel et al. 2003). Consequently, the origin of the rhythmic activity of the longitudinal muscle needs further investigation.

The origin of LF contractions is not clear. Our results show that: (i) they can be recorded (at the same frequency) in strips oriented both circularly and longitudinally; (ii) they are present in strips without SMP; (iii) the relative amplitude and frequency between segments and orientation is conserved when the SMP is previously dissected and (iv) they can be recorded all along the colon in presence of the neural blocker TTX. This result proves that in rat-*in vitro*-studies the pacemaker responsible for LF contractions is non-neurogenic. On the contrary, in the mouse colon a neurogenic pacemaker has been very well documented and the corresponding mechanical activity has been called 'Migrating motor complex' (Lyster et al. 1995; Spencer et al. 1998; Brierley et al. 2001). Moreover, in aganglionic segments of mouse colon with intact ICC distribution the spontaneous electrical activity was impaired (Ward et al. 2002). The presence of myogenic contractions has been previously demonstrated in the rat colon (Pluja et al. 2001; Gonzalez & Sarna 2001a; Gonzalez & Sarna 2001b). These LF contractions are independent of the ICC-SMP and their properties in terms of frequency and amplitude are present in small strips without the neural integrity of the colon. In agreement with this result two plausible hypotheses regarding the origin of this mechanical activity can be considered: the ICC-AP network, or alternatively, stretch-activated mechanisms might originate these LF contractions. In 1983, Huizinga and co-authors suggested that presence of rhythmic electrical and mechanical activity in porcine colon might have a stretch origin (Huizinga et al. 1983). Unstretched preparations from the longitudinal muscle did not develop spontaneous electrical activity but when gradually increasing the stretch the frequency of spike bursts and corresponding contractions progressively increased. This might be due to stretch induced depolarization of smooth muscle cells. In this case, stretch sensors should be present and peg and socket junction have been postulated to accomplish this function (Thuneberg & Peters 2001). We cannot rule out the possibility that this mechanism is involved in the origin of LF contractions in the rat colon. It is possible that distension *in vivo* caused by the presence of pellets might induce this motility pattern. Further investigations are needed to evaluate this hypothesis.

No differences in the frequency between both contraction types were found when comparing strips oriented circularly and longitudinally. Although we cannot

demonstrate it, this result suggests that the mechanical activity might have a common origin in the circular and longitudinal segment studied (ICC hypothesis) or alternatively, two pacemaker systems with the same frequency affect both layers simultaneously. In both cases it is reasonable to hypothesize that the longitudinal and circular muscle layers contract synchronously in the whole colon but simultaneous measurements from both layers are needed to prove it.

In this paper we have studied the distribution of *c-kit* positive ICCs along the rat colon because ICCs are the pacemaker cells responsible for spontaneous electrical and mechanical activities. Our aim was to characterize cell density and morphology to find a structural basis for the different mechanical behaviours. To quantify cell density we used semitransparent whole mounts and a stereological method that included unbiased sampling, and by using a three-dimensional dissector probe we avoided counting the cells more than once. To our knowledge this procedure has not been used in quantifying ICCs and NO nerves before. However, we did not find major differences between proximal, mid and distal colon in the density of *c-kit* positive ICCs measured in whole mounts. In a previous paper Ward and co-workers (Ward et al. 2002) described a gradient (the ICC density was greatest in the proximal colon and decreased along its length) in the number of ICCs in the mouse colon. However, cells were counted from cryostat cross-sections and not from whole mounts. In our work, constant values along the colon were obtained at the level of AP and SMPs. If the origin of HF contractions is the ICC-SMP plexus a ‘correlation’ between cell density and contraction cannot be calculated because constant values both in ICC density and frequency of contractions were obtained in all segments, suggesting that the same pacing frequency is driven by the same number of cells. If we assume that the ICC network located at the AP originates LF contractions there is no correlation between ICC density (constant) and frequency of contractions (decrease distally). However, in the proximal colon where the frequency of LF contractions is higher, more ramified processes (not cell density) were found. It is possible to hypothesize that more processes might increase contacts between ICCs and smooth muscle cells. An increase in the number of contacts can facilitate the rate of depolarization of the smooth muscle cells increasing the frequency of contractions and in this case a relationship between morphology and function can be

established. This is true if we assume that the origin of LF contractions is the ICC-AP network but this is still unclear. The question that it is still not solved is how different types of ICCs can drive different frequencies; this could be related to structural data or alternatively to intrinsic metabolic pathways in the ICCs may be the origin of such differences.

Another issue is that LF and HF contractions are differently influenced by neural inputs. HF contractions are quite constant and poorly modified by TTX, L-NNA or apamin. Moreover, these contractions are less affected when EFS is applied. In fact, in circular muscle strips from proximal colon, EFS abolished LF contractions and HF contractions are clearly recorded. This suggests that HF contractions are less influenced by neural inputs. In contrast, LF contractions are abolished when EFS is applied suggesting that inhibitory neurotransmitters such as ATP or nitric oxide (Pluja et al. 1999) hyperpolarize the smooth muscle causing the cessation of these muscle activity. Experiments done in a microelectrode setup show that regular inhibitory junction potential (IJP) of high amplitude can abolish spontaneous motility contractions (data not shown).

If we compare the effect of TTX, L-NNA and apamin on the circular and longitudinal muscle, the amplitude of LF contractions is increased more in the circular compared to the longitudinal muscle layer. This result suggest that the tonic release of inhibitory mediators affects the circular more than the longitudinal layer as it has previously been reported (Mule et al. 1999). This result is consistent with presence of a dense innervation of nNOS positive fibers in the circular but not in the longitudinal muscle layer.

In all areas of the colon (proximal, mid and distal) TTX increased the amplitude of LF contractions in circular muscle strips. This result suggests the presence of an inhibitory neural tone all along the colon. However, the highest density of nNOS positive cells was found in the mid area. This is consistent with the effect of TTX and L-NNA in the mid colon where the increase in the amplitude of contractions was higher. In a previous paper, regional differences in the nitrergic innervation in the rat colon have also been described (Takahashi & Owyang 1998). Our results are similar to those

reported if we assume that what they considered ‘proximal colon’, is mid colon in the present paper (they rejected the first 1-3 cm). It is important to define precisely the anatomical criteria (see Figure 1) to properly locate the different areas studied.

In this paper, we have demonstrated the presence of two types of contractions in the rat colon that vary in relation to the orientation of the muscle strip and the area studied. The movement of a pellet inside the colon probably needs a strong contraction with a LF; consequently LF contractions might be related to propulsion. In the proximal colon high amplitude LF contractions are very active in the circular muscle and are probably used to retain the content in the colon. In the mid colon LF contractions are very active in both circular and longitudinal muscle strips, whereas in the distal area, LF contractions are more prominent in strips oriented longitudinally to help in the movement of the content to the rectum. In contrast HF contractions can be responsible for mixing and possibly water absorption and are present all along the colon. This motility is probably responsible for formation and transit of pellets including mixing, water absorption and propulsion. The origin of both contractions is myogenic and suggests the presence of two pacemakers that might be related to the ICC distribution. ICC-SMP are found in the proximal, mid and distal colon at equal density (between 50 and 60 cells/mm^2) and this network is probably responsible for HF contractions. In contrast, LF contractions can be originated in the ICC-AP network (about 100 cells/mm^2) or alternatively it is an intrinsic property of the smooth muscle itself (i.e., stretch/distension activated mechanism). Colonic circular smooth muscle cells are under inhibitory tone, which probably involves nitric oxide and apamin-sensitive mediators such as ATP. This is consistent with the presence of nNOS positive fibers in the circular and to a small extent in the longitudinal muscle layer.

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