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Chapter 3

Interstitial cells of Cajal are involved in the
afferent limb of the RAIR

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submitted

Abstract

Background

Interstitial cells of Cajal (ICC) have been shown to be involved in nitrergic neurotransmission of the lower esophageal sphincter and pylorus.

Aim

Here, we studied the role of ICC and nitric oxide (NO) in the inhibitory neurotransmission of the murine internal anal sphincter (IAS).

Methods

The rectoanal inhibitory reflex, rectal compliance and relaxation of isolated IAS to electrical stimulation were measured in controls, KIT^W / KIT^{Wv} mice and neuronal NO synthase (nNOS)-deficient mice. In addition, we evaluated the effect of blockade of nNOS using L-NAME. The distribution of nNOS positive neurons and ICC in the IAS was assessed immunohistochemically.

Results

KIT-positive ICC were present in a dense network in the IAS of controls, but not in KIT^W / KIT^{Wv} mice. Relaxation of IAS muscle strips induced by electrical stimulation was diminished in nNOS^{-/-} mice, but not in KIT^W / KIT^{Wv} mice. Blockade of NOS reduced the relaxation of IAS muscle strips in both mice. Relaxation of the IAS to rectal distension was significantly diminished in KIT^W / KIT^{Wv} mice and nNOS-deficient mice. In concert, *in vivo* blockade of NOS attenuated the relaxation of the IAS in controls. No significant difference in compliance was found.

Conclusion

The inhibitory innervation of the IAS and the RAIR are mediated by NO and a RAIR requires an intact network of ICC in the IAS. Thus, both a loss of nitrergic innervation and a deficiency of ICC lead to an impaired anal relaxation and may play an important role in rectal evacuation disorders.

Introduction

Normal defecation depends on the complex interplay between colonic motility, rectal sensation and anal sphincter function. When stool arrives in the rectum, filling of the rectum leads to stretch of the rectal wall with subsequent triggering of a transient relaxation of the internal anal sphincter (IAS), also called the rectoanal inhibitory reflex (RAIR). Abnormalities in this RAIR have been shown to be involved in defecation disorders and severe constipation as in Hirschsprung's disease^{1,2}.

The RAIR is a motor pattern mediated by intrinsic enteric nerves. Previous animal studies have shown that the inhibitory neurons relaxing the IAS mainly release nitric oxide (NO)^{3,4}. Blockade of the NO biosynthesis reduced the relaxation of muscle strips of the IAS and impaired the RAIR^{3,5}. Recently, evidence was provided that the nitrergic innervation is dependent on the presence of an intact network of interstitial cells of Cajal (ICC)^{6,7}. ICC function as pacemaker cells coordinating the electromechanical activity of the gut^{8,9}. In addition, ICC located in the muscular layer have been shown to mediate the nitrergic neurotransmission in the stomach and lower esophageal sphincter (LES)⁷. To what extent ICC are also involved in the nitrergic innervation of the IAS is unknown. Therefore the present study was designed to evaluate the role of ICC in the nitrergic innervation of the IAS and in the triggering of the RAIR.

Materials and Methods

Animals

Adult KIT^W / KIT^{Wv} mice (20 - 30 g) and their wild-type ($KIT^W / +$) controls as well as nNOS-deficient mice (18-28 g) and their respective wild-type controls (site-bred C57BL/6J) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). For immunohistochemical studies, transgenic mice were used that carry the KIT^{W-lacZ} allele ("lacZ-positive controls")¹⁰⁻¹³. KIT^{W-lacZ} / KIT^{Wv} have a lack of ICC¹⁴, similar to the more extensively studied KIT^W / KIT^{Wv} animals^{9,15}, and will be referred to as "ICC-deficient animals".

All animals were maintained under controlled conditions and were used at 8-12 weeks of age. Experiments were approved by the Ethical Animal Research Committee of the University of Amsterdam, The Netherlands, and by the Faculty Committee for Use of Laboratory Animals of the Faculté de Médecine, Université Libre de Bruxelles, Belgium.

Immunohistochemistry

Fresh frozen anorectal specimen of control and KIT^W / KIT^{Wv} mice were harvested for longitudinal cryosections, while paraformaldehyde-fixed specimen of control ($KIT^{W-lacZ} / +$) and KIT^{W-lacZ} / KIT^{Wv} mice were harvested for transverse and longitudinal cryosections as well as for whole mount preparations, as described previously^{12,13}.

Whole mounts - preparation and immunohistochemistry

After preincubation with 10% normal mouse serum, samples were incubated overnight with the KIT goat and the nNOS rabbit antisera (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in Tris buffered saline-Triton-X (TBS-TX) containing 1% normal horse serum (NHS), rinsed in TBS, incubated in the dark for 1 hour at Room Temperature (RT) in TBS containing donkey anti-rabbit antiserum coupled to FITC (Jackson ImmunoResearch Laboratories, West Grove, PA) and biotinylated donkey anti-goat antiserum (Jackson) diluted 1/200 and 1/100 (v/v) respectively, rinsed in TBS and finally incubated in the dark for one hour at RT with Streptavidin-Texas-Red (Jackson) diluted 1/400 (v/v) in TBS. Specimens were then transferred onto a glass slide coated with 0.1% Poly-L-lysine and were cut into two equal parts. Coverslips were mounted with "Slow Fade Light" antifade mounting medium

(Molecular Probes, Eugene, OR) before viewing on a confocal microscope (MRC 1024, Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) fitted on an inverted microscope equipped with a Plan-Neofluar 40x/1.3 oil immersion objective (Axiovert 100, Zeiss, Oberkochen, Germany). As KIT-ir appeared fairly weak in ICC-deficient animals with the fluorescent detection system, the more sensitive detection system using peroxidase was additionally applied.

Sections – preparation and immunohistochemistry

Specimens were fixed overnight in fresh 4 % paraformaldehyde solution in PBS, pH 7.4 at 4°C, cryopreserved in graded solutions of sucrose (10-30 %), embedded in Tissue-Tek OCT compound (Miles, Elkhart, IND.), oriented either transversely or longitudinally, and snap-frozen in 2-methyl butane at -80°C. Sections (15 μ m thick) were cut on a cryostat and mounted on slides coated with 0.1 % poly-L-lysine.

After preincubation with NHS the sections were exposed overnight at RT to KIT goat antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1/500 in TBS-TX containing 1% NHS, rinsed in TBS, incubated for 1 hour at RT in TBS containing biotinylated donkey anti-goat antiserum (Jackson) diluted 1/200 (v/v) followed by incubation for 1 hour at RT in a solution containing an avidin biotin-complex (ABC Vectastain®, Vector, Burlingame, CA). The immunoreaction was visualized with the chromogen 3,3'-diaminobenzidine (DAB) (Dako, Denmark).

Xgal histochemistry on *KIT*^{W-lacZ} / + and *KIT*^{W-lacZ} / *KIT*^{Wv} transgenic mice was performed as previously reported¹⁰.

In vitro studies

Organ bath Experiments

KIT^W / *KIT*^{Wv} mice, nNOS^{-/-} and control mice were killed by cervical dislocation. The perianal skin was excised and the anus and terminal rectum were quickly removed and placed in Krebs-Ringer's solution (KRS) (mmol/L: NaCl 118.3, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25, glucose 11.1). The solution was maintained at 37°C and aerated with a mixture of 95% O₂ and 5% CO₂.

A ring segment comprising 2 mm of the terminal rectum including the anal orifice was removed and opened. One circular muscle strip was cut and mounted in an organ bath (25 mL) filled with KRS. Each muscle strip was connected to a metal rod and attached to a strain gauge transducer (Statham UC2, Quincy, MA)

for continuous recording of the isometric tension. A resting force of 600 mg was applied on the strips. Platinum electrodes were placed parallel to the tissue in the organ bath for application of electrical field stimulation. Electrical impulses (0.25 – 8.0 Hz, 1 ms, pulse trains lasting 10 s) were provided by a Grass stimulator (Quincy, MA) and a direct current amplifier. Nonadrenergic noncholinergic (NANC) conditions were obtained by the addition of 1 μ mol/L atropine and 1 μ mol/L guanethidine. After each contraction, the muscle strips were washed at least three times every 5 min.

In a first series of experiments, the effect of electrical stimulation was investigated and the effect of sodium nitroprusside (SNP, 2.5 mmol/L) and 5'-adenosine triphosphate (ATP, 1.0 mmol/L) were tested on the circular muscle strips of the murine IAS.

In a second series, the effects of N-nitro-L-arginine methyl ester (L-NAME, 200mmol/L), the blocker of the neuronal conductance tetrodotoxin (2.0 mmol/L), the blocker of the P₂ purinergic receptor suramin (100mmol/L), and the combination of L-NAME and suramin were investigated on the responses to electrical impulses, to ATP (1.0 mmol/L) and SNP (2.5 mmol/L). L-NAME and suramin were added at least 20 minutes before experimentation.

In vivo studies

Anorectal Manometry

Anorectal perfusion manometry was performed with a purpose-built micro-manometric anorectal catheter (outer diameter 0.8 mm). The polyimide catheter incorporated an array of 4 side-holes spaced 0.5 mm apart for measurement of anal sphincter pressure and basal pressure within the rectum. All side-holes were perfused with sterile degassed water at a rate of 0.02 mL/min. A polyethylene balloon was located proximally to the catheter to distend the rectum.

Protocol

Anorectal manometry was performed in anesthetized mice. Mice were anesthetized by an intraperitoneal (i.p.) injection of a mixture of fentanyl citrate / fluanisone (Hypnorm; Janssen Pharmaceuticals, Beerse, Belgium) and midazolam (Dormicum; Roche, Mijdrecht, The Netherlands) at 0.07 mL/10g (Fentanyl 0.32 mg/ml, fluanisone 10 mg/ml, midazolam 5 mg/ml). The catheter was positioned with at least 2 side-holes straddling the anal sphincter high-pressure zone and 1 side-hole in the rectum.

After positioning, basal anal sphincter pressure was recorded. Thereafter a polyethylene rectal balloon was introduced and positioned aside the manometric catheter. After an equilibration time of 15 minutes, graded rectal distension of 10 seconds was applied using the polyethylene balloon (0.25 – 0.40 mL air insufflation) to evoke IAS relaxations. Consecutive rectal distensions were performed at 1-minute intervals and distensions were repeated three times at each volume. This distension protocol was repeated after addition of L-NAME (100 mg/kg, i.p.).²³

In a separate series of experiments, rectal compliance was measured with a non-compliant (polyethylene) balloon with a maximum capacity of 0.45 mL. The balloon was fixed on a silicone catheter with a diameter of 1 mm. This catheter was connected to a pressure transducer measuring intra-balloon pressure during stepwise volume-controlled distension (0.04-0.40 mL).

Data analysis

Basal IAS pressures were obtained from the baseline period preceding the distension protocol. A drop in anal sphincter pressure of at least 5 mmHg during 2 to 5 seconds was identified as a RAIR. If rectal distension resulted in a RAIR, the amplitude of each relaxation was determined as the percentage decrease of basal pressure. This was calculated from the mean anal sphincter pressure before distension to the minimal anal sphincter pressure during the reflex. The duration of the reflex was measured from the onset of the relaxation until the pressure returned to its basal level.

Chemicals

All chemicals were from Sigma (St. Louis, MO), unless otherwise stated. The following drugs were used: ATP; L-NAME; suramin; tetrodotoxin and SNP. All chemicals were dissolved in distilled water and diluted in Krebs-Ringer solution to the stated final concentrations.

Statistical Analysis

Group data are expressed as mean \pm SEM. Differences in the data were all evaluated by Mann-Whitney U test or Wilcoxon signed ranks test were indicated. $P < 0.05$ was considered as statistically significant. The n values reported refer to the number of animals used in each protocol.

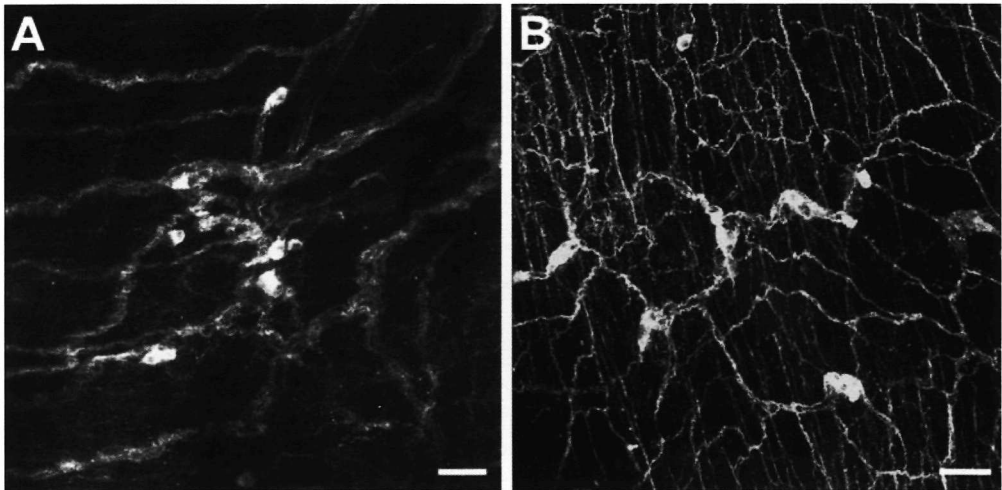
Results

Immunohistochemistry

nNOS-ir in the region of the IAS

In both control ($KIT^{W-lacZ} / +$) (not shown) and KIT^{W-lacZ} / KIT^{Wv} mice (figure 1 a & b), a dense network of nNOS-ir nerve fibers was present in the circular and longitudinal muscle layers of the distal rectum including the IAS. Within the intermuscular plane of the IAS both nNOS-ir neurons and nerve bundles were present but the myenteric ganglia contained fewer neurons and the nerve bundles were coarser when compared to the distribution in the proximal rectum (not shown). Conversely, the density of nNOS-ir nerve fibers and neurons of the submucosal plexus within the region of the IAS was similar to the proximal rectum.

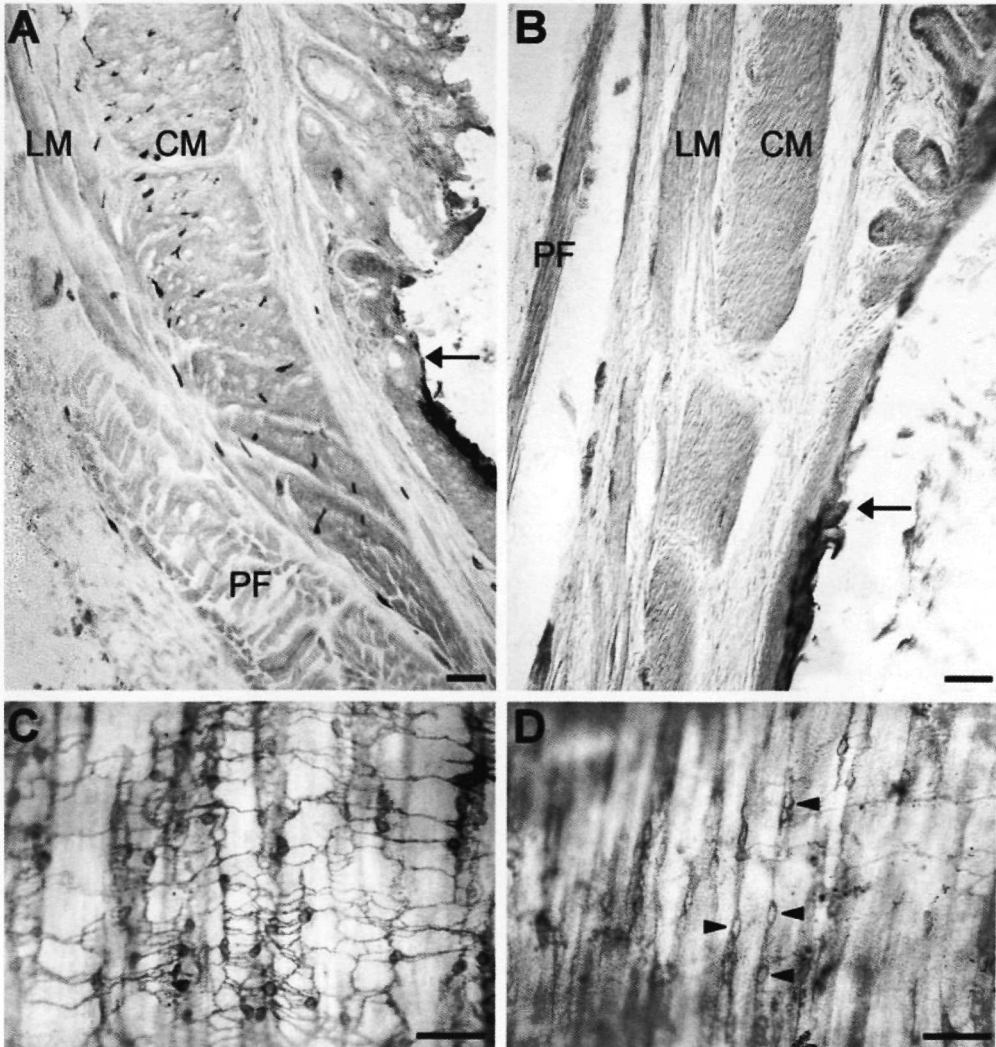
Figure 1. nNOS-ir distribution in the anorectum of a KIT^{W-lacZ} / KIT^{Wv} ICC-deficient mouse. **A:** 8 μ m thick projection of the myenteric region, showing a few nNOS-ir myenteric neurons and numerous large nerve bundles. **B:** 16 μ m thick projection of the circular muscle layer and submucosal plexus, showing abundant nNOS-ir nerve fibers in the circular muscle layer and submucosal nNOS-ir fibers and neurons. Orientation: oral: left, aboral: right. Scale bars: 50 μ m.



KIT-ir in the region of the IAS

In controls, (figure 2 a) spindle shaped KIT-ir ICC were present in both muscle layers, parallel to the smooth muscle cells. Stellate KIT-ir ICC were abundant surrounding the myenteric ganglia. At the level of the submuscular plexus, KIT-ir ICC formed chains parallel to the circular muscle layer. These chains connected each other by numerous delicate longitudinally processes. ICC at the submuscular

Figure 2. Distribution of KIT-ir ICC in the IAS region of control $KIT^{W-lacZ} / +$ and ICC-deficient KIT^{W-lacZ} / KIT^{Wv} mice. A, B: longitudinal sections across the IAS region of a control $KIT^{W-lacZ} / +$ mouse (A) and an ICC-deficient KIT^{W-lacZ} / KIT^{Wv} mouse (B). The arrows in the lumen at right indicate the muco-cutaneous transition. The various populations of KIT-ir ICC are abundant in the control (A) while KIT-ir ICC are essentially lacking in the ICC-deficient animal (B). C, D: wholemount preparations showing KIT-ir ICC in the submuscular plexus of a control $KIT^{W-lacZ} / +$ mouse (C) and an ICC-deficient KIT^{W-lacZ} / KIT^{Wv} mouse (D). KIT-ir ICC form chains running parallel to the circular muscle layer and interconnected by multiple processes in the submuscular plexus of a control $KIT^{W-lacZ} / +$ mouse (C), while in the submuscular plexus of an ICC-deficient KIT^{W-lacZ} / KIT^{Wv} mouse (D), chains of KIT-ir ICC appeared only faintly stained (arrowheads) and lack most longitudinal interconnecting processes. Orientation in C, D: oral at left, aboral at right. Abbreviations used: CM, circular muscle layer; LM, longitudinal muscle layer; PF, striated muscle of the pelvic floor. Scale bars : 50 μ m.



plexus exhibited a weaker KIT-ir compared to the other populations of KIT-ir ICC and were best viewed on wholemounts (figure 2 c). In the jejunum of the same animals, KIT-ir was detected in ICC-MP and ICC-DMP, as expected (not shown).

In ICC-deficient animals mice, KIT-ir ICC were lacking in the muscle layers and around the myenteric ganglia (figure 2 b), while faint KIT-ir ICC at the level of the submuscular plexus were still present (figure 2 d). However, they were less abundant and apparently lacked the normal branching pattern (compare with figure 2 c). In the jejunum of these animals a few ICC-DMP exhibited a weak KIT-ir, as expected in these ICC-deficient animals (not shown). Due to the extreme lack of KIT-expressing ICC in the region of the IAS of ICC-deficient animals, no quantification was attempted.

Xgal histochemistry in KIT^{W-lacZ} / KIT^{Wv}

While Xgal histochemistry readily detected the nuclei of KIT-expressing ICC-DMP in the jejunum, no Xgal deposit at all was observed in the proximal rectum, as well as in the region of the IAS, (not shown).

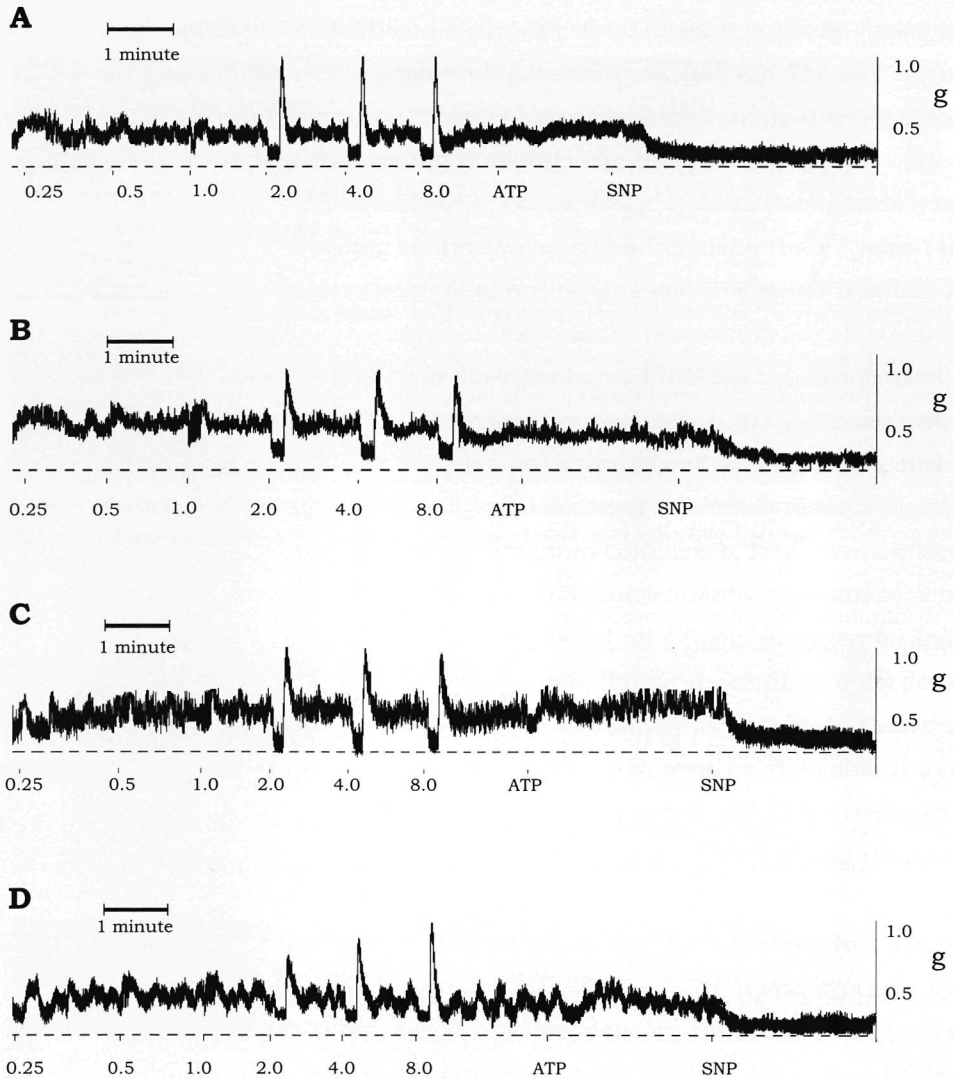
Organ bath studies

Involvement of NO in the inhibitory innervation of the IAS

In the presence of atropine and guanethidine, electrical stimulation (0.25-8.0 Hz, 1 ms, pulse trains lasting 10 s) induced a frequency-dependent relaxation of circular muscle strips of the murine IAS during the period of stimulation followed by an off-contraction (figure 3a and c). The maximal relaxation of KIT^W / + controls was obtained at 4 Hz (0.16 ± 0.05 g) (figure 4a) and of nNOS controls at 2 Hz (0.32 ± 0.14 g) (figure 4b). The resting (active) tone of the muscle strips of KIT^W / + controls (0.5g), KIT^W / KIT^{Wv} mice (0.5g), nNOS control (0.6g) and nNOS^{-/-} mice (0.6g) were comparable.

Both the relaxation and the off-contraction were abolished by tetrodotoxin, a blocker of the neuronal conductance. SNP induced a sustained relaxation (0.15 ± 0.01 g for control mice) of the IAS muscle strips. The relaxation to SNP was resistant to TTX. In contrast to SNP, ATP failed to relax the IAS preparation (figure 3).

Figure 3. Representative recordings showing the NANC inhibitory responses to electrical impulses (0.25 - 8.0 Hz, 1 ms, pulse trains lasting 10 seconds), ATP (1.0 mM) and SNP (2.5 mM) in muscle strips of a KIT^W / + control (panel A), KIT^W / KIT^{Wv} (Panel B), nNOS control (panel C) and nNOS^{-/-} mouse (panel D).



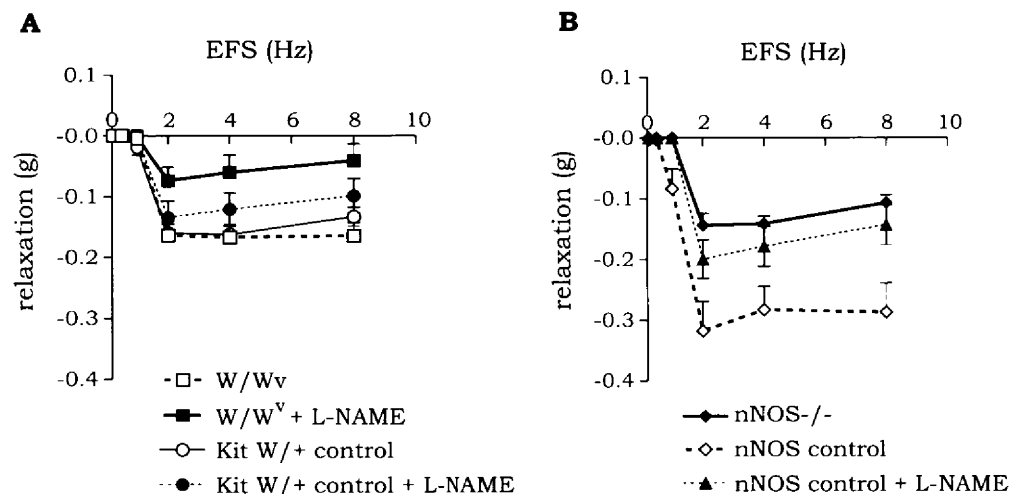
Effect of L-NAME in control mice

Inhibition by L-NAME reduced the relaxations in KIT^W / + controls (n=8, figure 4a) and in nNOS control mice (n=6, figure 4b) ($p < 0.001$). L-NAME had no effect on the relaxations to SNP. The combination of L-NAME and suramin did not further reduce the relaxations n=4) ($p = 0.19$) (data not shown).

Figure 4a. Effect of L-NAME (200 μ mol/L) on relaxations induced by electrical field stimulation in KIT^W / KIT^{Wv} (dotted line, squares) and control mice (straight line, circles).

4b. Relaxations of IAS muscle strips evoked by EFS in control mice (dotted line, diamond) and $nNOS^{-/-}$ mice (straight line, triangles). The addition of L-NAME (200 μ M) reduced the relaxations of WT muscle strips (striped line, triangle).

Values shown are average \pm SEM (n=6) ($p < 0.001$, Wilcoxon Signed Ranks Test).



Studies in $nNOS^{-/-}$ mice

To further evaluate the role of nitrgic neurotransmission in the relaxation of the IAS, mice deficient in $nNOS$ were studied. Electrical stimulation of the IAS of $nNOS^{-/-}$ mice and their respective controls induced a frequency-dependent relaxation during the period of stimulation followed by an off-contraction (figure 3d). However, relaxations were also significantly reduced in $nNOS^{-/-}$ deficient mice compared to their controls (n=6) ($p < 0.001$) (figure 4b).

Evaluation of the role of ICC in the inhibitory innervation of the IAS.

We next determined the NANC inhibitory response of IAS tissue of KIT^W / KIT^{Wv} mice. Electrical stimulation induced a frequency-dependent relaxation of circular muscle strips of the murine IAS during the period of stimulation followed by an off-contraction in KIT^W / KIT^{Wv} mice (figure 3b). The maximal relaxation was obtained at 4 Hz (0.17 ± 0.05 g, n=10) and did not differ from control mice (0.16 ± 0.05 g, n=10). In concert, the amplitude of the frequency-dependent relaxations in KIT^W / KIT^{Wv} mice was similar to their controls (figure 4a). Inhibition by L-NAME reduced the relaxations in KIT^W / KIT^{Wv} mice (n=8) ($p < 0.001$) (figure 4a).

Anorectal manometry

Involvement of NO in the inhibitory innervation of the IAS

The basal IAS pressure in $KIT^W/+$ control mice was 21 ± 3 mmHg ($n=10$) (figure 5) and 21 ± 2 mmHg ($n=10$) for nNOS control mice. Distension of the rectum elicited a volume-dependent relaxation or RAIR. Both in $KIT^W/+$ controls (figure 6) and nNOS controls the duration of the RAIR was independent of distension volume. The average duration was 14 ± 1 and 13 ± 1 s respectively.

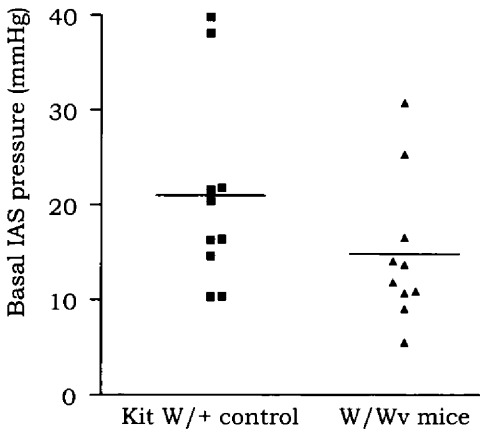


Figure 5. Basal internal anal sphincter pressure of $KIT^W/+$ control mice (squares) and KIT^W/KIT^{Wv} (triangles). Horizontal lines represent the mean values ($n=10$, n.s.).

Effect of L-NAME in control mice

Administration of L-NAME (ip) to nNOS controls reduced significantly the relaxations ($p<0.05$) (figure 7). In $KIT^W/+$ control mice, a comparable reduction was seen after administration of L-NAME (results not shown).

Studies in $nNOS^{-/-}$ mice

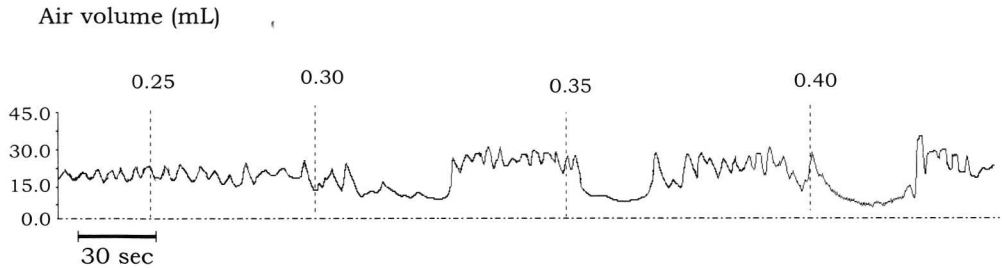
The basal IAS pressure in $NOS^{-/-}$ mice was comparable to their nNOS controls (21 ± 3 and 21 ± 2 mmHg resp.). However, the amplitude of the RAIR was significantly lower in $nNOS^{-/-}$ mice compared to nNOS controls ($p<0.01$) (figure 6 and 7). Notably, the average duration of the RAIR was increased in $nNOS^{-/-}$ mice, compared to their controls (24 ± 3 and 15 ± 1 s respectively, $p<0.01$).

Evaluation of the role of ICC in the inhibitory innervation of the IAS

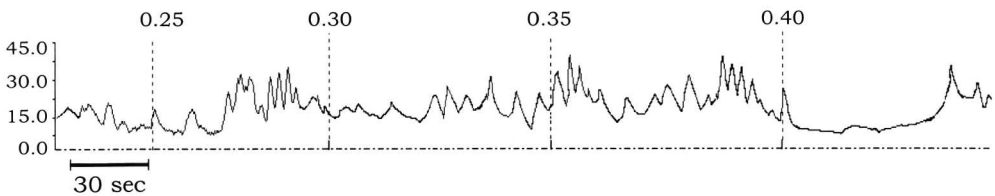
The basal IAS pressure in KIT^W/KIT^{Wv} mice was comparable to controls (15 ± 2 and 21 ± 3 mmHg respectively, $n=10$) (figure 5). Although the magnitude of the basal

Figure 6. Representative *in vivo* anorectal manometry recordings from a $KIT^W/+$ control (A), KIT^W / KIT^{Wv} (B) and $nNOS^{-/-}$ mouse (C). Clear RAIRs are observed upon incremental volumes of air insufflated (0.25-0.40 mL as indicated) in $KIT^W/+$ controls. In KIT^W / KIT^{Wv} mutant mice, aberrant RAIRs are only observed upon distension volumes of 0.40 mL. Clear RAIRs are also seen in $nNOS^{-/-}$ mouse, however, the amplitude of the RAIR was lower in $nNOS^{-/-}$ mice compared to $nNOS$ controls.

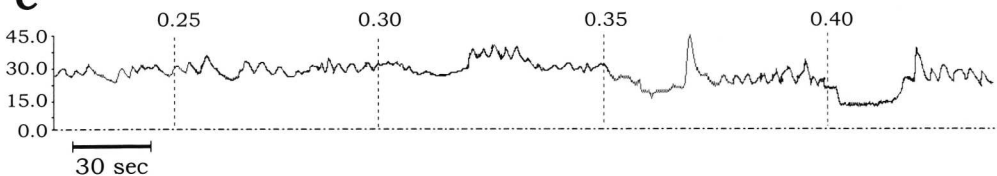
A



B

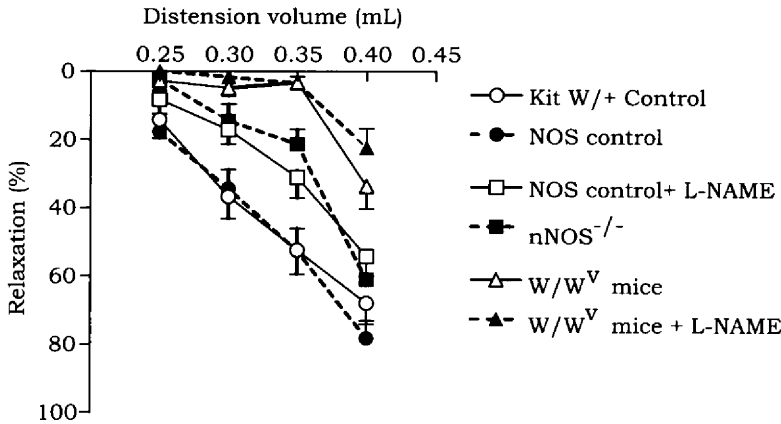


C



pressure was comparable, KIT^W / KIT^{Wv} mice showed a more irregular basal sphincter pressure pattern with twitch contractions compared to controls. Distension of the rectum elicited a volume-dependent relaxation in KIT^W / KIT^{Wv} mice. However, the RAIR amplitude was significantly attenuated in KIT^W / KIT^{Wv} mice compared to $KIT^W / +$ controls (figures 6 and 7). At high distension volume, the RAIR could be observed in KIT^W / KIT^{Wv} mice, though with variable duration and amplitude (figure 6 and 7). Administration of L-NAME (ip) to KIT^W / KIT^{Wv} mice had no effect (figure 7).

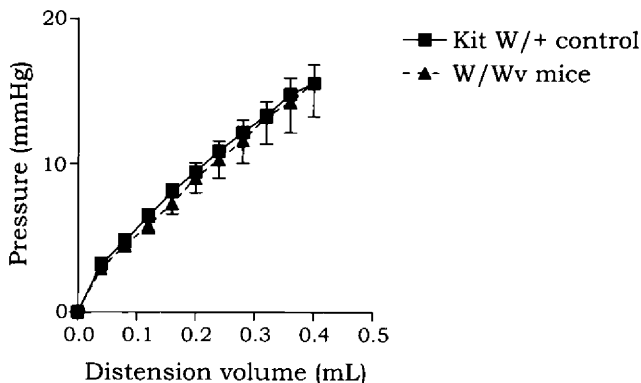
Figure 7. Amplitude of the RAIR in $nNOS^{-/-}$ mice (striped line, closed squares) and KIT^W / KIT^{Wv} (straight line, open triangle) is significantly decreased compared to $nNOS$ control mice (striped line, closed circle) and $KIT^W / +$ control mice (straight line, open circle) ($n=10$) ($p<0.05$, Wilcoxon Signed Ranks Test). Treatment of WT mice with L-NAME (100 mg/kg, ip; straight line, open squares) reduced the occurrence of RAIR to a similar extent. Treatment of KIT^W / KIT^{Wv} with L-NAME (100 mg/kg, ip; striped, closed triangle) slightly reduced the occurrence of RAIR (n.s.). Values shown are average \pm SEM.



Compliance in $KIT^W / +$ control mice and in KIT^W / KIT^{Wv} mice

As the differences in RAIR could result from changes in rectal compliance, we also determined this parameter in both control and KIT^W / KIT^{Wv} mice. Stepwise rectal volume distension revealed a similar increase in rectal pressure, demonstrating that rectal compliance was similar in control and KIT^W / KIT^{Wv} mice (figure 8).

Figure 8. Rectal compliance in KIT^W / KIT^{Wv} (dotted line, triangles) was not different from $KIT^W / +$ control mice (straight line, squares). Values shown are the average \pm SEM ($N=6$).



Discussion

NO is recognized as an important inhibitory neurotransmitter in the IAS. Previous studies showed that blockade of the NO biosynthesis reduced the relaxation of muscle strips of the IAS induced by electrical stimulation and impaired the RAIR^{3,5}. In the present study, we extended the role of NO as an inhibitory neurotransmitter to the murine IAS: blockade of NO biosynthesis resulted in a reduction of both the *in vitro* and *in vivo* relaxation of the IAS. These findings were further corroborated by experiments in nNOS^{-/-} mice. Isolated muscle strips relaxed significantly less whereas the RAIR was impaired compared to controls. It should be emphasized though that the IAS still relaxed in nNOS^{-/-} mice and that blockade of the NO biosynthesis in controls incompletely reduced the electrical stimulation-induced relaxation. These observations suggest that either the nitrgenic blockade was incomplete or, most likely, that another inhibitory neurotransmitter is involved. Previous studies in the rat IAS suggested ATP as a possible candidate¹⁶. However, in our study, high concentrations of ATP failed to relax the IAS. In addition, suramin had no effect on relaxation, excluding the possible involvement of ATP. To what extent VIP is involved remains to be investigated⁵.

ICC have been suggested to play a crucial role in normal gastrointestinal motility. In addition to their role as pacemaker cells^{17,7,9}, previous studies have suggested that ICC function as an intermediate between nerve fibers and smooth muscle cells mediating the nitrgenic inhibitory neurotransmission in the stomach, LES and pyloric sphincter⁶⁻⁸. Due to the absence of the intramuscular type of ICC, LES muscle strips from *KIT^W / KIT^{Wv}* mice contracted rather than relaxed in response to electrical stimulation⁷. Administration of L-NAME did not further change the response⁷. As immunohistochemistry staining showed that the nitrgenic innervation was intact, these findings indicated that ICC are important mediators of the nitrgenic innervation in these tissues. In the present study, we failed to confirm this observation in the IAS. Electrical stimulation of the IAS of *KIT^W / KIT^{Wv}* mice relaxed to the same extent as those from controls. In addition, blockade of NO biosynthesis greatly reduced these relaxations, indicating that the nitrgenic innervation was intact. The latter was further confirmed by immunohistochemical staining. Our results argue against a role for ICC as intermediate between nitrgenic nerves and smooth muscle cells. A similar conclusion was drawn by Sivarao et al.²¹, who showed that relaxations of the LES in response to swallowing or vagal

stimulation was unaffected in KIT^W / KIT^{Wv} mice.

How can the discrepancy between our data and those from Ward et al.⁷ be explained? One could argue that the electrical stimulation parameters used (1ms) were too intense resulting in overflow of NO to the smooth muscle which might still elicit responses in tissue lacking ICC²². However, in preliminary experiments, electrical stimulation with pulse trains of 0.5 ms failed to relax the IAS significantly. Furthermore, 1 ms pulse trains resulted in a normal frequency dependent response curve, excluding this possibility. Another explanation could be that the network of ICC of the IAS in contrast to the LES and pylorus is unaffected in KIT^W / KIT^{Wv} mice. However, similar to Ward et al., immunohistochemical assessment revealed that in KIT^W / KIT^{Wv} and KIT^{W-lacZ} / KIT^{Wv} mice, KIT-ir ICC were completely lacking in the muscle layers and around the myenteric ganglia.⁷ Alternatively, regional differences in the distance between nerve varicosities and smooth muscle cells, or differences in sensitivity of smooth muscle cells to NO could be involved, but clearly this needs to be further explored.

Although we showed that the inhibitory innervation of the IAS in vitro was intact in ICC-deficient mice, the relaxation of the IAS in response to rectal distension was clearly diminished in KIT^W / KIT^{Wv} mice. As it is not clear whether stretch or tension receptors are involved in the triggering of the RAIR, one might argue that changes in compliance could affect the IAS response to rectal distension. The rectal compliance in ICC deficient mice was however identical to that of control mice. This is in contrast with Ward et al. who described changes in gastric compliance in ICC-deficient mice²⁴. This discrepancy can be explained by the fact that in the stomach, ICC have been shown to be involved in nitrergic and cholinergic neurotransmission, most likely contributing to the described changes in compliance in mice lacking ICC. In our experiments, however, we were unable to confirm the involvement of ICC in the nitrergic neurotransmission of the IAS, which may explain why the compliance in the anorectal area is not altered. Our finding that the RAIR is strongly diminished in KIT^W / KIT^{Wv} mice in the presence of an unaltered rectal compliance suggests the involvement of ICC in the triggering of the RAIR.

Theoretically, ICC could play a role in the neural pathway mediating the RAIR either at the afferent limb in the detection of rectal distension, or at the efferent part¹⁸ by mediating the nitrergic neurotransmission¹⁹. Our data favor an involvement of ICC in the afferent limb, as both immunohistochemical and in vitro

studies showed a normal nitrergic neurotransmission in the IAS of KIT^W / KIT^{Wv} mice. One possible explanation could be that ICC act as mechanosensory receptors detecting rectal distension. Previous studies indeed showed that ICC make direct functional contact with intramuscular arrays of enteric nerves^{14, 18} and therefore could be involved in mechanical perception of distension. Alternatively, ICC may be required for the development of afferent nerves, especially as intramuscular arrays of vagal afferent fibers are significantly reduced in density in KIT^W / KIT^{Wv} mice compared to controls. This would suggest that abnormalities in the afferents secondary to the absence of a normal ICC network would underlie the impaired detection of stretch. However, the RAIR is considered to be mediated by a neural circuitry located within the enteric nervous system, and so far, no abnormalities in the enteric sensory neurons have been described in ICC deficient mice. To what extent the RAIR is impaired in KIT^W / KIT^{Wv} mice due to secondary changes in intrinsic sensory neurons or whether the ICC function as mechanoreceptors can not be determined from our experiments.

Impaired or absent relaxation of the IAS hampers the evacuation of stool leading to severe chronic constipation¹. The typical example is Hirschsprung's disease characterized by aganglionosis of the distal gastrointestinal tract with subsequent absence of the RAIR and delayed passage of meconium. Similarly, delayed evacuation of meconium is also present in another subgroup of neonates with abdominal distension and feeding problems²⁰. However, gastrointestinal symptoms resolve soon after birth. The exact reason for this delayed evacuation of meconium is unclear. Interestingly, a study showed that delayed maturation of ICC may lead to transient defecation problems in neonates²⁰. As we showed that a deficiency of ICC impairs the RAIR, a delayed maturation of ICC could be involved in the delayed passage of meconium in neonates. This hypothesis is currently investigated.

In conclusion, we showed that the inhibitory innervation of the IAS and the RAIR are mediated by NO. Moreover, a normal RAIR requires an intact network of ICC in the IAS. Thus, both a loss of nitrergic innervation and a deficiency of ICC lead to an impaired anal relaxation and may play an important role in rectal evacuation disorders.

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