Chapter 2

Pacemaker activity and neurotransmission in the colon of Ws/Ws rats

2.1. Summary

The aim of this study was to characterize functionally and morphologically the pacemaker activity and neurotransmission in the colon of Ws/Ws mutant rats, which harbour a mutation in the Kit gene that affects development of interstitial cells of Cajal (ICCs). Contractile activity, intracellular electrical activity, effects of enteric nerve stimulation and immunohistochemical studies (*c-kit* and nNOS) were performed. Unbiased stereological technique was used to quantify the density of *c-kit* and nNOS positive cells. In Ws/Ws rats, the density of *c-kit* positive cells at all three levels (Auerbach's plexus, intramuscular and submuscular plexus) was markedly reduced, whereas the densitiy of nNOS positive neurons was slightly reduced at the level of Auerbach's plexus region. Control +/+ rats showed slow waves (10-20 cycles/min) and cyclic depolarizations (1 cycle/min), which were associated with highly regular myogenic motor patterns at those frequencies. In Ws/Ws rats, the electrical activity consisted of an irregular pattern of action potentials, which trigger muscle contractions without a defined pattern. In both rat groups, TTX 1 μM or L-NNA 1 mM increased the mechanical activity. Stimulation of enteric nerves (under NANC conditions) resulted in abolishment of mechanical activity in both Ws/Ws and +/+ rats. Nerve stimulation in +/+ rats elicited a biphasic inhibitory junction potential (IJP) characterized by two components: an apamin-sensitive fast component and an L-NNA-sensitive sustained component. In Ws/Ws rats, a partial reduction in the sustained component was observed. We conclude that the presence of two ICC networks is necessary to have a regular pattern of electrical and mechanical activity. Despite the reduction in nNOS positive

neurons, Ws/Ws animals show an inhibitory nitrergic neural tone and a partial nitrergic IJP component, probably due to the lack of ICC-IM and/or to the reduction in the density of nNOS positive neurons. We cannot rule out a parallel pathway involving ICC-IM (in this case *c-kit* negative) and/or a direct relationship between nerve endings and smooth muscle cells.

2.2. Introduction

Interstitial cells of Cajal (ICCs) are now recognized as gut pacemaker cells and are also hypothesized to be mediators of enteric innervation (Huizinga et al. 2004; Ward et al. 2004; Daniel 2004). Evidence for these functions has been obtained mainly in the small intestine and stomach, but the role of ICCs in the colon is still poorly understood.

In the colon of several species including humans, ICCs are distributed at the level of Auerbach's plexus (ICC-AP), submuscular plexus (ICC-SMP) and they are located intramurally (ICC-IM). ICC-AP and ICC-SMP are multipolar cells with several branches, forming a network; whereas intramural ICCs are spindle-shaped cells, which run parallel to the muscle fibers (Christensen et al. 1992; Rumessen et al. 1993; Torihashi et al. 1994; Vanderwinden et al. 1996; Hagger et al. 1998; Torihashi et al. 1999). A similar distribution and morphology has been reported in the colon of rodents (Vanderwinden et al. 2000; Pluja et al. 2001).

A role for ICCs as pacemaker cells of the gut has been firmly established in the mouse small intestine at the tissue level (Ward et al. 1994; Huizinga et al. 1995; Malysz et al. 1996), at the cellular level (Koh et al. 1998; Thomsen et al. 1998; Koh et al. 2002) and *in vivo* (Der et al. 2000). In the small intestine, the main pacemaker area is the ICC network located near the AP. An intracellular mechanism involving calcium release from the endoplasmic reticulum and calcium uptake by mitochondria has been proposed as responsible for pacing ICCs. This mechanism (probably through gap junctions) activates voltage-gated channels causing slow waves in smooth muscle cells. The role of L-type calcium channels is poor in the pacemaker mechanism of the small intestine. In contrast, L-type calcium channel blockers inhibit cyclic depolarizations and slow

waves in the colon of rodents (Yoneda et al. 2003; Martin et al. 2004). The characteristics of colonic pacemaker activity and its cellular origin are not understood to the same degree as in the case of the small intestine. This is in part due to significant differences in pacemaker activity between species and it is not clear yet which animal is best suited to model human colonic activity (Huizinga & Daniel 1986). In the canine and porcine colon, the major slow wave activity is associated with the ICC-SMP network (Smith et al. 1987b; Liu et al. 1994), whereas the ICC-AP network is associated with the generation of "myenteric potential oscillations" with a higher frequency (Smith et al. 1987a). In rodents (mouse and rat), ICC-SMP generate slow wave activity at a frequency between 10-20 cycles/min (Pluja et al. 2001; Yoneda et al. 2001). In the rat colon, slow wave activity with superimposed cyclic depolarizations (with action potentials) has been associated with ICC-SMP and ICC-AP respectively (Pluja et al. 2001). Slow waves cause high frequency (HF) contractions, whereas cyclic depolarizations are the origin of low frequency (LF) contractions. LF contractions show a frequency gradient and present the highest values (about 2.5 cpm) in the proximal colon, whereas HF contractions are constant all along the colon (see Chapter 1). In humans, cyclic depolarizations have been described (Rae et al. 1998). These cyclic depolarizations are sensitive to nifedipine. Accordingly, the pacemaker activity and the cellular mechanisms underlying rhythmicity in the colon are not well characterized.

A role for intramural ICCs (ICC-IM) in neurotransmission was first proposed by Daniel and Posey-Daniel (Daniel & Posey-Daniel 1984) based on structural data of the opossum lower esophageal sphincter. Interstitial cells of Cajal are intercalated between nerves and smooth muscle cells and might mediate nerve responses (Daniel & Posey-Daniel 1984). In the colon of rodents, electron microscope studies show that ICC-IM are in close contact between nerves and smooth muscle cells (Wang et al. 2000). These contacts give morphological evidence that ICCs might also participate in neurotransmission. A physiological evidence that ICC-IM participate in neurotransmission is based on studies of the stomach of WWv mice, which lack ICC-IM (Burns et al. 1996). ICCs are thought to be essential for inhibitory neurotransmission, in particular nitrergic neurotransmission. In the gastric fundus of Sl/Sld mutant mice, ICC-IM were absent and, consequently, the inhibitory

neurotransmission and associated relaxations were impaired (Beckett et al. 2002). A similar conclusion was also reached for the lower esophageal and pyloric sphincters (Ward et al. 1998). This concept was challenged by Goyal and co-workers who showed that *in vivo*, swallow-induced nitrergic inhibition of the LES was normally present (Sivarao et al. 2001).

In the rat colon, the inhibitory junction potential (IJP) consists of a fast component (apamin-sensitive) followed by a slow component (L-NNA-sensitive). ATP might be responsible for the fast component and NO might mediate the sustained component (Pluja et al. 1999). In the gastric antrum of WWv mouse, which lack ICC-IM, the fast component (probably ATP-mediated) of the IJP is preserved, whereas the second component (probably nitrergic) is impaired (Suzuki et al. 2003). Subsequent studies continue finding evidences for a role of ICC-IM in inhibitory neurotransmission, although it becomes clear that the apamin-sensitive inhibitory responses are not affected by ICCs. Consequently, the role of ICC-IM in neurotransmission needs further investigation.

Accordingly, the role of ICCs regarding pacemaker activity and neurotransmission is not clear in the colon. Thus, the aim of the present work was to study the distribution of *c-kit* and nNOS positive cells and to characterize the pacemaker activity and neurotransmisssion in the colon of Ws/Ws and +/+ rats. Preliminary data from this work were presented at the Digestive Disease Week meeting of the American Gastroenterology Association (AGA), New Orleans, Louisiana, USA, 2004.

2.3. Methods

2.3.1. Animals and tissue preparation

Male Ws/Ws and sibling controls +/+ rats (Sumitomo Mitsui Banking Corporation, Japan), 7 weeks old, were used in the present study. Rats were kept individually and fasted for 16-18 h with *ad libitum* access to water. Animals were stunned before being decapitated and bled. The entire colon was removed from 1 cm below the ileocecal junction to the pelvic brim. To perform functional studies (mechanical and electrophysiological studies, Ws/Ws: n = 6; +/+: n = 6) the colon was placed in carbogenated Krebs solution. For morphological studies (Ws/Ws: n = 4; +/+: n = 4) the colon was placed in phosphate buffered saline (PBS) with nifedipine 1 µM (for 15 min to ensure relaxation). In both types of experiments, the colon was opened along the mesenteric border and pinned to a Sylgard base with the mucosa facing upwards. The colon was divided into three different parts: proximal, mid and distal colon (for more details see Chapter 1). The housing and handling of animals were approved by The Ethics Committee of the Universitat Autònoma de Barcelona.

2.3.2. Morphological studies

In rats used for morphological experiments, each segment (proximal, mid and distal) was cut into three pieces respectively. The first piece was frozen, and the second and third were prepared for whole mount immunohistochemistry using antibodies towards nNOS and *c-kit*. The mucosa was removed by sharp dissection in all preparations. Whole mount preparations were used to study the morphology and areal density (number of cells/mm^2: see stereological analysis) of nNOS and *c-kit* positive cells in the colon of Ws/Ws and +/+ rats. When using nNOS antibodies, whole mounts were fixed with 4% paraformaldehyde for three hours 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 hours. 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. After being rinsed in distilled water and 1% H_2O_2, tissue was 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 –80ºC. The sections where fixed in Zamboni before immunostaining. The rest of the procedure was similar to that used for the whole mounts.

2.3.3. Stereological analysis

An established stereological technique was used to count the number of nNOS positive cells and *c-kit* positive cells in the proximal, mid and distal colon of both Ws/Ws and +/+ rats. To determine the density of cells per region, the so-called fractionator technique (Larsen J.O. 1998; Gundersen 1986; Mayhew 1988) was used. The 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 stereological analysis of nNOS positive cells 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). nNOS positive cells were counted between Auerbach's and SMP plexuses, whereas all *c-kit* positive cells present in whole mounts were counted into two main groups: (i) at the level of the AP and (ii) adjacent to the submuscular plexus (SMP).

2.3.4. Motility studies

Circular muscle strips (full thickness) were obtained from proximal, mid and distal parts of the colon and were cut ~1 cm long and 0.3 cm wide. Preparations 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 ± 1ºC. The strips were tied to an isometric force

transducer (Harvard Apparatus Inc., Holliston, Massachusetts, USA) connected to a PC through an amplifier. Data were digitized (25 samples/s) and displayed with Data 2001 software (Panlab, Barcelona, Spain). Preparations were allowed to equilibrate for 1 h before experiments started.

Muscle bath experiments were performed to study: (i) the patterns of mechanical activity; (ii) the release of inhibitory mediators and (iii) the presence of an inhibitory neural tone. These experiments were performed both on +/+ and Ws/Ws animals in each segment (proximal, mid and distal) of the colon. To study the patterns of mechanical activity, strips were studied in normal Krebs solution (n = 6), NANC conditions (by adding atropine, propranolol and phentolamine 1 µM to the Krebs) (n = 6) and in the presence of TTX 1 µM (n = 4). When it was possible (see Results), the amplitude and frequency of spontaneous contractions were calculated. To study the release of inhibitory neurotransmitters, circular muscle strips were placed in a muscle bath under NANC conditions and were stimulated by electrical field stimulation (EFS: 28 and 40 V, 4 Hz, 0.3 ms, 2-3 min) through two platinum electrodes (n = 6). To study the presence of an inhibitory neural tone, the amplitude of contractions was measured in Krebs under NANC conditions and in the presence of TTX 1 µM (n = 4), L-NNA 1 mM (n = 5) and the small conductance calcium-activated K^+ channel blocker apamin 1 µM (n = 5). The amplitude of contraction was measured before and after drug addition. Due to the lack of a regular motor pattern in Ws/Ws rats (see Results), an average of the amplitude of irregular contractions was measured and compared before and after drug addition. In the case of +/+ rats, low frequency contractions were measured for this purpose.

2.3.5. Microelectrode studies

Strips from the mid colon were dissected with fine forceps by sharp dissection under a magnifying glass in order to remove the mucosa. These strips with both plexuses kept intact were obtained from Ws/Ws and +/+ rats (n = 6). The tissue was pinned with the serosa facing upwards in a Sylgard-coated chamber and was continuously perfused with carbogenated Krebs solution at 37 ± 1ºC. Strips were allowed to equilibrate for

approximately 1 h. Electrical recordings were obtained after impaling circular muscle cells with glass microelectrodes (40-60 Mq of resistance) filled with 3M KCl. Data were registered using a standard electrometer Duo 773 (WPI Inc., FL., USA), an oscilloscope 4026 (Racal-Dana Ltd., England) and simultaneously digitized (100 Hz) and collected using EGAA software coupled to an ISC-16 A/D card (RC Electronics Inc., Santa Barbara, CA, USA) installed in a personal computer. To study the pattern of electrical activity, the resting membrane potential (RMP) and the amplitude and frequency of slow waves and cyclic depolarizations were determined. To evaluate the release of inhibitory transmitters, electrical field stimulation (EFS) was performed: total duration 100 ms, frequency 20 Hz, pulse duration 0.3 ms and increasing amplitude strengths (5, 10, 12, 15, 17, 20 and 25 V). IJPs elicited by EFS were recorded in Krebs solution (n = 6), NANC conditions (n = 6) and in presence of nifedipine 1 µM (n = 6). To characterize the IJP, the amplitude and duration of the transient hyperpolarization were measured (both in Ws/Ws and +/+) and when L-NNA 1 mM and apamin 1 µM were consecutively added to the recording chamber. The durations were measured at the level of the resting membrane potential.

2.3.6. Solutions and drugs

a) *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.

b) *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 Nɣ-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.

2.3.7. Antibodies

Primary antibodies were rabbit anti-neuronal nitric oxide synthase (nNOS) (Chemicon, Temecula, CA) 1:500 and rabbit anti-*c-kit* receptor 1:500 (Santa Cruz, Sc 168, CA). Secondary antibody was biotin-conjugated donkey anti-rabbit $F(ab)_2$ (Jackson, Maine, 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.

2.3.8. Statistics

Data are expressed as means ∂ SE. A p value < 0.05 was considered to be statistically significant. Statistics were performed with GraphPad Prism v.3.0 (San Diego, CA, USA) software. Differences in the amplitude or duration of the IJPs before and after drug infusion were compared by analysis of variance for repeated measurements (two-way ANOVA) followed by Bonferroni post-test. A paired student's *t*-test or one-way analysis of variance (ANOVA) was used to compare mechanical activity in the absence and presence of drugs. In morphological studies, within each group of animals, a one-way ANOVA was used to test for regional differences of the three parts (proximal, mid and distal). *Post-hoc* comparisons 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. An unpaired *t*-test was used to test regional differences between +/+ and Ws/Ws animals.

2.4. Results

2.4.1. Immunohistochemistry and stereology

Neuronal nitric oxide synthase (nNOS) positive neurons were distributed between the circular and the longitudinal smooth muscle layers at the level of Auerbach's plexus (AP) forming a network of nerve strands and ganglia, in both Ws/Ws and +/+ rats (Figure 1). nNOS positive cells displayed dark somata and non-reacting nuclei. Auerbach's ganglia were interconnected by nerve strands of variable length and thickness. Regional differences were found between the three parts of the colon both in +/+ and Ws/Ws animals (Figure 2). The highest values in the density of nNOS positive cells (ANOVA $p < 0.05$) were found in the mid colon for both control and mutant animals (Figure 2). The density of nNOS positive cells was higher in control (+/+) than in Ws/Ws animals, with the exception of the proximal colon (Table 1). Using whole mount immunohistochemistry, *c-kit* positive cells were found in +/+ rats at the level of Auerbach's plexus (AP), the submuscular plexus (SMP) and intramurally (IM). At the level of the AP, *c-kit* positive cells were multipolar cells with long branching processes, which formed a network (Figure 3) and sometimes extended into the circular and longitudinal muscle layers. The processes at the circular muscle side seemed to be in contact with cells in the outer circular muscle layer (Vanderwinden et al. 2000). Intramuscular *c-kit* positive cells were spindle-shaped cells and ran parallel to the muscle fibers. At the level of SMP, ramified *c-kit* positive cells formed a network of cells (not shown). In Ws/Ws rats, a reduced number of *c-kit* positive cells was observed at Auerbach's plexus of the proximal colon. At the level of the SMP and within the muscle layers, *c-kit* positive cells were rare. In the mid and distal colon, *c-kit* positive cells were rarely seen at any level (Figures 2 and 3). From the whole mount preparations, *c-kit* positive cells were counted at the level of AP and SMP. In control +/+ rats, ANOVA analysis did not indicate any difference in the areal density between proximal, mid and distal colon at the level of AP and SMP (Figure 2). However, in mutant rats, ANOVA analysis indicated differences between the proximal colon *versus* mid and distal colon at AP (Figure 2). In Ws/Ws rats, *c-kit* positive cells were rare

(more than a 90% loss) at all other locations (Figure 2 and Table 1). Frozen sections were used to confirm this distribution of *c-kit* positive cells throughout the thickness of the colon in both Ws/Ws and +/+ rats, as shown in Figure 4.

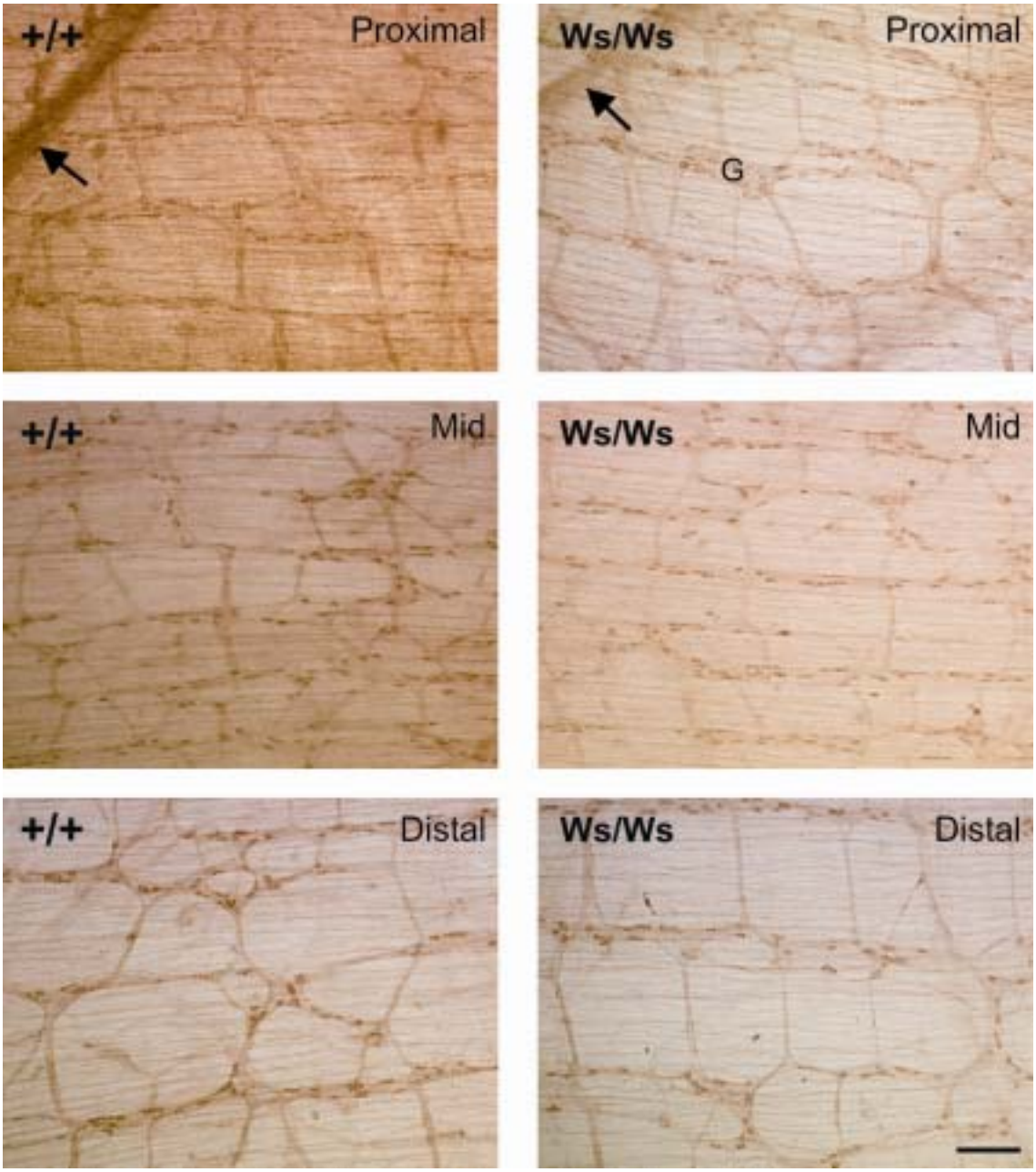

Figure 1. Whole mount preparations showing nNOS positive cells in the proximal, mid and distal colon from +/+ (left) and Ws/Ws (right) rats. The arrows represent the characteristic folds in the proximal colon. Auerbach's ganglia (G) were interconnected to each other by nerve strands. Scale bar applied to all figures: 200 µm.

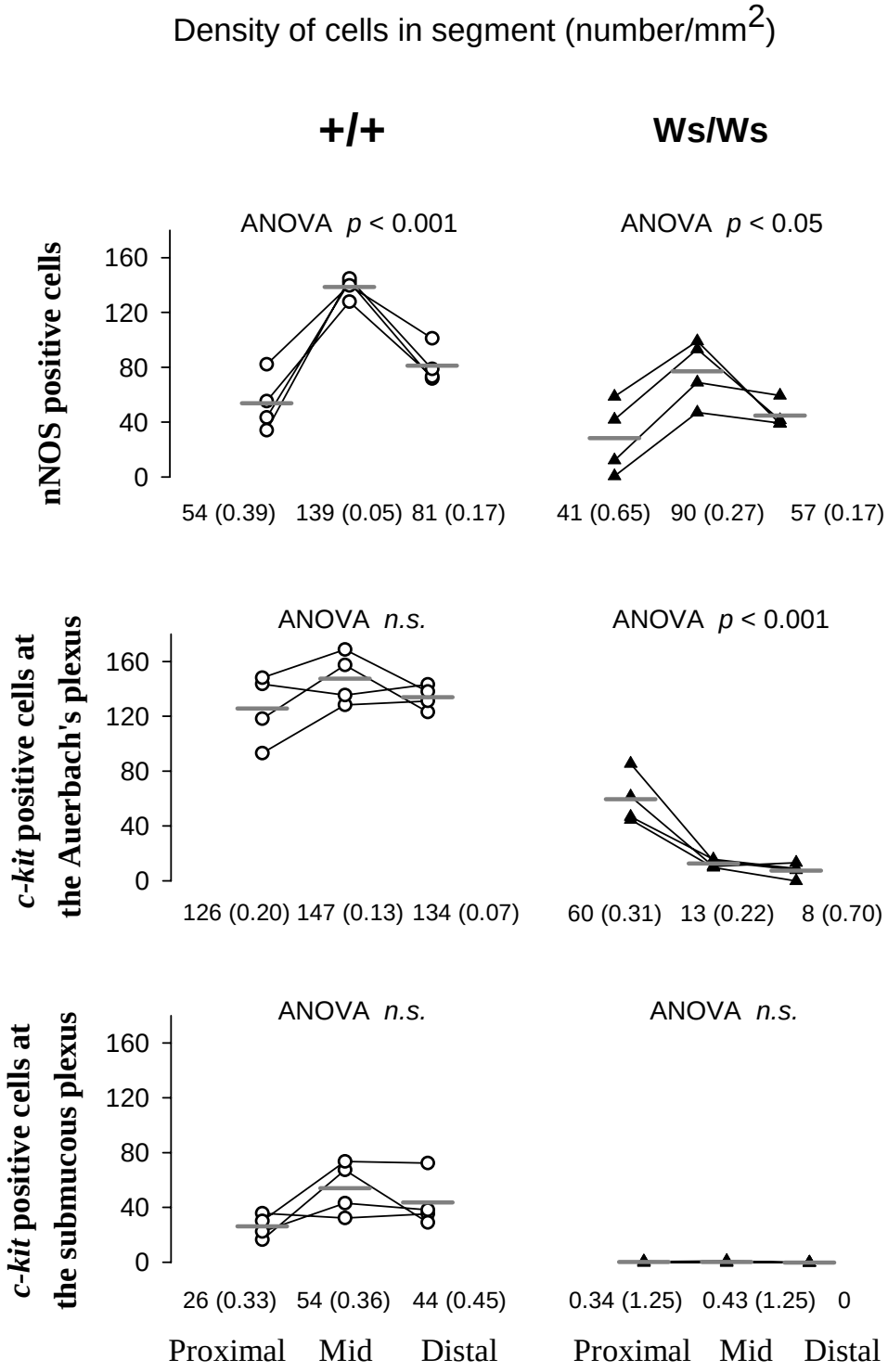

Figure 2. Areal densities of nNOS positive cells and *c-kit* positive cells in the colon of +/+ (left) and Ws/Ws (right) rats. The horizontal lines show group mean. The values show areal density means of nNOS positive cells and *c-kit* positive cells (means: number of cells/mm^2 surface area). Coefficients of variation (CV=SD/group mean) are shown in parenthesis.

	Colon	Unpaired *t*-test	% Loss
nNOS positive cells at AP	Proximal	*n.s.*	None
	Mid	$p < 0.01$	35%
	Distal	$p < 0.05$	29%
***c-kit* positive cells at AP**	Proximal	$p < 0.01$	52%
	Mid	$p < 0.0001$	91%
	Distal	$p < 0.0001$	94%
***c-kit* positive cells at SMP**	Proximal	$p < 0.001$	99%
	Mid	$p < 0.001$	99%
	Distal	$p < 0.01$	100%

%Loss = [(+/+) – (Ws/Ws)] x 100 / (+/+)

Table 1. Comparison of the density of nNOS and *c-kit* positive cells between +/+ and Ws/Ws rats in each part of the colon. The percentage of loss was calculated using the areal density in Ws/Ws rats and considering +/+ values as 100%.

2.4.2. Patterns of spontaneous mechanical activity in the rat colon

Circular muscle strips from +/+ rats with AP and SMP intact showed a regular pattern of spontaneous mechanical activity characterized by low frequency (LF) and high frequency (HF) contractions in proximal, mid and distal colon (Figure 5A). In circular muscle strips from +/+ rats, both LF and HF contractions were of higher amplitude in the proximal colon compared to those from the mid and distal colon (Table 2). LF contractions had higher frequency in proximal segments and decreased distally (Table 2), whereas HF contractions showed a steady frequency (between 10.5 and 15.3 cpm, n = 6) along the colon. In Ws/Ws animals, we could not establish a regular pattern of spontaneous contractions. In the majority of our recordings, irregular contractions characterized by low amplitude and irregular frequency were recorded in Krebs solution (Figure 5B). In the presence of TTX (n = 4), the same patterns were maintained both in +/+ (Figure 6) and Ws/Ws rats (not shown), suggesting a myogenic origin of these contraction types.

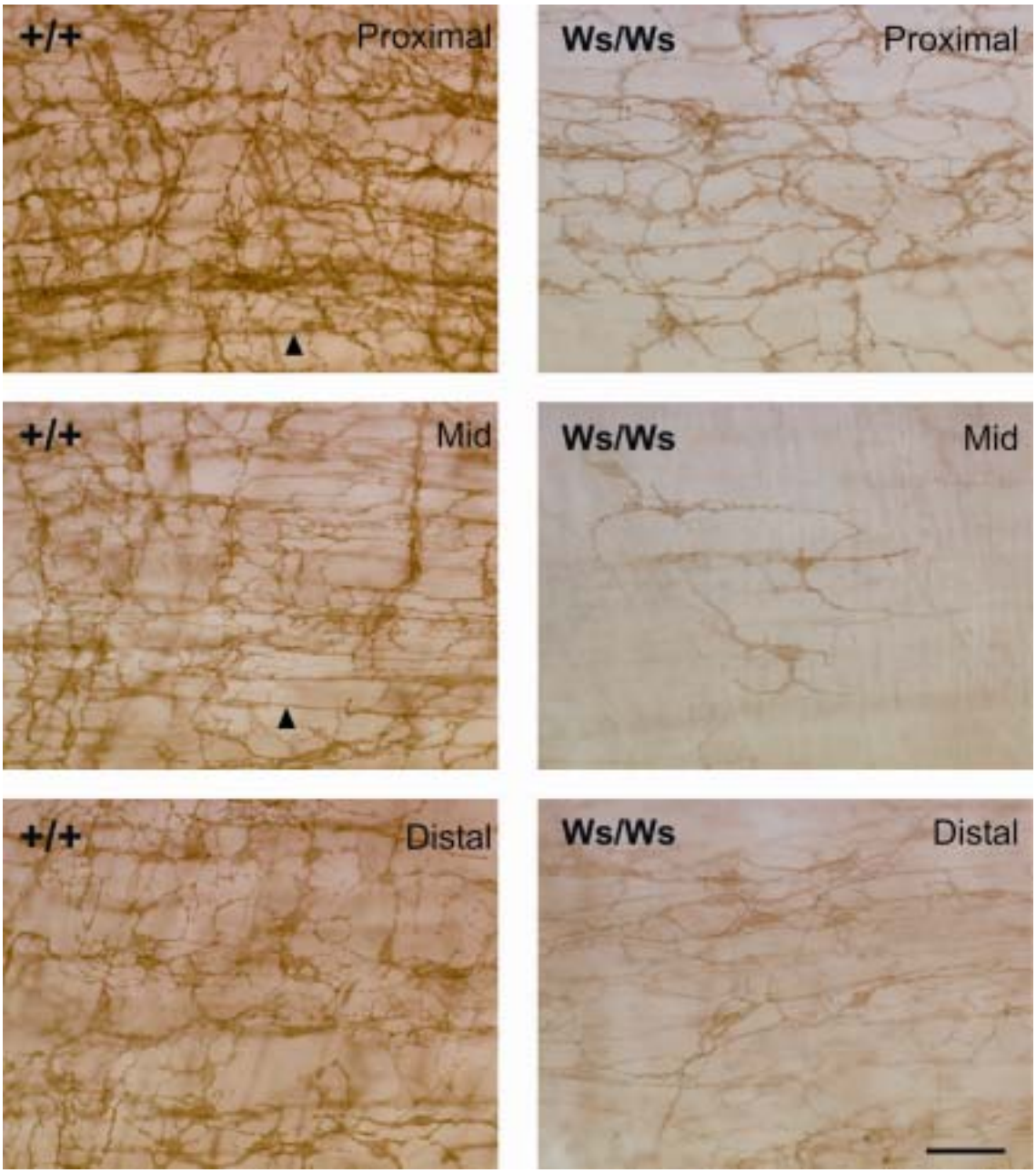

Figure 3. Whole mount preparations showing *c-kit* positive cells in the proximal, mid and distal colon from +/+ (left) and Ws/Ws (right) rats. Arrowheads have been added on ICC-IM (out of focus) near the Auerbach's plexus in the circular muscle layer. Scale bar applied to all figures: 50µm.

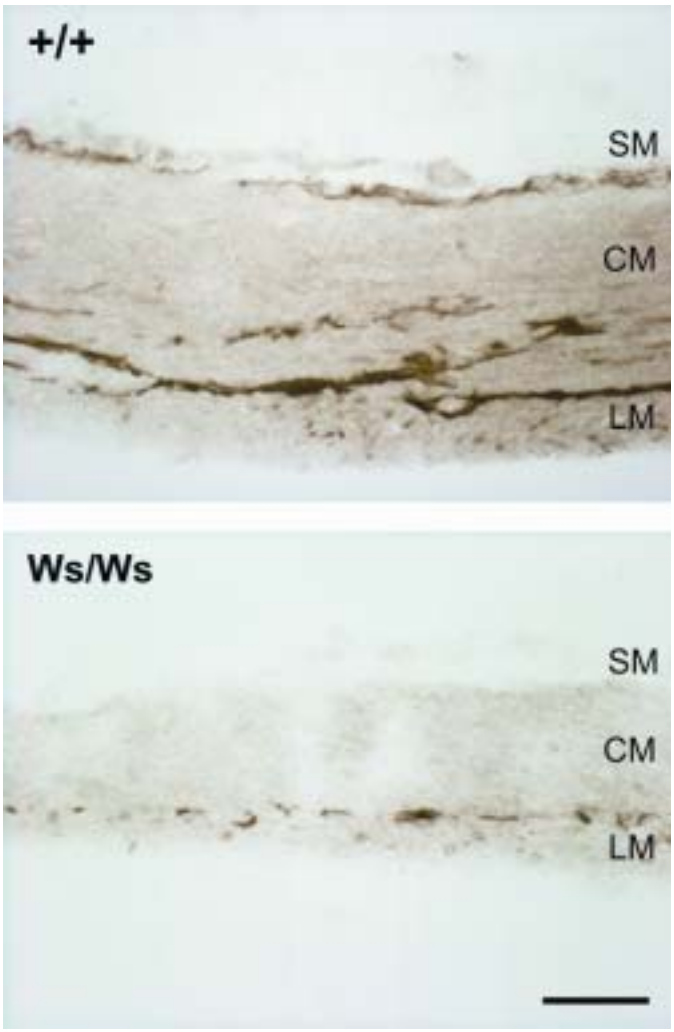

Figure 4. Frozen sections from the proximal colon of +/+ (top) and Ws/Ws (bottom) rats. Notice the presence of *c-kit* positive cells at the Auerbach's plexus (between circular and longitudinal muscle layers), submucosal (SM) border and intramurally in +/+ rats. Mutant rats only showed *c-kit* positive cells at the Auerbach's plexus. CM: circular muscle layer; LM: longitudinal muscle layer. Scale bar: 50 µm.

	LF contractions		**HF contractions**	
	Amplitude (mg)	Frequency (cpm)	Amplitude (mg)	Frequency (cpm)
Proximal	1109 ± 219	1.53 ± 0.12	223 ± 29	11.26 ± 0.48
Mid	843 ± 228	0.54 ± 0.04	119 ± 23	14.15 ± 1.19
Distal	410 ± 124	0.74 ± 0.08	101 ± 18	12.99 ± 1.38
Anova	$p < 0.05$	$p < 0.0001$	$p < 0.01$	*n.s.*

Table 2. Motility patterns observed in colonic muscle strips from +/+ rats (n = 6), with both plexuses intact in Krebs solution. Cpm: contractions per minute; Anova: differences between proximal, mid and distal colon.

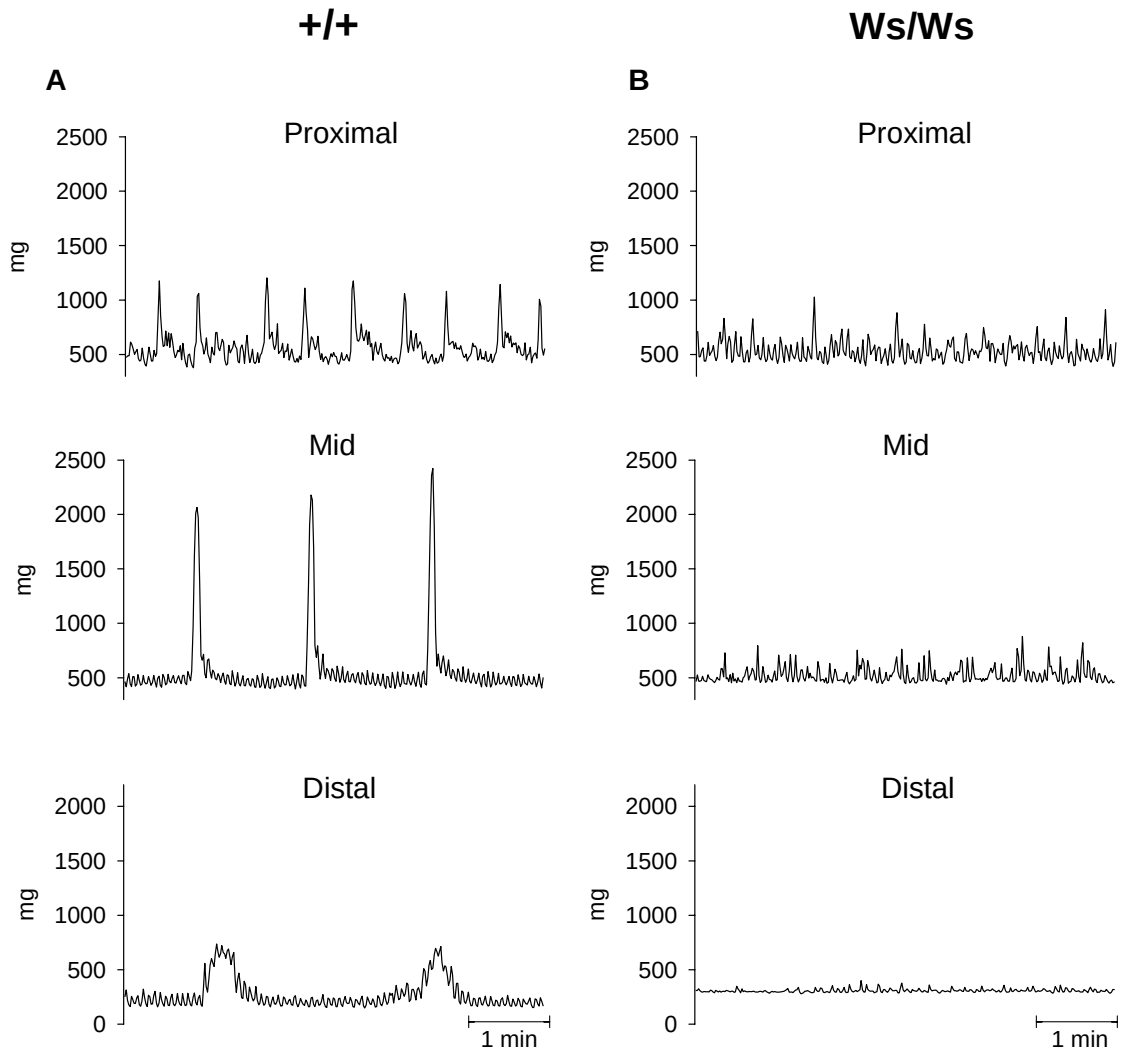

Figure 5. Muscle bath recordings showing the spontaneous cyclic mechanical activity displayed by circular muscle strips from the proximal, mid and distal colon of +/+ (A) and Ws/Ws (B) rats. Tissue was mounted with preserved Auerbach's plexus (AP) and submuscular plexus (SMP) and incubated in Krebs solution.

2.4.3. Patterns of electrical activity in the mid colon

Intracellular electrical activity of circular smooth muscle cells from +/+ animals displayed a resting membrane potential of -53.5 ± 1.5 mV. The electrical recordings were dominated by cyclic depolarizations at 1.2 ± 0.1 cpm (n = 6) with marked superimposed action potentials with an amplitude of 24.0 ± 1.3 mV (Figure 7). In

addition, slow waves were recorded at 19.6 ± 1.4 cpm (n = 6) with an amplitude of 3.5 ± 0.3 mV.

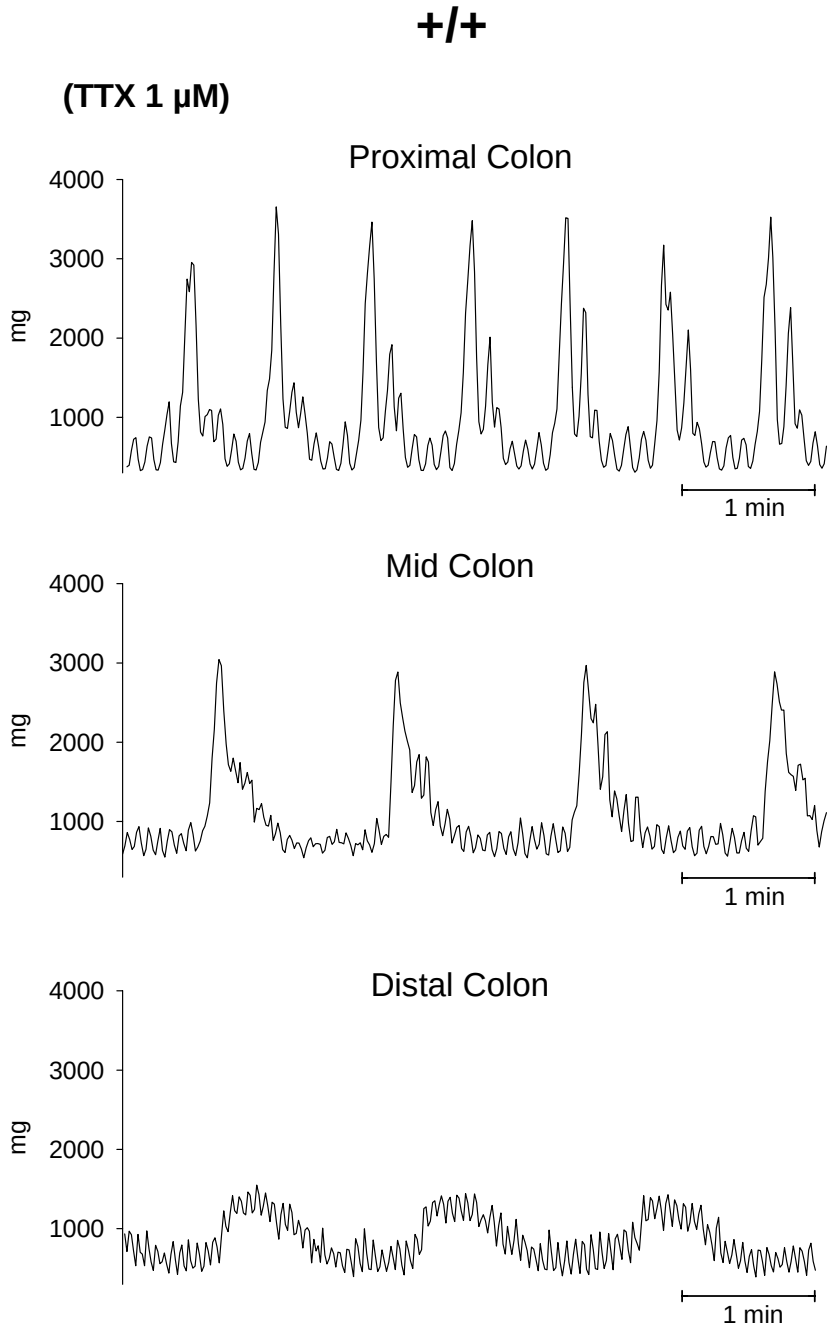

Figure 6. Mechanical recordings showing the spontaneous cyclic mechanical activity displayed by circular muscle strips from +/+ rats in the presence of the neural blocker TTX 1 µM. Colonic preparations were from proximal, mid and distal parts with preserved Auerbach's plexus (AP) and submuscular plexus (SMP). Notice the presence of myogenic high frequency and low frequency contractions.

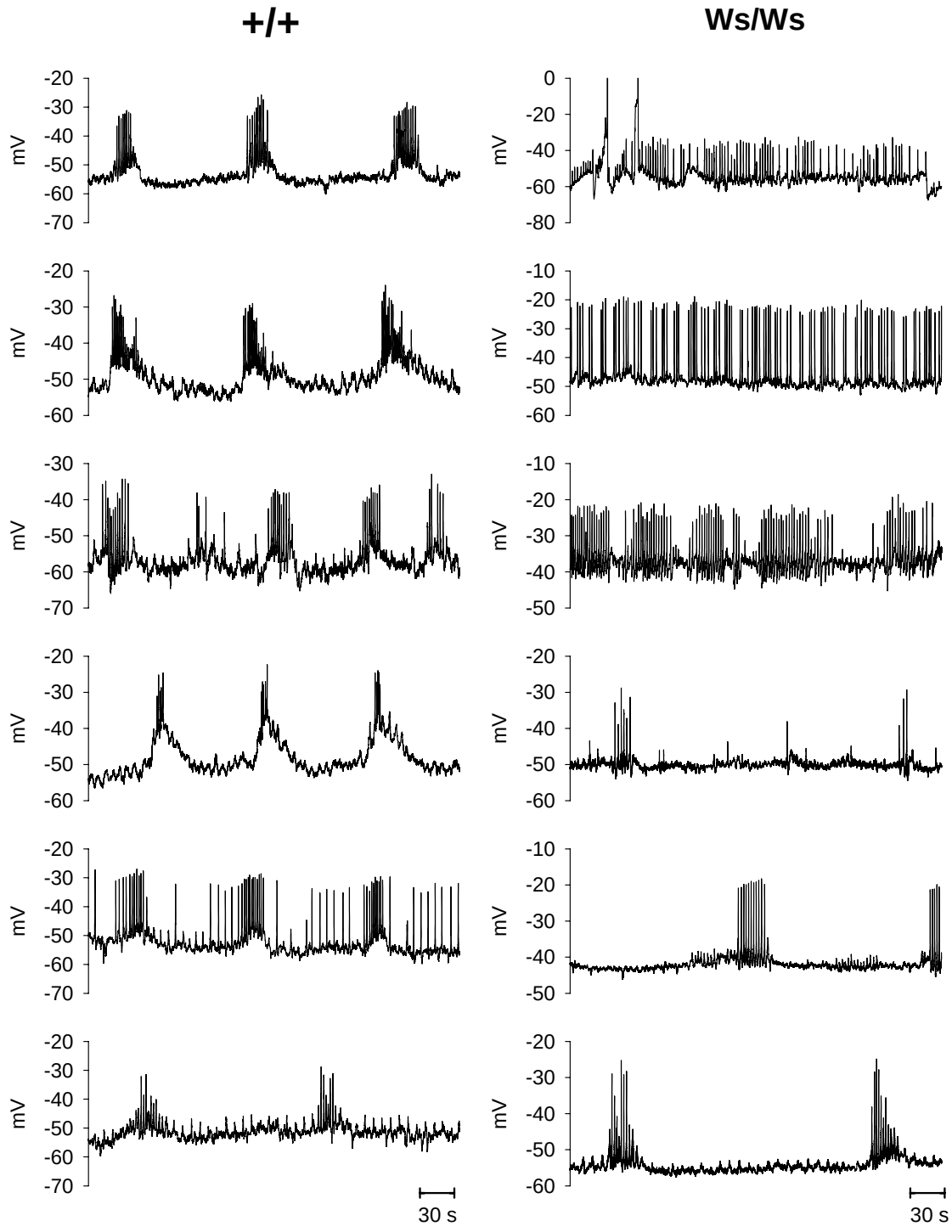

Figure 7. Intracellular microelectrode recordings showing the spontaneous electrical activity displayed by circular muscle strips from the mid colon with both plexuses kept intact in +/+ (left recordings) and Ws/Ws (right recordings). Each graph is from a different animal (n = 6).

The resting membrane potential of the circular muscle cells from Ws/Ws rats was -49.3 ± 3.2 mV. In 5 out of 6 mutant animals, the regular pattern observed in +/+ rats was absent (the cyclic depolarizations with superimposed action potentials were not observed) and action potentials occurred either continuously or in irregular bursts. In one preparation, however, regular cyclic depolarizations occurred. Action potentials occurred at amplitudes of 25.7 ± 2.6 mV (n = 6) (Figure 7), not significantly different from control tissue. The slow wave activity was not prominent in Ws/Ws tissues.

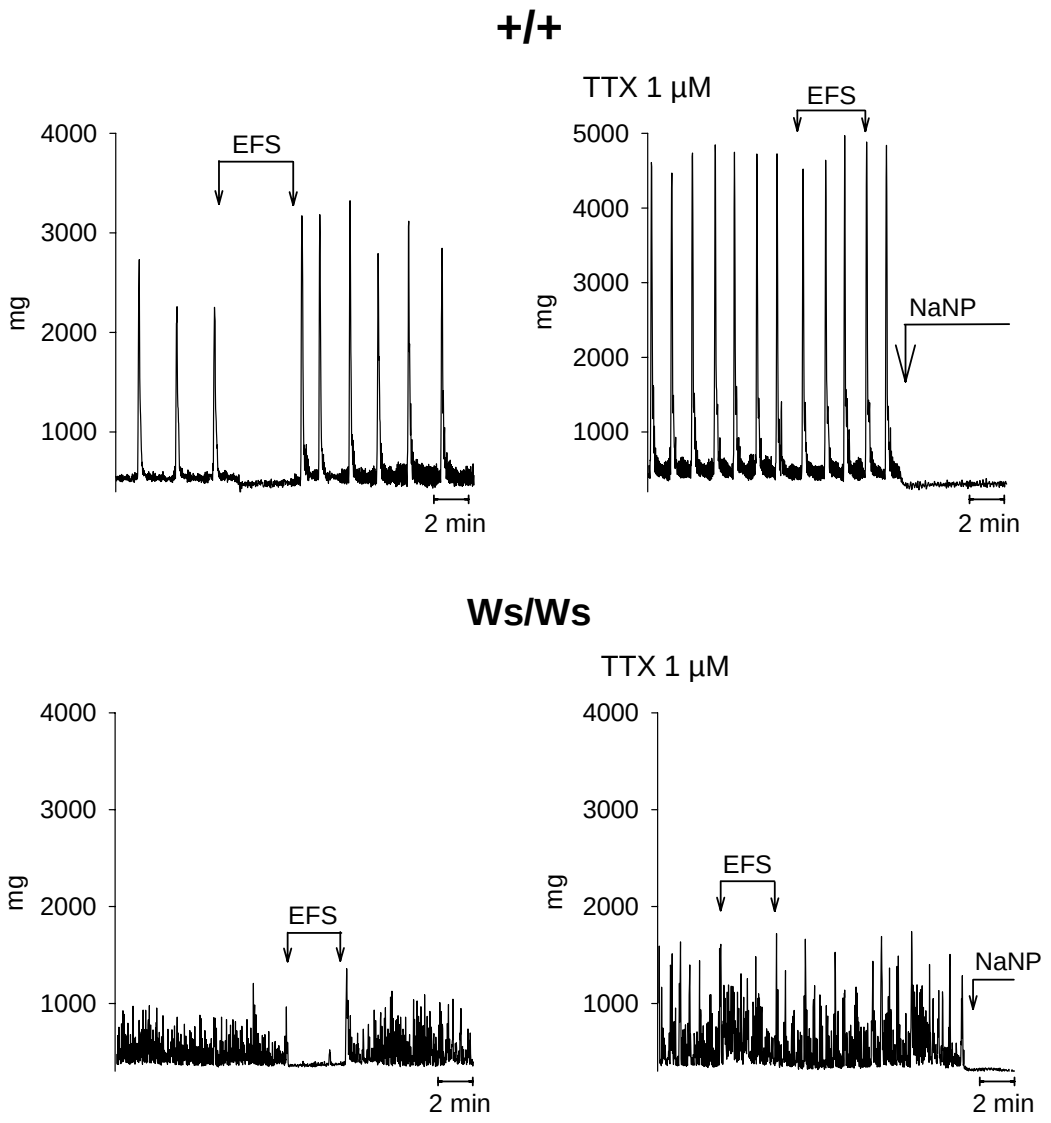

Figure 8. Mechanical recordings showing the effect of electrical field stimulation (EFS, 28 to 40 V, 4 Hz, 0.3 ms, 2-3 min) on the spontaneous cyclic mechanical activity displayed by the circular muscle in mid colon preparations. Recordings obtained from NANC condicions (left panels) and in the presence of TTX 1 µM (right panels), both in +/+ rats (top) and Ws/Ws rats (bottom). Sodium nitropruside (NaNP 10 µM) was added at the end to check muscle relaxation. EFS: electrical field stimulation.

2.4.4. Effect of inhibitory neurons on the spontaneous activity

In the presence of atropine, phentolamine and propranolol, nerve stimulation caused inhibition of the spontaneous mechanical activity in both control (n = 6) and Ws/Ws (n = 6) rats (Figures 8 and 9). This inhibition was completely prevented by prior addition of TTX 1 µM (Figure 8) or by prior addition of L-NNA 1 mM plus apamin 1 µM (Figure 9, n = 5).

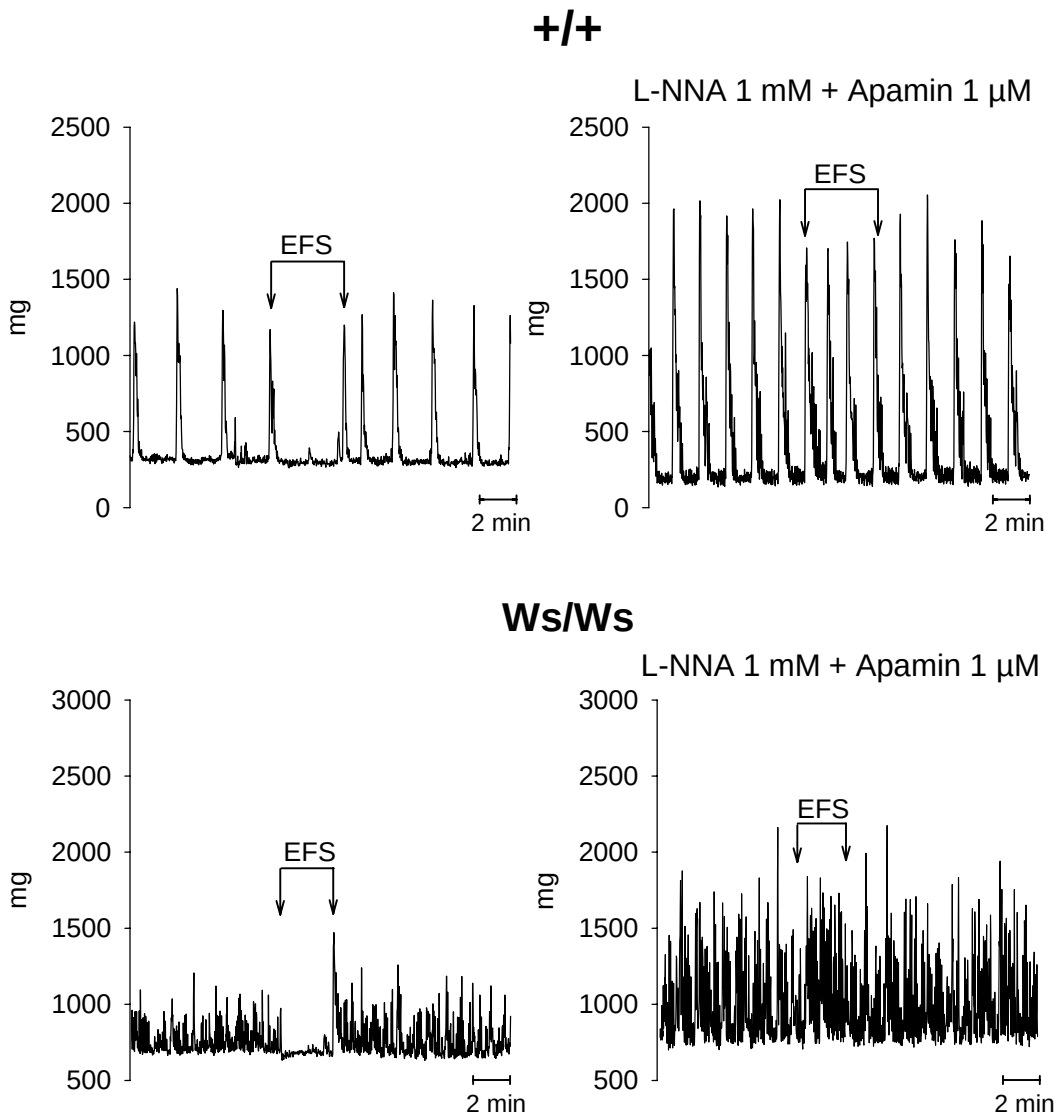

Figure 9. Mechanical recordings showing the effect of electrical field stimulation (EFS, 28 to 40 V, 4 Hz, 0.3 ms, 2-3 min) on the spontaneous cyclic mechanical activity displayed by mid colon circular muscle strips. Recordings obtained from NANC conditions (left panels) and in the presence of L-NNA 1 mM plus apamin 1 µM (right panels), both in +/+ (top) and Ws/Ws (bottom) rats. EFS: electrical field stimulation.

When measuring intracellular electrical activity, nerve stimulation caused an inhibitory junction potential (IJP) in both +/+ and Ws/Ws animals (Figures 10 and 11, n = 6). When the stimulus strength was gradually increased, the amplitude and duration of the IJP was progressively increased.

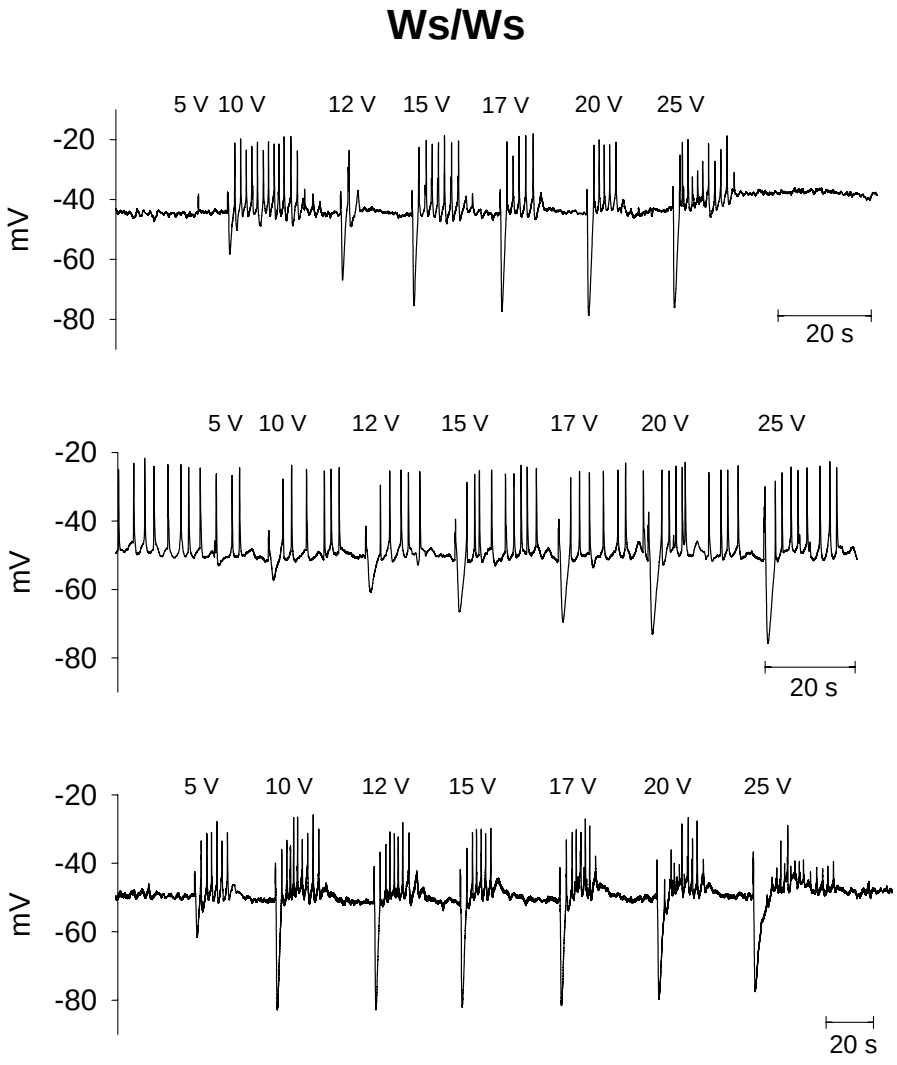

Figure 10. Intracellular recordings showing inhibitory junction potentials (IJPs) elicited by electrical field stimulation (EFS) at different stimulus strengths (5, 10, 12, 15, 17, 20 and 25 V) in three different Ws/Ws rats.

Figure 11C shows the relationship between the stimulus strength (voltage) and the amplitude (mV) and duration (s) of the IJPs in Krebs solution, non-adrenergic, non-cholinergic conditions (NANC) and with nifedipine 1 µM. In Krebs and under NANC conditions, an off response with spiking activity was recorded after the IJP, both in control and mutant animals (Figure 10). When nifedipine was added to the chamber, the spiking activity was abolished. In +/+ animals, the IJP clearly showed two components: an initial fast hyperpolarization and a sustained hyperpolarization (Figures 11A and 12A). There was no significant difference in the amplitude of IJPs between +/+ and WsWs rats. The IJP duration however, measured at the base of the IJP, was smaller in the colon of WsWs animals (Figure 11C). L-NNA 1 mM caused a reduction in the duration of the IJP in both +/+ and WsWs rats (Figure 12B). Apamin 1 µM (in the presence of L-NNA) caused a reduction in the duration and the amplitude of the IJP (Figures 12C and 13).

The addition of TTX 1 µM in muscle bath preparations increased the amplitude of spontaneous contractions in both control and mutant rats (Figure 14), indicating the presence of an inhibitory tone in both groups of animals. The addition of L-NNA 1 mM increased the amplitude of spontaneous contractions (Figure 14), indicating marked presence of spontaneous release of NO. When apamin was added in the presence of L-NNA, a slight increase in contractile activity occurred (not shown) indicating that colonic muscle preparations from +/+ and Ws/Ws rats have intrinsic spontaneous activity of nitrergic and purinergic nerves.

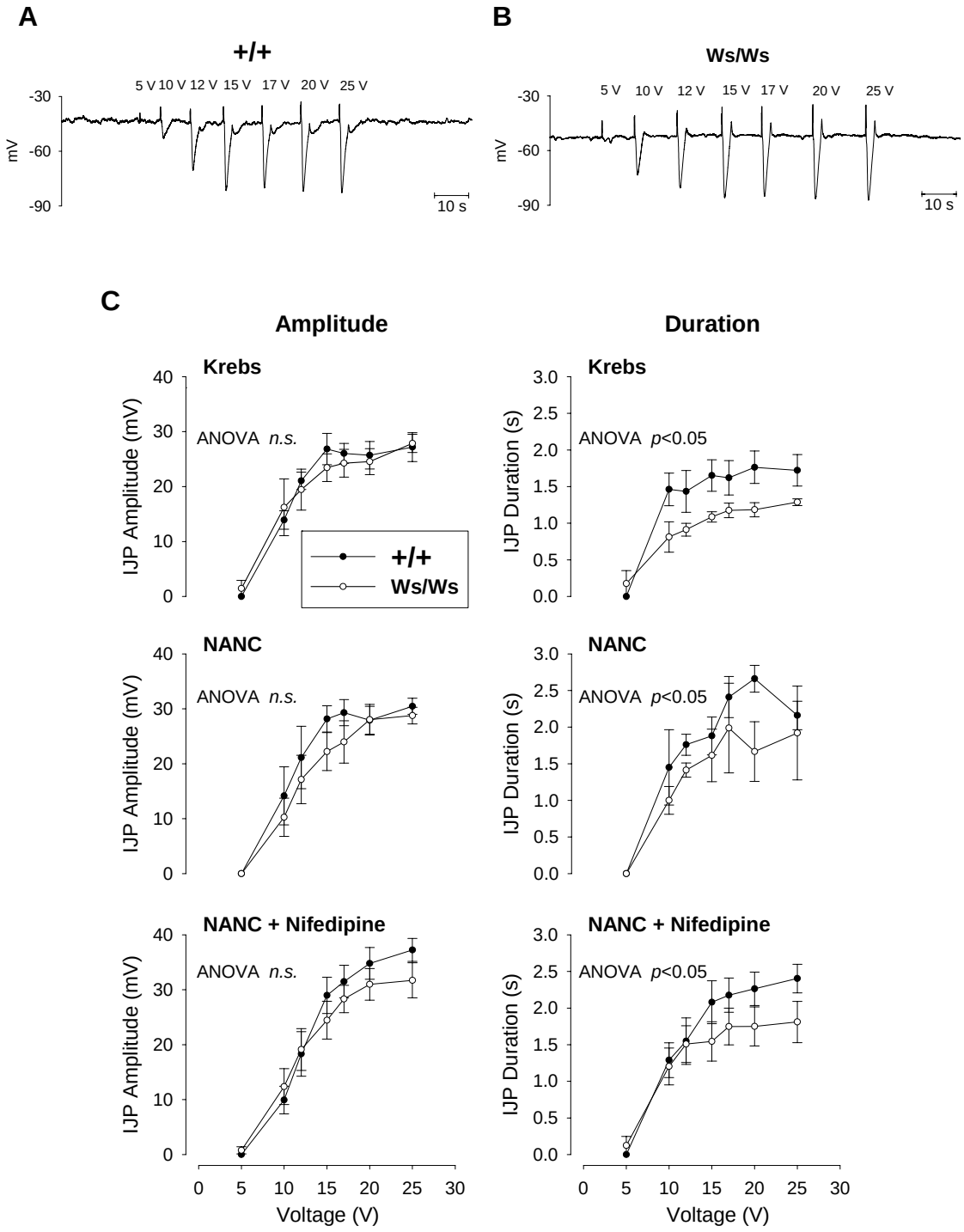

Figure 11. Intracellular recordings showing IJPs elicited by electrical field stimulation (EFS) increasing amplitude strengths (5, 10, 12, 15, 17, 20 and 25 V) in +/+ (A) and Ws/Ws (B) rats, under NANC conditions and in the presence of nifedipine 1 µM. (C) Graphs representing the amplitude (left) and duration (right) of the IJPs elicited by EFS in circular muscle strips in Krebs solution (top), under NANC conditions (mid) and in the presence of nifedipine 1 µM (bottom), both in +/+ (black circles) and Ws/Ws rats (white circles). Notice that mutant animals present a decrease in the duration (not the amplitude) of the IJP (Anova $p < 0.05$) in Krebs, NANC conditions and in presence of nifedipine 1 µM. IJP: inhibitory junction potential.

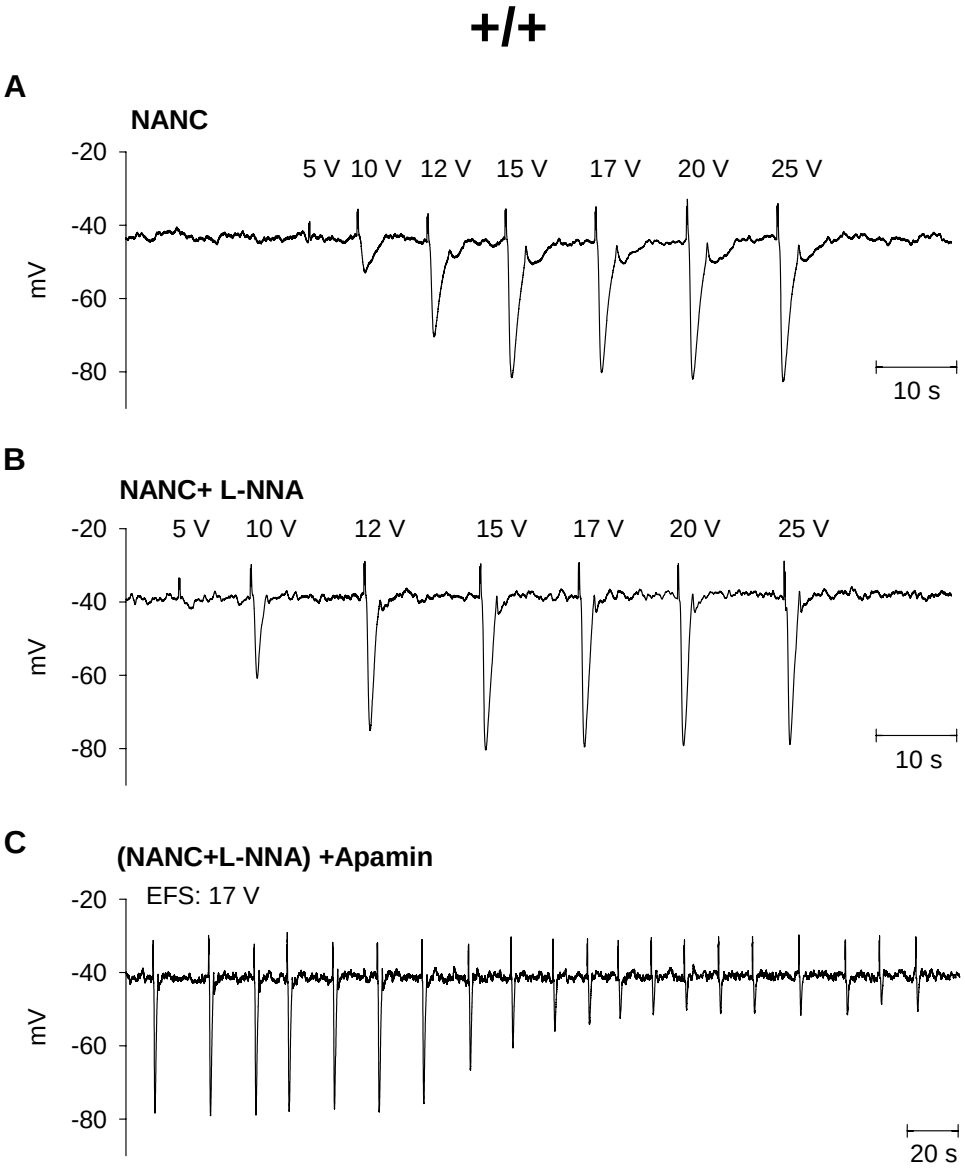

Figure 12. Intracellular recordings showing IJPs elicited by EFS at increased voltage strength of stimulation (5, 10, 12, 15, 17, 20 and 25 V) in +/+ rats under NANC conditions (A) and in the presence of L-NNA 1 mM (B). (C) Intracellular recording showing the effect of apamin 1 µM on the IJPs elicited by a repetitive stimuli of 17 V (in the presence of L-NNA). All these recordings were done in presence of nifedipine 1 µM. IJP: inhibitory junction potential; EFS: electrical field stimulation.

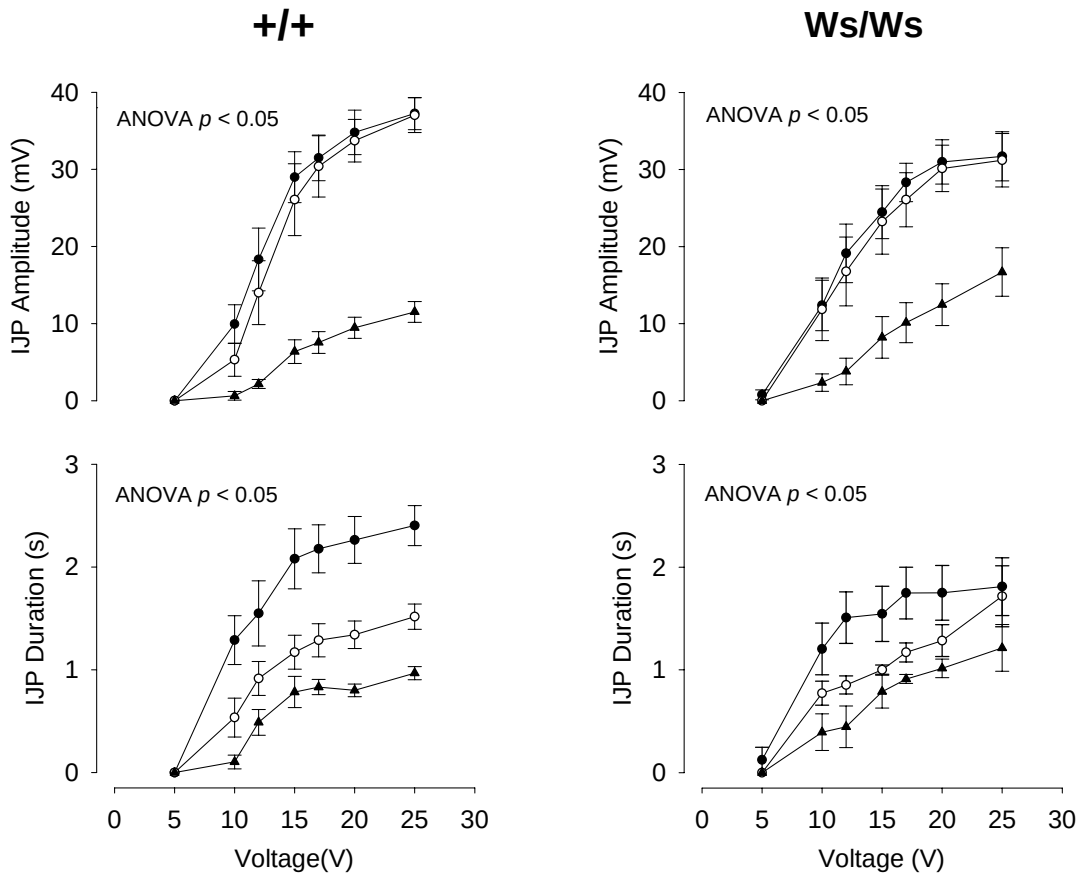

Figure 13. Graphs representing the effect of L-NNA 1 mM and L-NNA 1 mM plus apamin 1 µM on the amplitude (top panels) and duration (bottom panels) of IJPs elicited by electrical field stimulation (EFS) in circular muscle strips from +/+ (left) and Ws/Ws (right) rats. All these recordings were done in presence of nifedipine 1 µM and under NANC conditions. IJP: inhibitory junction potential.

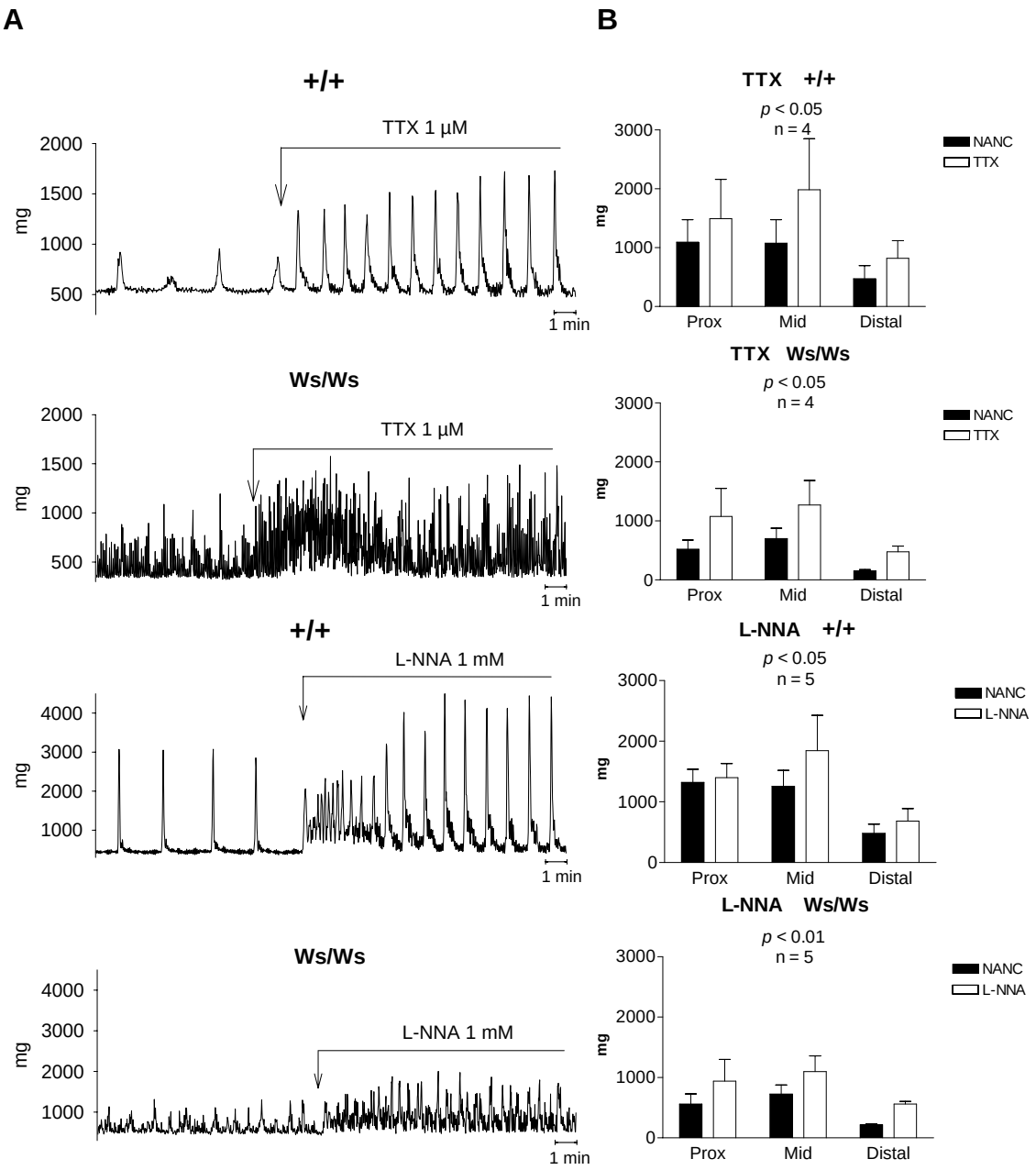

Figure 14. (A) Muscle bath recordings under NANC conditions from mid colon circular muscle strips showing the effects of TTX 1 µM (top) and L-NNA 1 mM (bottom) on the spontaneous mechanical activity in +/+ and Ws/Ws rats. (B) Graphs representing the differences in the amplitude of spontaneous contractions before and after the addition of TTX and L-NNA to the muscle bath.

2.5. Discussion

The pacemaker activity and neurotransmission was characterized in the colon of Ws/Ws rats compared to +/+ control animals. The colon of Ws/Ws rats shows an impairment of the regular motility pattern, a strong reduction in *c-kit* positive cells but still a partial functional neurotransmission.

Our first approach was to perform a morphological study combined with a stereological analysis to measure the density of *c-kit* positive cells and nNOS positive neurons. Stereology has been widely used to quantify the loss of neurons in the brain in several conditions, such as in aging (Long et al. 1999). This methodology has been previously used to quantify the distribution of *c-kit* and nNOS positive cells in the colon of Sprague-Dawley rats (see Chapter 1). The combination of morphological and stereological techniques was essential to quantify the density of nitrergic neurons and *c-kit* positive ICC cells in the colon of Ws/Ws and +/+ animals.

In the colon of +/+ rats, *c-kit* positive cells are found at the level of Auerbach's plexus (ICC-AP), submucosal border (ICC-SMP) and intramurally within both muscle layers (ICC-IM). ICC-AP and ICC-SMP are multipolar cells with several branches, forming a network; whereas intramural ICCs are spindle-shaped cells and run parallel to the muscle fibers. We have previously reported a similar distribution of *c-kit* positive cells in Sprague-Dawley rats (see Chapter 1). In contrast, in Ws/Ws rats very few *c-kit* positive cells are found. At the level of AP, a reduction between 52% (proximal colon) and 94% (distal colon) is estimated. An absence of *c-kit* immunoreactivity is observed at the level of the submucosal border (ICC-SMP) and within muscle layers (ICC-IM). The absence of *c-kit* positive cells has been reported at the level of AP in the small intestine of mutant rodents such as Ws/Ws rats (Isozaki et al. 1995), WWv mouse (Ward et al. 1994; Malysz et al. 1996) and Sl/Sld mouse (Mikkelsen et al. 1998). As it has been reported in the introduction of this chapter, interstitial cells of Cajal might be the pacemaker cells of the colon (Pluja et al. 2001; Yoneda et al. 2002; Yoneda et al. 2003) and ICC-IM probably participate in neurotransmission (Burns et al. 1996; Ward 2000; Ward & Sanders 2001). Accordingly, a functional study was performed to evaluate a possible impairment of both functions.

To evaluate the pacemaker activity, a double study using muscle bath and microelectrode techniques was performed. For technical reasons (8 muscle bath chambers and 1 microelectrode set up) the mechanical activity was studied in strips from the proximal, mid and distal colon, whereas the electrical activity was evaluated in the mid colon. Our recent studies have described a regular pattern of motility in Sprague-Dawley rats where: (i) the amplitude of contractions (both HF and LF contractions) is higher in strips from proximal colon compared to more distal segments and (ii) LF contractions show higher frequency in strips obtained from proximal colonic regions, whereas the frequency of HF contractions remains steady all along the colon (see Chapter 1).

The spontaneous electrical activity in Sprague-Dawley rats consists of slow waves superimposed with cyclic depolarisations forming a regular pattern (Pluja et al. 1999; Pluja et al. 2001). These results are identical to those found in our control +/+ rats. Moreover, the pacemaker responsible for this activity has a myogenic origin because in the presence of TTX the motor pattern is still preserved. In contrast, in Ws/Ws rats the electrical and mechanical patterns are impaired, showing that the reduction in *c-kit* positive cells is an important element that contributes to the alteration of pacemaker activity. Mechanical recordings consist of an amount of irregular contractions without a clear difference between HF and LF contraction types. Hence, smooth muscle contractile activity is preserved but it has become irregular. Recordings from microelectrode setup show that the consequence of the impairment of the regular pattern of activity is irregular bursts of action potentials, as it has been described in the small intestine of different mutant animals, which lack ICCs (Malysz et al. 1996; Mikkelsen et al. 1998). In the colon of Ws/Ws rats, high frequency slow wave activity is also more difficult to distinguish. This is likely the result of loss of *c-kit* positive ICCs at the submuscular plexus (SMP) with which this electrical activity is functionally coupled (see Chapter 1). In a previous study performed on Ws/Ws rats, rhythmic spontaneous contractions were recorded in colonic strips (Yoneda et al. 2001). These rhythmic spontaneous contractions are probably similar to the irregular muscle contractions described in the present study. The presence of *c-kit* negative interstitial cells has been suggested as responsible for the residual motor pattern (Yoneda et al. 2001). However,

c-kit negative interstitial cells have been characterized in the stomach of Ws/Ws rats and were considered fibroblast-like cells (Ishikawa et al. 1997). These cells do not present the accumulation of abundant mitochondria found in normal ICCs but, unlike fibroblast, they have multiple gap junctions with smooth muscle cells. Accordingly, the role of these *c-kit* negative interstitial cells is unknown but it is unlikely that they participate as pacemaker cells if the basis of pacing is related to the calcium reuptake from mitochondria. In contrast, *c-kit* negative interstitial cells can participate in neurotransmission because: (i) cell to cell communication, due to the presence of gap junctions that couple these cells to smooth muscle cells has been described and (ii) close association between *c-kit* negative interstitial cells have been reported. Consequently, a putative role in neurotransmission but not as a pacemaker cells can be attributable to *c-kit* negative interstitial cells.

Both in +/+ and Ws/Ws rats, nNOS positive neurons are found in the colon between circular and longitudinal muscle layers forming a network of nerve strands and ganglia. Moreover, fine fibers are found running parallel to the long axes of smooth muscle cells, especially in the circular muscle layer. The density of nNOS positive neurons is not homogeneous along the colon, being highest in the mid area in both +/+ and Ws/Ws rats. This highest density of nNOS positive neurons is consistent with a major nitrergic tone found in the mid colon of Sprague-Dawley rats (see Chapter 1). Comparing the density of nNOS positive neurons between Ws/Ws and +/+ rats, a significant decrease in the number of nNOS positive neurons is estimated in the mid and distal colon (35% and 29% respectively). These results were not expected at the beginning of the experiments, but the decrease in the density of nNOS positive neurons in mutant rats could be one possible explanation for the reduction in the second component of the inhibitory junction potential in these animals.

The inhibitory neurotransmission was evaluated with the muscle bath and microelectrode set up. Electrical field stimulation (EFS) causes inhibition of the spontaneous mechanical activity and elicits inhibitory junction potentials (IJPs). We have previously shown in Sprague-Dawley rats, that the inhibitory mediators released by enteric motor neurons are probably ATP and nitric oxide (Pluja et al. 1999). In these animals, the IJP shows two components: a fast component followed by a sustained

hyperpolarization. The fast component of the IJP is apamin-sensitive and is probably mediated by ATP. In contrast, the sustained component is apamin-insensitive but L-NNA-sensitive and it is probably due to nitric oxide release (Pluja et al. 1999). A biphasic IJP has also been described in mouse colon (Shuttleworth et al. 1997), guinea-pig colon (Hirst et al. 2004) and human jejunum (Stark et al. 1993). In the present paper, we report a similar result in +/+ rats, suggesting that the inhibitory neurotransmitters in these animals are also ATP and nitric oxide.

The colon of Ws/Ws rats displays inhibitory junction potentials and mechanical relaxations induced by EFS. This was an unexpected result and was not consistent at all with the absence of ICCs within muscle layers. In other areas of the gastrointestinal tract, in WWv mice, the absence of ICC-IM causes impairment of inhibitory neurotransmission (Christensen et al. 1992; Burns et al. 1996; Beckett et al. 2002). Similarly to what we found in +/+ rats, the IJPs in Ws/Ws animals show two components. Assuming that the fast IJP is mediated by ATP (Pluja et al. 1999; Xue et al. 1999), the present study shows that purinergic neurotransmission is likely independent of *c-kit* positive ICC-IM, as observed in the stomach of WWv mouse (Hirst et al. 2002). A role for ICC-IM in NO-mediated inhibition cannot be excluded however, since the slow component of the IJP was reduced in the colon of Ws/Ws rats and hence, possibly related to the strong reduction in the number of ICC-IM. The reduced slow component of the IJP can also be related to the reduced number of nitrergic nerves. When nNOS is blocked, an increment in the mechanical activity is found indicating the presence of ongoing release of nitric oxide. These results are consistent with a functional nerve-muscle interaction. The marked presence of NO and ATP mediated inhibition despite a strong reduction in *c-kit* positive cells suggests two possible hypotheses: (i) *c-kit* positive ICC-IM might not be essential for neurotransmission; this possibility should be considered because direct and indirect (via ICC) innervation has been ultrastructurally described in the stomach (Mitsui & Komuro 2002) or (ii) that neurotransmission might be accomplished by *c-kit* negative interstitial cells that are in close association with nerve varicosities and form gap junctions with smooth muscle cells (Ishikawa et al. 1997). It is important to consider that some studies have reported that not all ICCs identified with the electron microscopy are *c-kit* positive. *c-kit* negative

ICCs have been reported in the deep muscular plexus of the human small intestine. In this area, the cells have a weak or no immunoreactivity using several *c-kit* antibodies that stain ICC-AP in the same specimen, but have ultrastructural properties similar to ICCs (Wang et al. 2003).

We conclude that the presence of two ICC networks is necessary to have a regular pattern of electrical and mechanical activity. The neurotransmission is partially functional in Ws/Ws rats despite a reduction in *c-kit* positive ICCs. Two hypotheses are plausible: (i) neurotransmission is independent of *c-kit* positive ICCs (so the reduction in the density of nNOS positive neurons might be responsible for the altered nitrergic neurotransmission) and (ii) *c-kit* negative interstitial cells mediate nerve-muscle interaction.

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Chapter 3

Effect of 4-aminopyridine (4-AP) on the spontaneous activity and neuromuscular junction in the rat colon

3.1. Summary

Two pacemakers are involved in the generation of the spontaneous electrical activity in the rat colon. Slow waves, originated near the submuscular plexus, elicit high frequency contractions whereas low frequency contractions are related to cyclic depolarizations, which might be originated near the Auerbach's plexus. Inhibitory junction potentials (IJPs) in the rat colon show a fast component followed by a sustained NO-mediated hyperpolarization. Since K^+ channels are involved in the repolarization of both neurons and smooth muscle cells, we intended to study the changes induced by 4-AP (5 mM and 10 mM) on the spontaneous activity and on the inhibitory neurotransmission in colonic preparations from Sprague-Dawley rats. We used muscle bath and microlectrode techniques to characterize such effects. 4-AP inhibited the repolarization of smooth muscle cells and caused a transient (for 4-5 minutes) raise of membrane potential (9.7 ± 3.1 mV; n = 4) and repetitive spikes. The mechanical activity correlated with these electrical changes consisted of a transient increase in tone without cyclic activity followed by long lasting high amplitude cyclic contractions. To avoid smooth muscle cyclic depolarizations, nifedipine 1 µM was added to the microelectrode set up. 4-AP 5 mM hyperpolarized (-11.4 ± 2.1 mV, n = 5) the smooth muscle and induced spontaneous IJPs. This effect was not observed when the tissue was preincubated with TTX 1 µM (n = 7) and L-NNA 1 mM (n = 4). We conclude that 4-AP inhibits repolarization of smooth muscle cells and induces release of NO from nerve endings.

This effect might be due to inhibition of K^+ channels both in neurons and smooth muscle cells.

3.2. Introduction

Potassium channels are crucial elements controlling excitability and therefore the gastrointestinal motility. The opening of K^+ channels stabilizes the membrane potential closer to potassium equilibrium and far from firing threshold. The diversity of potassium channels described in gastrointestinal smooth muscles is high including delayed-rectified potassium channels (Carl 1995), K_A channels (Vogalis & Lang 1994), calcium-activated potassium channels (Xiong et al. 1995) and ATP-sensitive potassium channels (Pluja et al. 1998), among others. Potassium channels are involved in the repolarization of excitable cells (both neurons and smooth muscle cells) and in the inhibitory junction potential caused by the release of inhibitory mediators.

Gastrointestinal smooth muscle cells develop slow wave activity, which is originated by interstitial cells of Cajal (ICCs). In smooth muscle cells, slow waves are mediated by several ionic conductances carried by a diversity of voltage-gated channels (Farrugia 1999). Slow waves consist of a fast depolarization, a plateau phase and a repolarization. The plateau phase of electrical slow waves, in phasic gastrointestinal smooth muscle cells, is critical for excitation-contraction coupling. This plateau depends on the balance between inward Ca^{2+} current and outward K^+ current that is sustained for several seconds (Thornbury et al. 1992). When slow waves reach the calcium channel open threshold, calcium enters inside smooth muscle cells and triggers a muscular contraction. This inward of calcium is through L-type calcium channels. Outward currents responsible for repolarization of slow waves are also important factors in rhythmicity. In canine colonic circular smooth muscle cells, 4-aminopyridine (4-AP), a potassium channel blocker, increased the amplitude and prolonged the plateau phase of slow waves, suggesting that a voltage-dependent K^+ current participates in repolarization of slow waves (Thornbury et al. 1992). The mice colon presents cyclic depolarizations, which trigger cyclic contractions (Spencer 2001). 4-AP blocks the

repolarization and induces a repetitive spiking period (Koh et al. 1999). A rapidly inactivating K^+ current (A-type current), sensitive to 4-AP, has been characterized. This current is carried by Kv4 channels (Amberg et al. 2002).

Neurotransmitter release involves action potentials in enteric motor neurons. The release of inhibitory mediators causes a transient hyperpolarization of smooth muscle cells called inhibitory junction potential (IJP). Potassium channels participate in the repolarization of enteric neurons. Blockade of potassium channels might increase the neurotransmitter release after electrical field stimulation. Accordingly, the release of non-adrenergic, non-colinergic inhibitory transmitters is increased by 4-AP in the guinea-pig duodenum (Ohkawa 1984) and the release of nitric oxide is enhanced by 4-AP in the canine ileocolonic junction and lower esophageal sphincter (De Man et al. 1993; Daniel et al. 2000).

In the rat colon, we have previously demonstrated the presence of two rhythms: cyclic depolarizations with action potentials that cause low frequency (LF) contractions and slow waves, probably originated in the network of ICCs close to submuscular plexus, which trigger high frequency (HF) contractions ((Pluja et al. 2001), see chapter 1). Moreover, the IJP in the rat colon consists of two different components: a fast component and a slow sustained component. The first component is blocked by apamin 1 μM and the second component is blocked by L-NNA 1 mM, showing that a purinergic transmitter (such as ATP) and a nitrergic mediator (such as NO) might be inhibitory neurotransmitters in this preparation (Pluja et al. 1999).

Thus, the aim of this study is to demonstrate a dual effect of 4-AP: (i) a putative post-junctional effect on the electrical and mechanical rhythm and (ii) a pre-junctional effect on the neurotransmitter release in colonic circular smooth muscle cells from Sprague-Dawley rats.

3.3. Methods

3.3.1. Animals

Male Sprague-Dawley rats 300-350 g (Iffa-Credo, Lyon, France) and 8-10 weeks old were used in the present study. Animals were kept with 12 h light/12 h dark at a constant temperature (19-21ºC) and humidity (60%) in groups of three animals and had unlimited access to water and food. Before the *in vitro* 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. This procedure and the animal management were approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

3.3.2. Tissue preparation

The entire colon was carefully removed and placed into a dish containing carbogenated Krebs solution. The mesenteric fat was removed and the colon was opened along the mesenteric border. A piece of mid colon 1 cm long and 0.4 cm wide was pinned to a Sylgard-base with the mucosa facing upwards. According to a previous work (Pluja et al. 2001), the following circular muscle preparations were studied: (i) full thickness strips with Auerbach's and submuscular plexuses (to study the spontaneous electrical and mechanical activity) and (ii) muscle strips without submucosa (only for electrical studies: effect of drugs on the IJP and on the resting membrane potential).

3.3.3. Intracellular microelectrode recording

Preparations were pinned to the base of a Sylgard-coated chamber and continuously perfused with carbogenated Krebs solution at 37 ± 1ºC. Strips were allowed to equilibrate for approximately 1 h before recording. Circular smooth muscle cells were impaled with glass microelectrodes filled with 3 M KCl (30-60 Mq of resistance). The membrane potential was measured with a standard electrometer Duo 773 (WPI Inc., FL., USA), displayed on a digital storage oscilloscope 4026 (Racal-Dana Ltd.,

England), and simultaneosly digitized (100 Hz) and collected using EGAA software coupled to an ISC-16 A/D card (RC Electronics Inc., Santa Barbara, CA, USA) installed in a personal computer. Electrical field stimulation (EFS) that induced IJPs was performed using two silver chloride plates placed perpendicular to the longitudinal axis of the preparation. Train stimulation of EFS had the following parameters: duration 100 ms, frequency 20 Hz, pulse duration 0.3 ms and increasing amplitude strengths (5, 10, 12, 15, 17, 20 and 25 V). The amplitude and duration of elicited IJPs were measured under control NANC conditions and after infusion of the drug.

3.3.4. Spontaneous mechanical activity

Muscle bath technique was used to study the effect of 4-AP on the spontaneous mechanical activity. Circular muscle strips were attached with 2/0 silk threads to an isometric force transducer (Harvard UF-1 Apparatus Inc., Holliston, MA, USA) that was connected to a computer through an amplifier. The muscle bath (10ml) was filled with carbogenated Krebs solution at 37 ± 1ºC. Data were digitized (25Hz) and simultaneously displayed and collected using Datawin1 software (Panlab, Barcelona, Spain). A tension of 1 g was applied and the tissue was allowed to equilibrate for 1 h. After this period, circular muscle strips showed spontaneous phasic contractions. The amplitude and frequency of contractions were measured before and after drug addition.

3.3.5. Solutions and drugs

Krebs solution (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 $MgSO_4$ 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: 4-aminopyridine (Sigma Chemical, St. Louis, MO), apamin and tetrodotoxin (Latoxan, Valence, France), sodium nitroprusside (NaNP) (Research Biochemicals International, RBI), atropine sulphate (Merck, Darmstadt, Germany), phentolamine, *N*ϖ-nitro-L-arginine (L-NNA) and nifedipine (Sigma Chemical, St. Louis, MO, USA), propranolol (Tocris, Tocris Cookson Ltd., Bristol, UK). All drugs were prepared as stock solutions in distilled

water, except for nifedipine (1 mM) which was disolved in 100% ethanol, L-NNA, which was disolved in Krebs solution and prepared before using and tetrodotoxin, which was diluted in 1% glacial acetic acid.

3.3.6. Data analysis and statistics

The differences in the amplitude and duration of the IJPs before and after drug infusion were compared by analysis of variance (two-way ANOVA) for matched values followed by Bonferroni's post-test. The difference in the resting membrane potential (RMP) before and after drugs was compared by a *t*-test with a single column of values. A paired student's *t*-test or one-way analysis of variance (ANOVA) was used to compare mechanical activity in the absence and presence of drugs. Data are expressed as means ∂ SE. Differences were considered as statistically significant when $p < 0.05$.

3.4. Results

3.4.1. Effects of 4-aminopyridine on the spontaneous activity

The spontaneous electrical and mechanical activities (Figures 1A and 2A) of intact rat colonic smooth muscle cells have been previously described in detail (Pluja et al. 2001). Briefly, the spontaneous electrical activity is composed of: (i) slow waves (5.7 ± 0.4 mV; 14.6 ± 0.8 slow waves/min; 3.7 ± 0.3 s), which correlate with high frequency (HF) contractions at about 10-15 contr/min and (ii) cyclic depolarizations with action potentials at the top (12.8 ± 1.9 mV; 1.3 ± 0.2 cyclic depolarizations/min; 23.2 ± 1.1 s; 16.6 ± 2.6 spikes/cyclic depolarization), which induce low frequency (LF) contractions at 0.5-1 contr/min. The spontaneous mechanical activity is shown in detail in Figure 2 and data are reported in the non-adrenergic, non-cholinergic, NANC (control) rows of tables 1 and 2.

In the microelectrode set up, 4-AP 5 mM caused a transient depolarization (9.7 ± 3.1 mV; n = 4) of smooth muscle cells and induced repetitive spikes (Figure 1B). After 4-5 minutes exposed to 4-AP 5 mM, cyclic depolarizations with repetitive spikes were recorded (Figure 1C).

The effect of 4-AP on the spontaneous mechanical activity was investigated at two different concentrations (5 mM and 10 mM). In presence of 4-AP 5 mM, mechanical recordings show a transient increase of the baseline muscle strength (168.2 ± 43.4 mg, n = 9) accompanied by an abolition of the spontaneous contractions during an interval between 10-20 minutes (959.1 ± 132.4 s, n = 9) (Figure 2). Higher concentrations (4-AP 10 mM) showed the same effect: increase of baseline muscle strength: 964.7 ± 196.8 mg, n = 6; period of inhibition: 873.4 ± 172.1 s, n = 6. After that period of inhibition, the spontaneous mechanical activity started again progressively (Figures 2 and 3). The amplitude of LF and HF contractions was dose-dependently increased by the incubation with 4-AP. Regarding the frequency, an opposite result was found between HF and LF contractions, i.e. an increase of the frequency was observed for LF contractions and a slight decrease was measured for HF contractions (Tables 1 and 2).

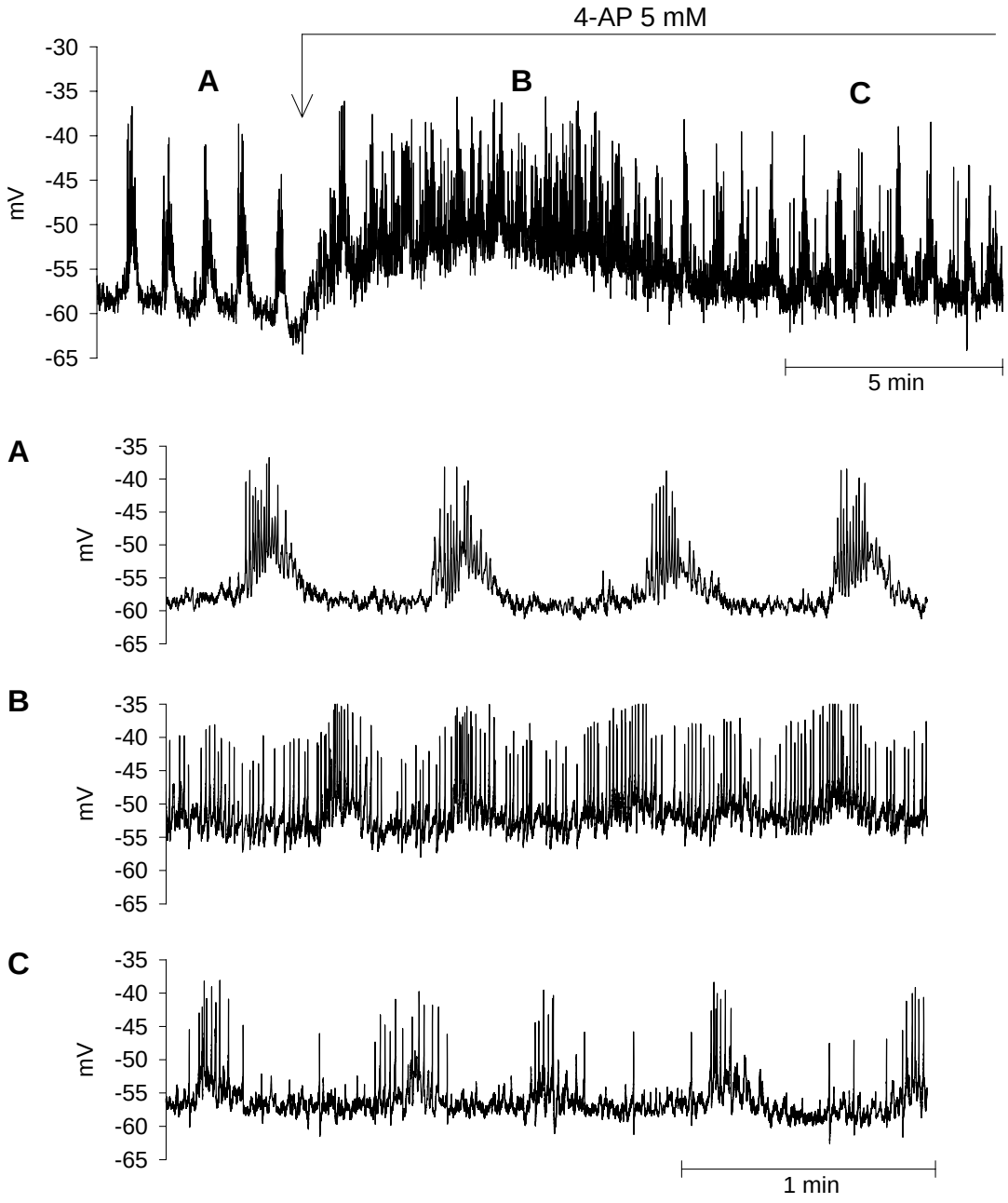

Figure 1. Effect of 4-aminopyridine (4-AP) on the spontaneous electrical activity of colonic circular smooth muscles with Auerbach's and submuscular plexuses kept intact. (A) Typical tracing showing slow waves and cyclic depolarizations. (B-C) Recordings showing the effect of 4-AP 5 mM on the spontaneous electrical activity at two different moments.

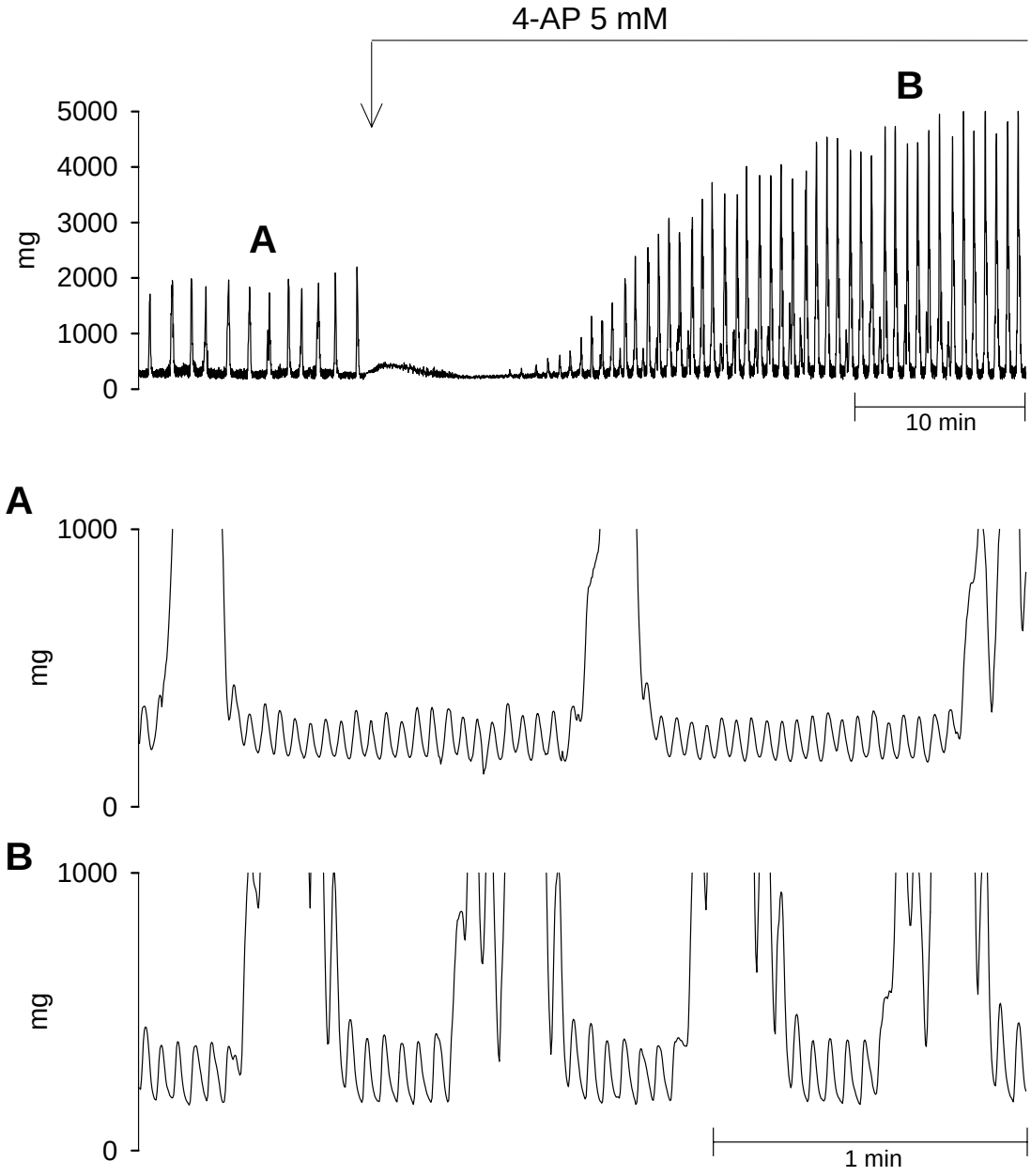

Figure 2. Effect of 4-aminopyridine (4-AP) on the spontaneous mechanical activity of intact rat colonic circular smooth muscle strips (top). Tracings showing in more detail high frequency contractions in NANC conditions (A) and in presence of 4-AP 5 mM (B).

Parameter / Contr. type		**Amplitude** (mg)	**Freq.** (cpm)	**Duration** (s)
Low frequency contractions	NANC	2351.1 ± 380.6	0.65 ± 0.06	29.3 ± 3.2
	4-AP 5 mM	3683.4 ± 637.2	0.92 ± 0.12	32.7 ± 3.7
	t-student	$p < 0.01$	$p < 0.05$	$p < 0.05$
High frequency contractions	NANC	120.7 ± 13.6	11.87 ± 1.26	6.1 ± 1.1
	4-AP 5 mM	213.6 ± 48.8	10.37 ± 0.94	6.4 ± 0.8
	t-student	*n.s.*	$p < 0.01$	*n.s.*

Table 1. Characterization of the mechanical activity of intact rat colonic circular muscle strips in NANC conditions and in the presence of 4-aminopyridine 5 mM (n = 9). Cpm: contractions per minute.

Parameter / Contr. type		**Amplitude** (mg)	**Freq.** (cpm)	**Duration** (s)
Low frequency contractions	NANC	3004.7 ± 814.6	0,60 ± 0,08	30.6 ± 2.5
	4-AP 10 mM	6524.0 ± 1500.9	1.05 ± 0,03	33,1 ± 0.6
	t-student	$p < 0.01$	$p < 0.01$	*n.s.*
High frequency contractions	NANC	130.4 ± 23.1	13.58 ± 0.36	4.4 ± 0.1
	4-AP 10 mM	397.1 ± 90.4	11.28 ± 0.62	5.2 ± 0.2
	t-student	$p < 0.05$	$p < 0.01$	$p < 0.01$

Table 2. Characterization of the mechanical activity of intact rat colonic circular muscle strips in NANC conditions and in the presence of 4-aminopyridine 10 mM (n = 6). Cpm: contractions per minute.

3.4.2. Contribution of pre-junctional potassium channels to the effect of 4-AP

In order to understand the role of pre-junctional potassium channels, microelectrodes recordings were performed in presence of nifedipine 1 µM and the inhibitory effect of 4-AP on the mechanical activity was investigated. 4-AP 5 mM caused a transient hyperpolarization of smooth muscle cells of 11.35 ± 2mV (n = 5, p<0.005) and induced an unstable membrane potential and spontaneous IJPs (Figure 4A), under NANC conditions. The RMP was unaffected when 4-AP was added in presence of both TTX 1µM (n = 7) and L-NNA 1 mM (n = 4) (Figures 4B and C). In presence of TTX 1 µM and L-NNA 1 mM the interval of quiescence induced by 4-AP was reduced (Figures 3 and 5).

3.4.3. Effects of 4-aminopyridine on the neurotransmission

The ability of potassium channels to influence neurotransmission and smooth muscle cells was investigated using microelectrode and muscle bath techniques.

In microelectrodes set up under NANC conditions and in presence of nifedipine 1 µM, electrical field stimulation (EFS) induced a transient membrane hyperpolarization called inhibitory junction potential (IJP) (Shuttleworth et al. 1997; Pluja et al. 2001). In the rat, IJPs are characterized by two phases: a fast hyperpolarization followed by a sustained hyperpolarization (Figure 6A). The fast hyperpolarization characterizes the amplitude of the IJP and the sustained hyperpolarization characterizes the duration of the IJP. Both parameters increased when increasing voltages were applied (5, 10, 12, 15, 17, 20, and 25 V) (Figure 6A). 4-AP 5 mM did not affect the amplitude of the IJPs (n = 7) but markedly reduced the duration (p < 0.0001; n = 7) (Figures 6B and C). Inhibition of the nitric oxide synthase by L-NNA 1 mM did not affect the amplitude (n = 7) but decreased the duration of the IJPs (p < 0.0001, n = 8). Addition of 4-AP 5 mM, after L-NNA perfusion, did not modify neither the duration nor the amplitude of the IJP (n = 8; Figure 6D).

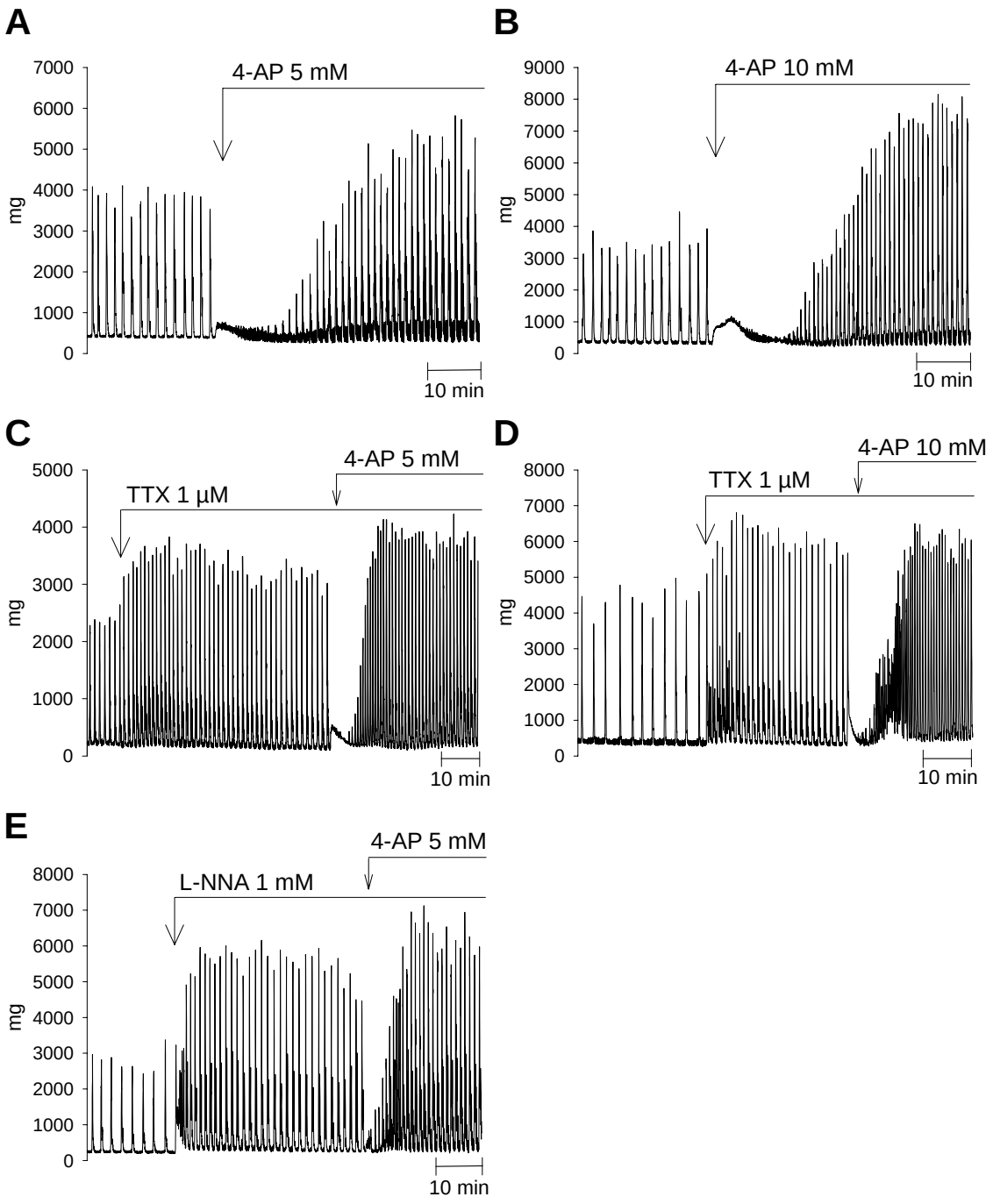

Figure 3. Recordings showing the effect of 4-aminopyridine (5 mM and 10 mM) on the spontaneous mechanical activity in NANC conditions (A and B), in the presence of TTX 1 µM (C and D) and in the presence of L-NNA 1 mM (E).

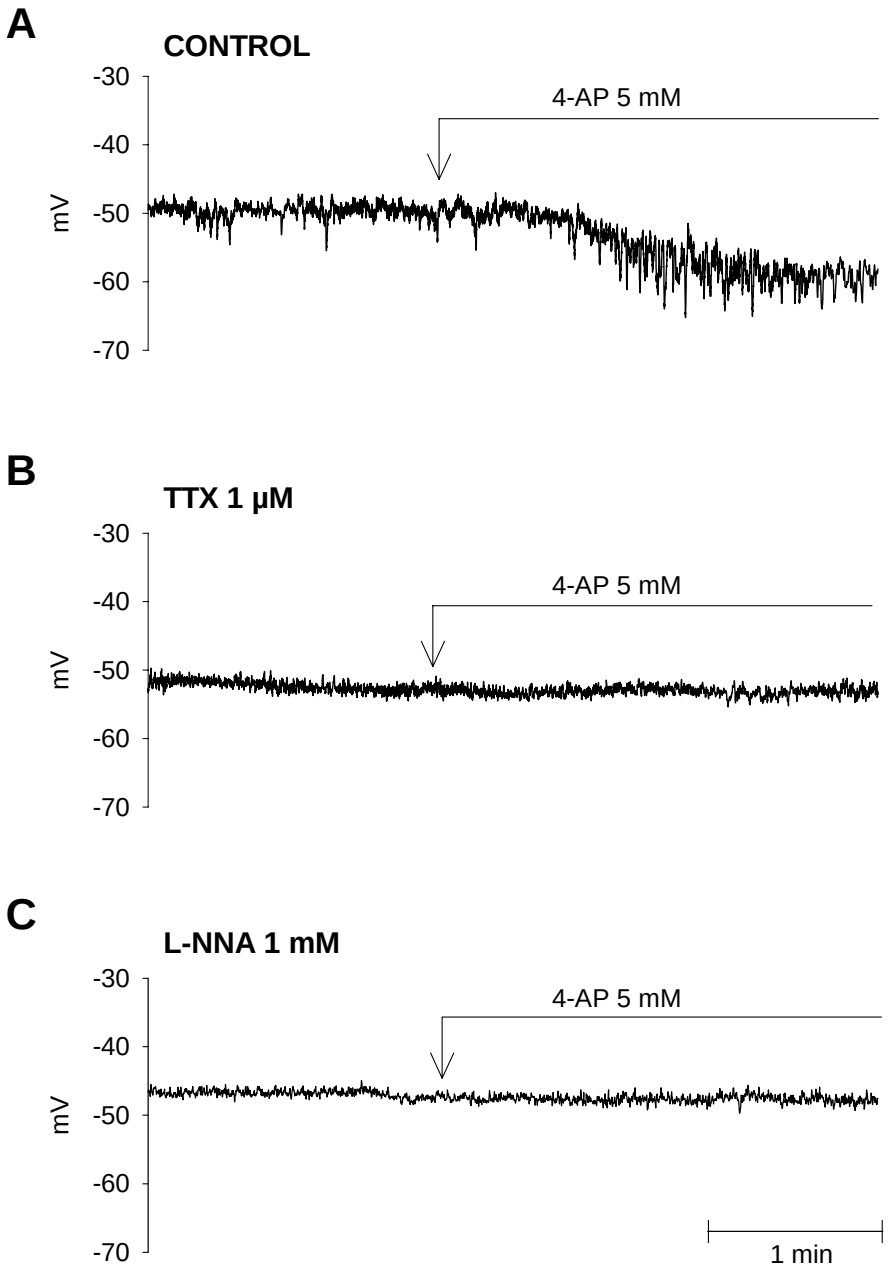

Figure 4. Intracellular microelectrode recordings showing the effect of 4-aminopyridine 5 mM on the membrane potential in NANC conditions (A) and when adding TTX 1 µM (B) or L-NNA 1 mM (C) into the chamber. All intracellular tracings were obtained in the presence of nifedipine 1 µM.

In the muscle bath, the nitric oxide donor sodium nitroprusside abolished the spontaneous activity in presence of TTX and 4-AP (n = 6), showing that 4-AP do not block the inhibitory effect of NaNP (Figure 7). Moreover, a similar result was obtained when the tissue was preincubated with 4-AP and apamin (n = 6; Figure 7). These results show that these potassium channel blockers are not involved in the NO pathway in smooth muscle cells.

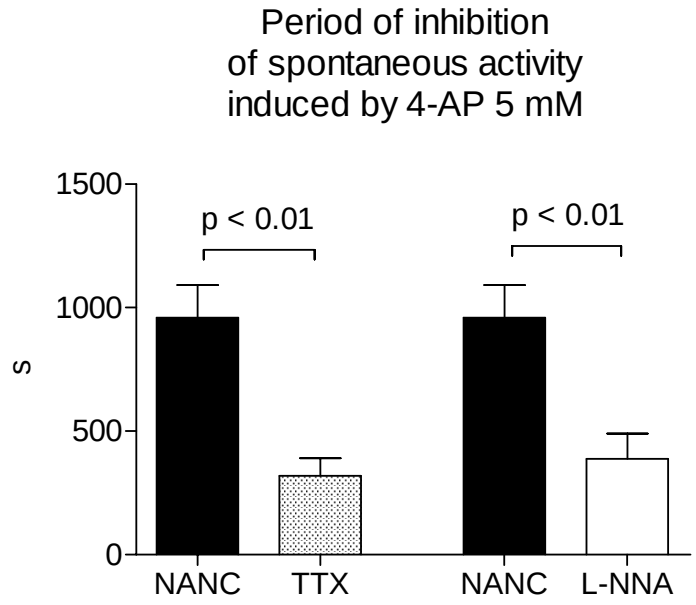

Figure 5. Bar diagrams showing the period of inhibition caused by 4-aminopyridine 5 mM on the spontaneous mechanical activity in NANC conditions and in presence of TTX 1 µM and L-NNA 1 mM.

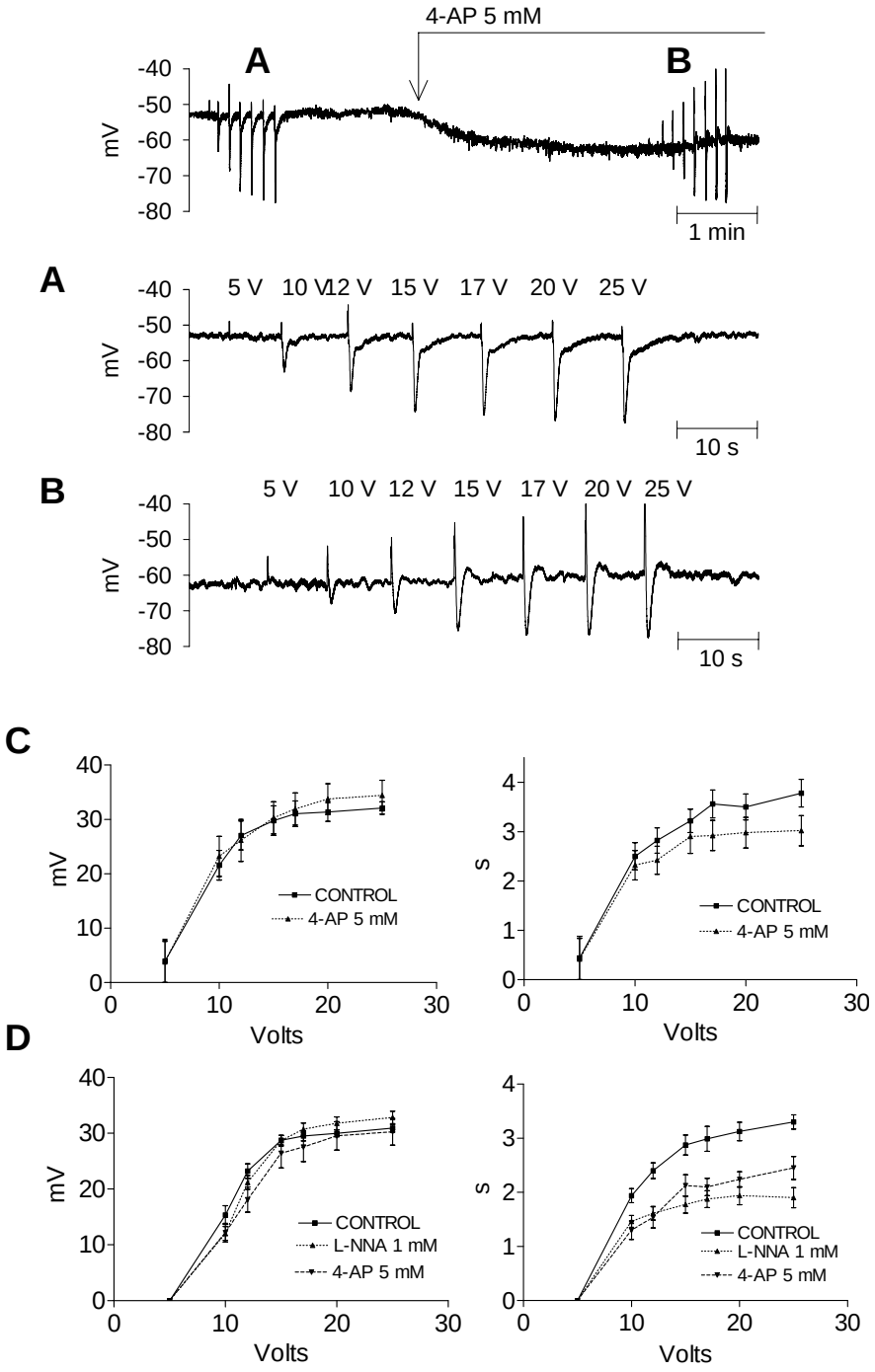

Figure 6. Intracellular microelectrode recordings showing the effect of 4-aminopyridine 5 mM on the inhibitory junction potentials (IJPs) and on the resting membrane potential (top) in one colonic circular smooth muscle cell. (A) Train of IJPs under NANC conditions increasing the voltage of stimulation. (B) Train of IJPs in presence of 4-AP. Effect of 4-AP 5 mM on the amplitude (left) and duration (right) of the IJPs obtained at different stimulus strength in NANC conditions (C) and in the presence L-NNA (D). All the recordings were done in presence of nifedipine 1 µM.

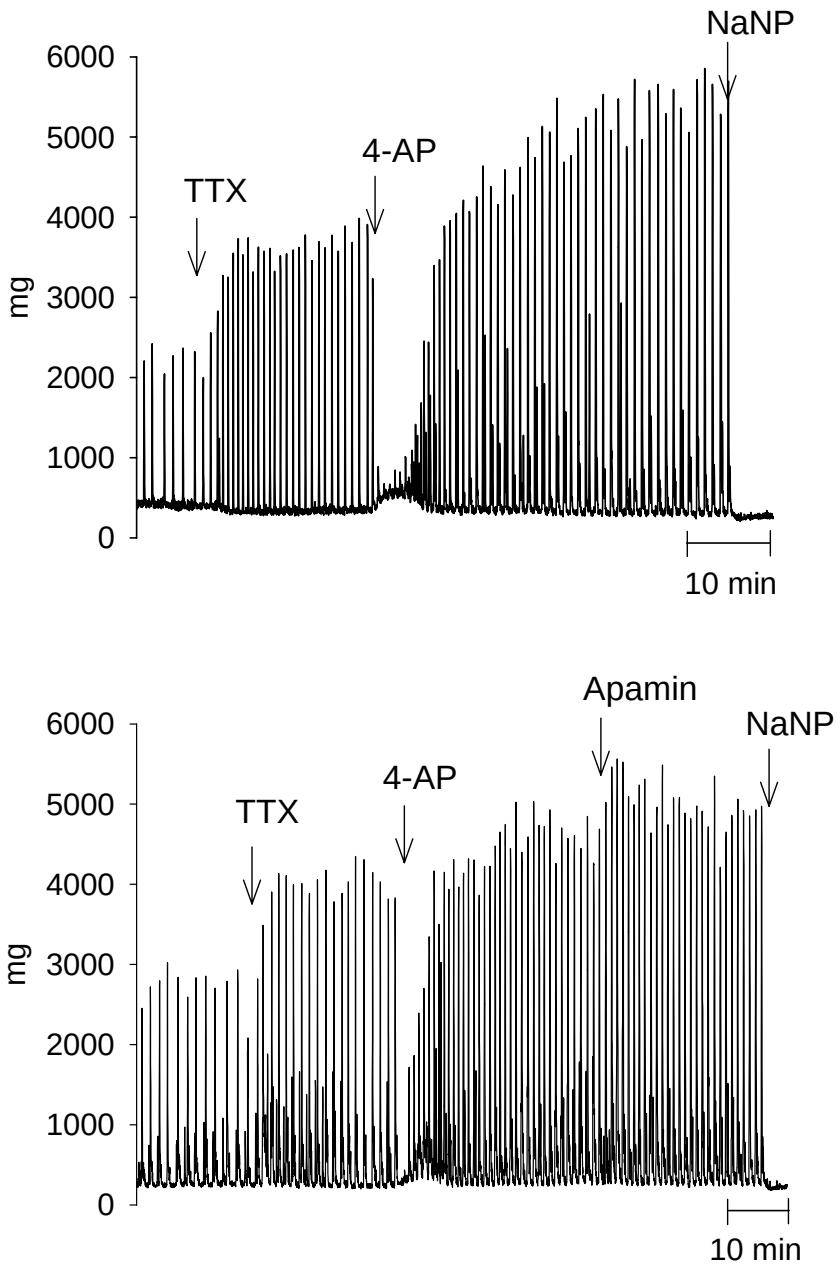

Figure 7. Mechanical recordings showing the inhibitory effect of sodium nitroprusside (NaNP 10 µM) after the addition of: (i) TTX 1 µM, plus 4-aminopyridine 5 mM (top) and (ii) TTX 1 µM, plus 4-aminopyridine 5 mM and plus apamin 1 µM (bottom).

3.5. Discussion

The results found in the present work show a dual effect of the non-selective potassium channel blocker 4-aminopyridine (4-AP) in the Sprague-Dawley colon: (i) a pre-junctional effect that causes the release of nitrergic neurotransmitters and (ii) a post-junctional effect that inhibits the repolarization and leads to repetitive spiking activity. These effects are due to blockade of 4-AP-sensitive channels pre and post-junctionally and demonstrate a role of these channels in the repolarization of smooth muscle cells and inhibitory motor neurons.

In circular colonic smooth muscle cells from Sprague-Dawley rats, 4-AP causes a transient depolarization of the membrane potential followed by repetitive spiking. This repetitive spiking activity is probably related to the increase in the baseline observed in our mechanical experiments. In the mouse colon, 4-AP causes repetitive spiking activity and inhibits the repolarization of colonic smooth muscle cells (Koh et al. 1999). This suggests that cyclic depolarizations found in the colon of rodents depend on a channel that is affected by 4-AP. However, 4-AP shows a poor selectivity (Mathie et al. 1998). 4-AP blocks calcium-independent transient outward currents (Koh et al. 1999). These currents are probably carried by Kv4 channels that might be responsible for the repolarization phase of colonic smooth muscle cells in the mouse (Amberg et al. 2002). However, data from patch-clamp experiments (not shown) demonstrate that 4-AP is also able to slow the inactivation of calcium-activated potassium currents. In the rat colon, this transient-inactivated potassium current is calcium dependent because it cannot be recorded in 0 calcium or in presence of nifedipine (not shown). Comparing cyclic depolarizations of colonic smooth muscle cells and slow wave activity of the small intestine, a major difference regarding the sensitivity of dihidropyridines occurs. In the small intestine, slow wave activity is L-type calcium channel independent. Accordingly, intestinal slow wave activity can be recorded in the presence of nifedipine (Cayabyab et al. 1996) but in the colon, cyclic depolarizations and slow waves are abolished in the presence of dihidropyridine derivatives (Pluja et al. 2001; Yoneda et al. 2003). This suggests that L-type calcium channels are important in the depolarization of colonic smooth muscle cells. Depolarization and subsequent

increase of intracellular calcium can activate several potassium channels including delayed-rectifier potassium channels, calcium-activated potassium channels and transient-activated calcium-independent potassium channels leading repolarization. 4-AP can be acting through these channels inhibiting the repolarization and inducing repetitive spiking activity.

As we previously reported, the colon of rats showed two mechanical activities: (i) high frequency contractions (10 and 15 contr/min) caused by slow wave activity related to the ICC-SMP network and (ii) low frequency contractions (0.5 and 2 contr/min) caused by cyclic depolarizations, which might be related to the ICC network located at the Auerbach's plexus ((Pluja et al. 2001), see chapter 1). In this study we show that after a period of quiescence, both type of contractions still occur but with higher amplitude and duration. This suggests that 4-AP-sensitive channels participate in both types of contractions. Similar to these results, 4-AP increases the plateau phase of slow waves in canine colonic circular smooth muscle cells (Thornbury et al. 1992). Accordingly, both cyclic depolarizations and slow wave activity are sensitive to 4-AP.

To avoid a post-juntional effect, nifedipine is added to the microelectrode set up and the cyclic activity is abolished (Pluja et al. 2001). In the presence of nifedipine, 4-AP causes hyperpolarization of smooth muscle sensitive to TTX and L-NNA. This is not consistent with a post-junctional effect and shows that 4-AP is causing the release of inhibitory nitrergic transmitters. A similar effect was previously demonstrated in the canine ileocolonic junction and lower oesophageal sphincter (De Man et al. 1993; Daniel et al. 2000). It is interesting to notice that in the rat colonic smooth muscle cells the two major inhibitory transmitters are ATP and NO (Pluja et al. 1999). The fact that the hyperpolarization induced by 4-AP is fully blocked by L-NNA might suggest that NO and ATP are released by different subclasses of inhibitory motor neurons or alternatively the effect of 4-AP is able to increase enough calcium to induce the release of NO but not ATP. Electrical field stimulation causes an inhibitory junction potential characterized by two components: a fast hyperpolarization (apamin-sensitive, L-NNA-insensitive) followed by a sustained hyperpolarization (L-NNA-sensitive), indicating the release of ATP and nitric oxide (Pluja et al. 1999). A puzzling result is

that the second component measured with the duration (at the baseline) of the IJP is reduced in presence of 4-AP. This result suggests two possible hypotheses: (i) 4-AP is blocking post-juntionally the effect of NO or (ii) the hyperpolarization itself causes a reduction in the second component. Our results are not consistent with the first hypothesis because 4-AP hyperpolarizes the smooth muscle and also because in the presence of TTX plus 4-AP, exogenous nitric oxide is able to abolish the spontaneous activity. Nitric oxide acts on smooth muscle through several potassium channels (Koh et al. 1995) and 4-AP do not block the NO pathway in the rat duodenum (Martins et al. 1995). Our results also show that 4-AP and apamin sensitive channels are not involved in the NO pathway in smooth muscle cells.

We conclude that 4-AP has a dual effect on Sprague-Dawley colonic smooth muscle cells: (i) it delays repolarization of smooth muscle cells and (ii) it causes release of nitric oxide from nerve endings. This dual mechanism is responsible for the transient increase in the baseline found in mechanical recordings, followed by a quiescent period and a posterior increase in the mechanical activity.

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General discussion

Interstitial cells of Cajal (ICCs) are pacemaker cells that generate electrical slow waves in gastrointestinal smooth muscle cells (Huizinga et al. 1995; Torihashi et al. 1995; Ward et al. 1995; Daniel 2001; Huizinga 2001; Ward & Sanders 2001). Moreover, certain classes of ICCs may mediate neurotransmission between enteric motor neurons and smooth muscle cells in the gastrointestinal tract (Burns et al. 1996; Ward et al. 1998; Ward et al. 2000). Close apposition between nerve endings and ICCs has been previously demonstrated (Daniel & Posey-Daniel 1984). This communication between ICCs and smooth muscle cells may involve electrical or metabolic coupling through gap junctions, or secretion of nitric oxide or carbon monoxide (Huizinga 2001). These observations support a functional pathway involving nerve endings, ICCs and smooth muscle cells.

The role of ICCs in the stomach and intestine are becoming increasingly clear. In the small intestine, ICC-AP originate slow wave activity and ICC-DMP might participate in neurotransmission. In the colon, however, the different functions of ICCs are less well-known (Vanderwinden et al. 2000). Thus, in this study we have investigated the mechanisms by which ICCs play a role in the pacemaker activity and in neurotransmission in the rat colon. To assess these objectives, we studied the colon of Sprague-Dawley rats, Ws/Ws mutants and its siblings +/+ rats. Mutant rats present a spontaneous mutation in the *Ws* locus that affects the normal activity of Kit receptor. The *c-kit* proto-oncogene encodes Kit, the receptor tyrosine kinase for stem cell factor (SCF). The SCF-Kit signalling pathway is crucial for the differentiation and proliferation of ICCs and consequently, these mutant animals present underdevelopment of certain classes of ICCs.

Spontaneous myogenic activity in the rat colon

The rat colon presents spontaneous electrical and mechanical activities. In the presence of the neural blocker tetrodotoxin, the spontaneous activity is still preserved, suggesting

a myogenic origin. This myogenic spontaneous activity has been described in other works on the rat colon (Pluja et al. 2001; Gonzalez & Sarna 2001b). However, this conclusion seems to contrast with studies that reported a neurogenic origin of the spontaneous activity in the mouse colon since TTX abolished this activity. These neural-mediated electrical and mechanical activities have been called migrating myoelectric complexes or migrating motor complexes, respectively (Lyster et al. 1995; Spencer et al. 1998; Brierley et al. 2001). This is supported by studies in aganglionic segments of mouse colon with intact ICC distribution, where the spontaneous electrical activity was impaired (Ward et al. 2002). Conversely, in the present study we have found that the rat colonic spontaneous activity: (i) is myogenic in origin and that (ii) is present in small strips of tissue and do not depend on the entire colon. Our data show that two functional pacemakers are found along the rat colon being responsible for slow wave activity and cyclic depolarizations. This electrical activity triggers high frequency (HF) and low frequency (LF) contractions, respectively. Other works found similar results in the rat colon (Pluja et al. 2001; Gonzalez & Sarna 2001a; Gonzalez & Sarna 2001b). Based on the hypothesis that ICCs are pacemaker cells, our first aim was to characterize the network of ICC cells in the colon of Sprague-Dawley, Ws/Ws and +/+ rats.

Morphology and distribution of interstitial cells of Cajal in the rat colon

The distribution and morphology of ICCs were studied in semitransparent whole mounts using a stereological analysis along the colon. Kit immunoreactivity (c-*kit* antibodies) has been widely used to label ICCs in several species (Maeda et al. 1992; Ward et al. 1994; Huizinga et al. 1995; Torihashi et al. 1995; Sanders 1996). In the colon of Sprague-Dawley and +/+ rats, *c-kit* positive cells are found at the level of the Auerbach's plexus (ICC-AP), submucosal border (ICC-SMP) and within circular and longitudinal muscle layers (ICC-IM). ICC-AP and ICC-SMP are multipolar cells and form a dense network. These two ICC networks can be the origin of the two types of electrical and mechanical activities described in this study. In contrast, intramural ICCs are spindle-shaped cells arranged in parallel with smooth muscle cells and might

participate in the neurotransmission between nerve endings and smooth muscle cells. This distribution of ICCs is very similar to those described in other species including human (Faussone-Pellegrini et al. 1990; Rumessen et al. 1993), dog (Berezin et al. 1988; Berezin et al. 1990) and mouse (Faussone-Pellegrini 1987). Regarding the density of ICCs, no major differences are found between proximal, mid and distal colon at the AP (about 100 cells/mm^2) and SMP (between 50 and 60 cells/mm^2). Similar results are obtained from both Sprague-Dawley and +/+ rats. In contrast, Ws/Ws rats present very few *c-kit* positive cells. At the level of the AP, a reduction between 52% (proximal colon) and 94% (distal colon) is estimated. A lack of *c-kit* positive cells is found both at the SMP plexus and within smooth muscle layers. An absence of *c-kit* positive cells at the AP has been reported in Ws/Ws rat small intestine (Isozaki et al. 1995), WWv mutant mice (Ward et al. 1994; Malysz et al. 1996) and Sl/Sld mutant mice (Mikkelsen et al. 1998). These results demonstrate that the mutation in the *c-kit* gene impairs the development of *c-kit* positive ICCs in the colon of Ws/Ws rats. As a result, the pacemaker activity and neurotransmission is analyzed in Sprague-Dawley, Ws/Ws and +/+ rats.

Spontaneous electrical and mechanical activity

Spontaneous electrical activity from circular colonic strips from Sprague-Dawley rats consists of slow waves superimposed with cyclic depolarizations forming a regular pattern (Pluja et al. 2001). These results are consistent with the presence of two functional pacemakers. In the dog colon, intracellular recordings from circular smooth muscle cells clearly show two pacemaker frequencies: 6 cycles/min slow waves originated from the ICC-SMP network and 20 cycles/min oscillations, probably paced by the ICC-AP network (Smith et al. 1987). In the human colonic circular muscle layer, slow waves are identified near the submucosal edge generating mechanical activity at 2-4 cpm (Rae et al. 1998). In order to identify the pacemaker activity in the different areas of the colon, we performed a study with proximal, mid and distal colonic strips.

HF and LF contractions vary in relation to the orientation of the muscle strip (circular or longitudinal orientation) and the segment studied (proximal, mid and distal

colon). Regarding the amplitude and frequency of contractions, several mechanisms can be responsible for these differences.

In Sprague-Dawley and +/+ rats, the amplitude of both LF and HF contractions is higher in circular muscle strips from the proximal colon compared to more distal segments. In contrast, longitudinal muscle strips show higher amplitude of contractions in distal segments compared to proximal strips. The reasons for these differences between layers are not clear, but they could be related to different structural properties such as thickness, number of cells, coupling or contractile proteins and they are probably independent of the pacemaker mechanism.

LF contractions show higher frequency in proximal regions compared to distal segments. LF contractions are due to cyclic depolarizations observed from intracellular microelectrodes recordings (Pluja et al. 2001). These cyclic depolarizations allow the opening of calcium channels and consequently, muscular action potentials occur. Action potentials, which cause spiking activity, are recorded *in vivo* with electromyographic techniques. Accordingly, the basic pattern of colonic myoelectrical activity is characterized by spike bursts at a higher frequency in the proximal colon (0.9/min) than in the distal colon (0.5/min) (Ferre & Ruckebusch 1985). These results suggest that a gradient in the frequency of LF contractions can be recorded from both *in vitro* and *in vivo* studies and that the mechanism responsible for these differences is an intrinsic property of the strip. In contrast, neurogenic migrating motor complexes are constant all along the mouse colon (Brierley et al. 2001). The origin of LF contractions is still unknown but two hypotheses are plausible: (i) LF contractions might be originated by the ICC-AP network and/or by (ii) a stretch-activated mechanism, which might be partially ICC independent.

The hypothesis that the ICC-AP network originates LF contractions is supported by the fact that dissection of the submucosa and the associated ICC-SMP network preserves the LF activity. Thus, we could suggest that LF contractions cannot be originated by ICC-SMP. Regarding the differences in frequency found along the colon, our hypothesis is that the higher frequency of LF contractions found in proximal colon strips might be related to the greater number of ramifications found in *c-kit* positive cells at the AP from this colonic region. An increment in the number of contacts can

facilitate the rate of depolarization of smooth muscle cells and increases the frequency of contractions. In this case, a relationship between morphology and function can be established if we assume that LF contractions originate from the ICC-AP network.

The second hypothesis is that a stretch mechanism is the origin of this activity as it has been shown in the pig colon (Huizinga et al. 1983). This might be due to 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). It is possible that distension produced by pellets *in vivo* induces this motility pattern but this needs further investigation. However, preliminary data from our laboratory show that stretch does not modify the frequency of spontaneous activity in human colonic strips, suggesting that stretch does not originate or modulate this activity in humans.

HF contractions show a steady frequency all along the colon, for both longitudinal and circular orientations. In the Sprague-Dawley colon, after the dissection of the submuscular plexus (and its associated ICC-SMP), only LF contractions are recorded in both muscle layers, suggesting that the pacemaker responsible for HF contractions is the ICC network located at the submucosal border (Pluja et al. 2001). However, it is difficult to explain the presence of HF contractions in longitudinal muscle strips with both AP and SMP preserved. One possible explanation could be that the electrical activity originated in the ICC-SMP reaches the longitudinal muscle, but good coupling through gap junctions between both circular and longitudinal muscle layers has not been demonstrated. However, ICC cells from the canine colon can couple both muscle layers through close appositions (Liu et al. 1998). Another possibility is that mechanical interaction between layers would transfer an active event from one layer to another (Wood & Perkins 1970).

It is interesting to mention that no differences are found in the frequency of both contraction types when comparing strips oriented circularly and longitudinally. This could suggest that the mechanical activity might have a common origin in both muscle layers (ICC hypothesis) or alternatively, two pacemaker systems with the same frequency are affecting both layers simultaneously (Mule et al. 1999). In both cases, circular and longitudinal muscle layers contract synchronously, but simultaneous measurements from both layers are needed to prove it.

Thus, the findings of the current study show that the pattern of motility in the colon is complex. The circular muscle is very active (higher amplitude and frequency) in the proximal colon. This major activity of the circular muscle from proximal colon strips might participate in the movement of pellets and therefore in the propulsion function. In the mid colon, both layers contract spontaneously to help in the mixing, water absorption and propulsion. The longitudinal muscle layer, however, is very active in distal segments to let pellets move towards the rectum. HF contractions are steady along the colon and might be related to mixing and water absorption processes.

Role of L-type calcium channels and K^+ channels in the spontaneous activity

In the small intestine, slow wave activity follows a frequency gradient that decreases distally. These slow waves are insensitive to nifedipine, an L-type calcium channel blocker (Cayabyab et al. 1996). However, in the rat colon, our results show that nifedipine abolish the spontaneous activity (both cyclic depolarizations and slow waves), showing that L-type calcium channels play an important role in the generation of spontaneous activity. A similar result was found in the mice colon. In this case, slow waves were also sensitive to nifedipine (Yoneda et al. 2003). Therefore, it is possible that the mechanism responsible for the slow wave activity varies depending on the area of the gastrointestinal tract.

Potassium channels are crucial in the repolarization phase of slow waves. In the canine circular smooth muscle cells, electrical recordings show that 4-AP increases the plateau phase of slow waves (Thornbury et al. 1992). In the present study, we demonstrate that the non-selective potassium channel blocker 4-AP inhibits repolarization and causes a repetitive spiking activity. However, 4-AP has also pre-junctional effects, which cause the release of inhibitory mediators, as it has been also described in other areas of the gastrointestinal tract (Ohkawa 1984; De Man et al. 1993; Daniel et al. 2000).

It is therefore tempting to speculate that: (i) L-type calcium channels are important in the depolarization of colonic smooth muscle cells and that (ii) potassium channels, probably acting through transient-activated outward currents or

calcium-activated potassium channels, participate in the repolarization of smooth muscle cells. In the rat colon, the ionic basis underlying the slow wave activity and cyclic depolarizations are presently unclear.

Spontaneous electrical and mechanical activity in Ws/Ws and +/+ rats

In order to evaluate the role of ICCs in the spontaneous activity, we have performed a study on Ws/Ws and +/+ rats. It is interesting to notice that +/+ rats present a similar pattern of spontaneous activity than Sprague-Dawley rats including: (i) two types of electrical activities that trigger HF and LF contractions; (ii) a myogenic origin; (iii) a gradient of the amplitude values; (iv) a frequency gradient of LF contractions and (v) a steady frequency of HF contractions. These results show that the properties of spontaneous electrical and mechanical activities are identical in Sprague-Dawley and +/+ rats.

In contrast, Ws/Ws rats show an impairment of the regular motility pattern. In these mutants, the electrical pattern is impaired and consists of an amount of uncoordinated spikes without a regular pattern. Only one of the six mutants shows a more regular electrical pattern. Mechanical recordings from mutant rats consist of an amount of irregular contractions without a clear difference between HF and LF contractions. In these animals, smooth muscle contractile activity is preserved but it has become irregular. In a previous study performed on Ws/Ws rats, rhythmic spontaneous contractions were recorded in colonic strips (Yoneda et al. 2001). These rhythmic spontaneous contractions resemble the irregular pattern of muscle contractions described in the present work. These data demonstrate that the presence of two ICC networks is crucial to the pacemaker activity.

The presence of *c-kit* negative interstitial cells in Ws/Ws rats has been suggested as responsible for the residual motor pattern (Yoneda et al. 2001). In the stomach of Ws/Ws rats, *c-kit* negative interstitial cells were also characterized using electron microscopy and were considered fibroblast-like cells (Ishikawa et al. 1997). These cells did not present the accumulation of abundant mitochondria found in normal ICCs and consequently, these cells lacked the intracellular pacemaker machinery. Therefore, it is

possible that the reduction in *c-kit* positive cells contributes to the alteration of the pacemaker system.

Inhibitory neurotransmission in the rat colon

Regulation of smooth muscle excitability is provided by non-adrenergic, non-cholinergic (NANC) inhibitory motor neurons. Stimulation of these neurons elicits hyperpolarization of post-junctional smooth muscle membranes, termed inhibitory junction potentials (IJPs), and causes smooth muscle relaxation. The study of neuronal nitric oxide synthase (nNOS) positive neurons is included in the present work because NO is known to be an important inhibitory neurotransmitter of the gastrointestinal tract (Bult et al. 1990). In Sprague-Dawley, Ws/Ws and +/+ rats, nNOS positive cells are found in the colon between circular and longitudinal muscle layers forming a network of nerve strands and ganglia at the level of AP. The major density of nitrergic neurons is found in the mid colon. Comparing the density of nNOS positive neurons between Ws/Ws and +/+ rats, a significant decrease in the number of nNOS positive neurons is estimated in the mid and distal colon (35% and 29% respectively) in mutants. These results were not expected at the beginning of the experiments, but the decrease in the density of nNOS positive cells in mutant rats could be a possible explanation for the reduction in the second component of the inhibitory junction potential (IJP) found in these animals.

To functionally characterize the neurotransmission, we have used three different approaches: (i) we characterized the IJP with the microelectrode technique; (ii) we studied the inhibition of the spontaneous motility due to electrical field stimulation (EFS) and (iii) we evaluated the presence of a functional neural tone (the second and third approaches were performed with muscle bath technique).

(i) *Characterization of the inhibitory junction potential (IJP)*

Previous works in Sprague-Dawley rats showed that electrical field stimulation (EFS) of the colon elicits a biphasic IJP (Pluja et al. 1999). In Sprague-Dawley rats, IJPs present

two components: an apamin-sensitive fast component and an L-NNA-sensitive sustained component. The fast component is probably ATP-mediated, whereas the sustained component is due to the release of NO from enteric motor neurons (Pluja et al. 1999). Our data show similar results in +/+ rats. To check the hypothesis that ICCs are interface elements between nerve endings and smooth muscle cells, we studied the neurotransmission in Ws/Ws rats, which allegedly lack *c-kit* positive ICC-IM cells. Our results show that: (i) IJPs can be elicited in the absence of *c-kit* positive cells; (ii) IJPs present two components, suggesting a functional cotransmission between ATP and NO and (iii) a reduction in the duration of the second IJP component is observed in Ws/Ws colonic strips. Assuming that the fast component is mediated by ATP, purinergic neurotransmission is independent of *c-kit* positive ICC-IM, as observed in the stomach of WWv mice (Hirst et al. 2002). A role for ICC-IM in NO-mediated inhibition cannot be excluded since the sustained component of the IJP is reduced in mutant rats and this could be related to the important reduction in the number of *c-kit* positive ICC-IM. However, the reduction in the second IJP component in mutant rats could be also related to the decrease in the number of nNOS positive cells found in these rats. In other species, such as WWv mice, the absence of ICC-IM caused impairment of inhibitory neurotransmission (Burns et al. 1996; Beckett et al. 2002). Further experiments will be required to determine whether any of these hypotheses are responsible for the reduction in the second component of the IJP in Ws/Ws rats.

(ii) *Characterization of the mechanical relaxation induced by electrical field stimulation (EFS)*

Electrical field stimulation (EFS) inhibits the spontaneous motility due to the release of several mediators, such as NO and ATP (Pluja et al. 1999). In this study we find that LF and HF contractions are also differently influenced by neural inputs in Sprague-Dawley rats. In this sense, HF contractions are quite constant and poorly modified by EFS. In contrast, LF contractions are abolished by EFS. In +/+ and Ws/Ws rats, EFS induces a decrease in the spontaneous motility due to the release of ATP and NO. These results

demonstrate a functional neurotransmission in the colon that lacks *c-kit* positive ICC cells.

(iii) *Characterization of the neural tone*

The spontaneous mechanical activity is inhibited due to the presence of a neural tone that causes the ongoing release of inhibitory mediators. This can be demonstrated by perfusion of the strip with the neural blocker TTX, which causes an enhancement (higher amplitude and frequency) of the spontaneous motility. Moreover, apamin and L-NNA induce a similar effect, suggesting that the neural tone is caused by the release of both ATP and NO, respectively (Pluja et al. 1999). However, the presence of a neural tone varies according to the following parameters: (i) the contraction type, i.e. LF and HF contractions; (ii) the layer studied, i.e. circular vs. longitudinal and (iii) the segment studied, i.e. proximal, mid and distal colon. Consequently, in order to elucidate the role of ICCs cells in neurotransmission, we have studied the presence of a neural tone in Ws/Ws rats, which present a reduction in some types of ICCs.

In Sprague-Dawley rats, LF contractions are more influenced by a neural tone than HF contractions, showing that cyclic depolarizations can be more easily influenced by neural inputs than slow wave activity. Our studies in Sprague-Dawley rats show that circular and longitudinal muscle layers are differently influenced by the tonic release of inhibitory neurotransmitters. TTX, L-NNA and apamin increase the amplitude of LF contractions in the circular muscle layer to a major extent than in the longitudinal muscle layer. This is also described by other works in the rat proximal colon (Mule et al. 1999). This result is in agreement with our findings of a major presence of nNOS positive neurons in the circular than in the longitudinal muscle layer. Moreover, circular muscle strips show a higher inhibitory tone in the mid colon compared to proximal and distal segments. This is consistent with a highest density of nNOS positive neurons found in the mid colon. Previous works described regional differences in the nitrergic innervation in the rat colon (Takahashi & Owyang 1998). According to these results we can conclude that a neural tone is present in the colon of Sprague-Dawley rats but: (i) it influences the circular muscle layer to a major extent than the longitudinal muscle layer;

(ii) it affects the mid colon to a major extent than the proximal or distal colon and (iii) it modulates LF contractions to a major extent than HF contractions. These differences are due to a different inhibitory innervation (such as nNOS positive neurons) described in the present study and the ability to influence the spontaneous activity (HF vs. LF contractions).

We have studied the neural tone both in +/+ and Ws/Ws rats only in the circular muscle layer. Control rats show an ongoing neural inhibitory tone, which reduces the spontaneous activity. Due to previous results, we have studied the presence of the neural inhibitory tone by the analysis of LF contractions. Similarly to the results from Sprague-Dawley rats, we find a prominent neural inhibitory tone in the mid colon of +/+ rats. Ws/Ws rats present an inhibitory neural tone since perfusion of TTX increased the spontaneous irregular spontaneous activity. As we observed in Sprague-Dawley rats, a highest density of nNOS positive cells is observed in the mid colon of Ws/Ws and +/+ rats. This result is consistent with the presence of a neural inhibitory tone in the absence of *c-kit* positive ICCs.

Are ICCs involved in neurotransmission?

The results of the present work show that despite a strong reduction in *c-kit* positive interstitial cells in Ws/Ws rats: (i) IJPs and mechanical relaxation can be elicited by electrical field stimulation; (ii) a functional neural tone is present and (iii) both NO and ATP pathways are still present in Ws/Ws animals. Thus, three major hypotheses can be attributable to these results:

1. The first possibility is that ICCs are poorly involved in NO and ATP mediated neurotransmission. This is supported by structural data where direct and indirect (i.e., via ICCs) communication between nerve endings and smooth muscle cells has been described in the rat stomach (Mitsui & Komuro 2002). In this case, a double pathway (direct and indirect) exists and functional neurotransmission might be present despite the absence of ICCs. According to this hypothesis, ATP-mediated neurotransmission is functional in W/Wv mouse stomach, which lack ICC-IM (Hirst et al. 2002).

2. The second hypothesis is that *c-kit* negative interstitial cells mediate the interaction between nerve endings and smooth muscle cells. In this sense, *c-kit* negative interstitial cells were characterized in the stomach of Ws/Ws rats and were considered fibroblast-like cells. These cells do not present the accumulation of abundant mitochondria found in normal ICCs but, unlike fibroblasts, they have multiple gap junctions with smooth muscle cells. *c-kit* negative interstitial cells can participate in neurotransmission because: (i) cell to cell communication due to the presence of gap junctions that couple these cells to smooth muscle cells has been described (Ishikawa et al. 1997) and (ii) close associations between *c-kit* negative interstitial cells have been reported (Ishikawa et al. 1997). In consequence, a putative role in neurotransmission can be attributable to *c-kit* negative interstitial cells.

3. The third possibility involves a change in the phenotype due to the mutation with upregulation of certain receptors that accomplish a proper relaxation of the muscle. In this sense, upregulation of P2Y receptors has been described in the fundus of WWv mice, which lack ICC-IM (Sergeant et al. 2002). Consequently, supersensitivity to electrical field stimulation has been reported. In our work, we do not measure receptors involved in relaxation but nNOS positive neurons are still present in Ws/Ws rats. In contrast, the reduction observed in nNOS positive neurons can explain the reduction in the second component of the IJP. In the rat colon, supersensitivity to EFS is not observed but we cannot rule out a change in the expression of receptors involved in the inhibitory pathway.

Conclusions

In summary, the findings of the current study show the presence of a regular spontaneous electrical activity characterized by slow waves superimposed with cyclic depolarizations. These electrical events, found in Sprague-Dawley and +/+ rats, are the origin of high frequency (HF) and low frequency (LF) contractions, respectively. L-type calcium channels play an important role in the generation of this activity. Moreover, the non-selective potassium channel blocker 4-AP inhibits repolarization and causes repetitive spiking activity, suggesting that potassium channels are important in

rhythmicity. HF and LF contractions vary in relation to the orientation of the muscle strip (circular or longitudinal orientation) and the segment studied (proximal, mid and distal colon). The mechanical properties of each strip are responsible for the motor events that ensure transit, propulsion and mixing. The origin of both contraction types is myogenic and suggests the presence of two pacemakers that might be related to the ICC distribution. HF contractions might be originated at the ICC-SMP network that is distributed at equal density (between 50 and 60 cells/mm^2) along the colon. 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. In Ws/Ws rats, the density of *c-kit* positive cells is markedly reduced. Ws/Ws mutant rats show an impairment of the electrical and mechanical patterns and present irregular action potentials, which cause uncoordinated contractions. These results suggest that the integrity of these two networks of ICCs is necessary to have a regular pattern of spontaneous activity.

Regarding neurotransmission, electrical field stimulation causes an IJP and mechanical relaxations in colonic circular muscle cells. Circular smooth muscle cells are under inhibitory neural tone. Inhibitory neurotransmission probably involves nitric oxide and apamin-sensitive mediators, such as ATP. These results are observed in the three groups of animals: Sprague-Dawley, Ws/Ws and +/+ rats. However, Ws/Ws rats, show a reduction in the duration of the IJP. This might be due to a decrease in the number of *c-kit* positive interstitial cells or to a reduction in the density of nNOS positive neurons observed in Ws/Ws rats compared to control rats. We hypothesize that the presence of an inhibitory neurotransmission in Ws/Ws animals, which lack *c-kit* positive ICC-IM, might be due to the presence of *c-kit* negative interstitial cells; or that *c-kit* positive ICC cells are not essential for neurotransmission or alternatively, a change in the phenotype of Ws/Ws animals occurs.

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Conclusions

1. The rat colon shows a regular pattern of spontaneous electrical activity characterized by slow waves superimposed with cyclic depolarizations. This electrical activity triggers two types of spontaneous contractions termed high frequency (HF) and low frequency (LF) contractions, respectively. The origin of both contractions is myogenic and evidences the presence of two pacemakers that might be related to the interstitial cells of Cajal (ICC) distribution. HF and LF contractions vary in relation to the orientation of the muscle strip (circular or longitudinal orientation) and the segment studied (proximal, mid and distal colon). The different mechanical properties of each strip are responsible for the motor events that ensure transit, propulsion and mixing along the colon.

2. Two networks of ICCs are found along the proximal, mid and distal colon at equal density: (i) between 50-60 cells/mm^2 near the submuscular plexus (ICC-SMP) and (ii) about 100 cells/mm^2 at the Auerbach's plexus (ICC-AP). Intramural ICCs (ICC-IM) run between and parallel to smooth muscle cells. Similar results were obtained both in Sprague-Dawley and +/+ rats. In contrast, a strong reduction in *c-kit* immunoreactivity was found in Ws/Ws rats. This demonstrates that Ws/Ws rats are a suitable model to study the involvement of *c-kit* positive ICCs in the pacemaker activity and neurotransmission.

3. The electrical and mechanical activities found in +/+ rats are very similar to those described in Sprague-Dawley rats. In contrast, Ws/Ws mutant rats present an impairment of the electrical and mechanical patterns. The smooth muscle spontaneous activity consists of irregular action potentials that cause uncoordinated contractions. Thus, the presence of two ICC networks is necessary to coordinate motility patterns: (i) the ICC-SMP network is probably responsible for the slow wave activity that originates HF contractions and (ii) the ICC-AP network might be responsible for the cyclic depolarizations that trigger LF contractions or alternatively, this contraction type

is an intrinsic property of the smooth muscle itself (i.e., stretch/distension activated mechanism).

4. Nitric oxide synthase (nNOS) positive neurons form a network of nerve strands and ganglia at the AP region. Fine fibers run parallel to smooth muscle cells innervating to a greater extend the circular muscle layer. The mid colon is the most innervated segment (about 130 cells/mm^2). A slight reduction in nNOS positive neurons is observed in Ws/Ws rats.

5. Electrical field stimulation causes a biphasic inhibitory junction potential (IJP) and mechanical relaxation due to the release of inhibitory neurotransmitters such as ATP and NO. In Ws/Ws rats, IJPs and mechanical relaxations can be elicited despite a strong reduction in *c-kit* positive ICC-IM. However, Ws/Ws rats show a reduction in the second component of the IJP that might be due to a decrease: (i) in the nitrergic innervation and/or (ii) in *c-kit* positive ICC-IM. Assuming that the fast IJP is mediated by ATP, the present study shows that purinergic neurotransmission is likely independent of *c-kit* positive ICC-IM.

6. A functional neural tone due to the ongoing release of ATP and NO is present in the three groups of animals: Sprague-Dawley, Ws/Ws and +/+ rats.

7. The presence of NO and ATP mediated inhibition, despite a strong reduction in *c-kit* positive cells, suggests the following hypotheses: (i) *c-kit* positive ICC-IM might not be essential for neurotransmission; (ii) *c-kit* negative interstitial cells might mediate nerve-muscle interaction or (iii) a change in the phenotype in Ws/Ws animals may occur.

8. The non-selective potassium channel blocker 4-AP has a dual effect on Sprague-Dawley colonic smooth muscle cells: (i) a pre-junctional effect that causes the release of nitrergic neurotransmitters from nerve endings and (ii) a post-junctional effect that delays repolarization of smooth muscle cells and leads to repetitive spiking activity.

9. The study of the pacemaker activity and neurotransmission in animal models with specific mutations is an essential tool that can contribute to a better understanding of the human colonic motility in health and disease.