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Cell-specific effects of nitric oxide on the efficiency and frequency of long distance contractions in murine colon

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Abstract

Background: Nitric oxide (NO) mediates inhibitory neurotransmission and is a critical component of neuronal programs that generate propulsive contractions. NO acts via its receptor NO-sensitive guanylyl cyclase (NO-GC) which is expressed in smooth muscle cells (SMC) and interstitial cells of Cajal (ICC). Organ bath studies with colonic rings from NO-GC knockout mice (GCKO) have indicated NO-GC to modulate spontaneous contractions. The cell-specific effects of NO-GC on the dominant pan-colonic propulsive contraction, the long distance contractions (LDCs), of whole colon preparations have not yet been described.

Methods: Contractions of whole colon preparations from wild type (WT), global, and cell-specific GCKO were recorded. After transformation into spatiotemporal maps, motility patterns were analyzed. Simultaneous perfusion of the colon enabled the correlation of outflow with LDCs to analyze contraction efficiency.

Key Results: Deletion of NO-GC in both ICC and SMC (ie, in GCKO and SMC/ICC-GCKO) caused loss of typical LDC activity and instead generated high-frequency LDC-like contractions with inefficient propulsive activity. Frequency was also increased in WT, SMC-GCKO, and ICC-GCKO colon in the presence of L-NAME to block neuronal NO synthase. LDC efficiency was dependent on NO-GC in SMC as it was reduced in GCKO, SMC-GCKO, and ICC/SMC-GCKO colon; LDC efficiency was decreased in all genotypes in the presence of L-NAME.

Conclusions and Inferences: NO/cGMP signaling is critical for normal peristaltic movements; as NO-GC in both SMC and ICC is essential, both cell types appear to work in synchrony. The efficiency of contractions to expel fluid is particularly influenced by NO-GC in SMC.

KEYWORDS
colon, knockout mice, motility, nitric oxide, smooth muscle, spatiotemporal maps

Abbreviations: CMC, colonic motor complex; CMMC, colonic migrating motor complex; cpm, contractions per minute; GCKO, guanylyl cyclase knockout; GI, gastrointestinal; ICC, interstitial cells of Cajal; ICC-GCKO, ICC-specific guanylyl cyclase knockout; L-NAME, L-N^G-nitroarginine methyl ester; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NO-GC, nitric oxide-sensitive guanylyl cyclase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; SMC, smooth muscle cell; SMC/ICC-GCKO, smooth muscle-/ICC-specific guanylyl cyclase knockout; SMC-GCKO, smooth muscle-specific guanylyl cyclase knockout; TTX, tetrodotoxin; WT, wild type.

1 | INTRODUCTION

Contractile activity in the gastrointestinal (GI) tract is known to originate from the combination of pacemaker currents and excitatory and inhibitory innervation. Pacemaker cells generate oscillating membrane potentials called "slow waves." This electrical current spreads from the ICC network to smooth muscle cells (SMC) and promotes and orchestrates smooth muscle contraction. Myenteric ICC (ICC-MP) surround the myenteric plexus ganglia, whereas submuscular ICC (ICC-SMP) are located at the submucosal border of the circular muscle layer.[1-4] ICC-SMP generate slow waves to initiate high-frequency ripples in the circular muscle layer, whereas ICC-MP likely generate the slow phasic contractions in both circular and longitudinal muscle layers.[3,5-7] Both types of contraction occur in the presence of neuronal blockers such as tetrodotoxin (TTX) or hexamethonium indicating their myogenic origin.[8,9]

In the colon, several forms of contraction are generated to mix and propel colonic content. Ripples and slow phasic contractions are responsible for local mixing, whereas the propulsive contractions are generated to propel and, finally, expel feces. In the literature, the nomenclature for propulsive contractions is diverse; commonly used terms are "colonic migrating motor complex" (CMMC),[11,12] "colonic motor complexes" (CMC),[14] or "long distance contractions" (LDC)[15,16] of which the latter will be used in this manuscript to indicate a specific subtype of colonic motor complexes as described by Chen et al.[15] In the murine colon, these contractions occur every 2-4 minutes and show a duration of approximately 40-60 seconds. LDCs are characterized by an initial relaxation followed by a contraction which starts in the proximal part of the colon and propagates in the anal direction for at least two-thirds of the colon length.[11,18,19] A unique feature of LDCs is that the colon remains contracted while the contraction wave travels along the colon. LDCs have a neurogenic component as they are abolished by TTX[11]; they also have a myogenic component since they can be initiated in the presence of TTX by cholinergic agonists.[15,20] LDCs are most likely the mouse equivalent to the high amplitude propagating contractions (HAPC) of the human colon.[21]

Contractile activity is tuned by several excitatory and inhibitory factors. Colonic inhibitory signal transduction is mainly mediated by the neurotransmitter nitric oxide (NO).[15,22,23] Nitrergic nerves are also a critical component of neuronal programs that generate propulsive contractions as observed in the esophagus[24,25] and colon.[15] The main receptor for NO is NO-sensitive guanylyl cyclase (NO-GC).[26,27] Its expression has been shown in several GI cell types such as ICC and SMC indicating participation in the modulation of GI motility.[28,29] Our previous studies using colonic ring preparations provided evidence for an NO-dependent regulation of murine colon contractility[8]: Whereas ripples occur independent of NO, NO-GC in both ICC and SMC is involved in the modulation of slow phasic contractions as well as large neurogenic contractions of colon rings. However, as our previous study[8] evaluated the contractility of small colon rings, the analysis of propagating contractions was not possible. Thus, the present study focuses on the evaluation of NO-GC signaling in preparations of whole colon to allow comparison to the data obtained with colon rings. Therefore, video recordings of colons from global- and cell-specific knockout mice were transformed into spatiotemporal maps, and overall, contraction patterns including LDCs were evaluated. The results reveal a NO-dependent modulation of the LDC: The frequency of LDCs is modulated by NO-GC in both ICC and SMC, whereas the efficiency of LDC is to a large extent tuned by NO-GC in SMC.

Key Points

- Normal gastrointestinal motility involves signaling by nitrergic neurons. Thus, the major nitric oxide (NO) receptor, NO-sensitive guanylyl cyclase (NO-GC), is a key enzyme to study nitrergic modulation of gastrointestinal motility.
- We show that NO-GC in smooth muscle cells and ICC are required for normal peristaltic activity and that NO-GC in smooth muscle cells regulates propulsive efficiency by regulating force of contraction of the long distance contractions (LDCs), the murine equivalent of the high amplitude propagating contraction of the human colon.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

The animal procedures were performed according to the guidelines from directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. All experiments were approved by the local animal care committee.

2.2 | Animals

C57BL/6 mice were housed in standard mouse cages (267 × 207 × 140 mm; maximally 3 animals/cage) with woodchip bedding material and under conventional laboratory conditions (constant room temperature [22°C], humidity level [55%], a 12-hour light; 12-hour dark cycle [lights on at 6 AM]). Global GCKO and wild type (WT) littermates received a fiber-reduced diet containing omeprazole and bicarbonate, whereas all other mice received standard rodent diet (Altromin, Lage, Germany).[26] In order to alleviate the severe gastrointestinal phenotype, GCKO animals and the WT controls received a fiber-reduced diet supplemented with omeprazole and bicarbonate to reduce gastric acid production.[30] This diet strongly increases GCKO survival.

Guanylyl cyclase knockout animals and cell-specific knockout mice for ICC, SMC, and SMC/ICC were generated according to Friebe et al[26] and Beck et al[8] WT siblings were used as controls for GCKO animals (both receiving the same diet). Heterozygous (+/flox) animals

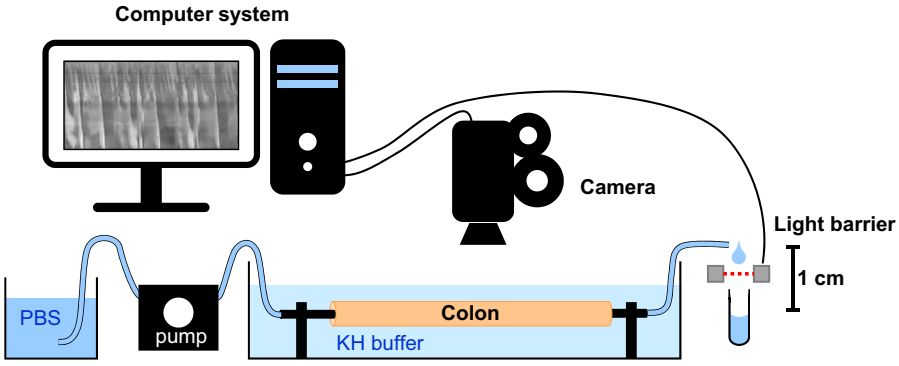

FIGURE 1 Experimental setup for video imaging of whole colon contractility. Colon was mounted in a chamber filled with Krebs-Henseleit (KH) buffer and continuously perfused with PBS. Colonic motility was recorded with a camera, a light barrier system detected outflow drops. Video recordings were transformed into spatiotemporal maps using a computer system with appropriate software

were used as controls for cell-specific KOs (either flox/flox or −/flox); both carried the respective Cre gene and were treated with tamoxifen. Comparison of WT animals with the controls used for the cell-specific KOs (which receive normal rodent chow) did not show any differences in colonic motility; thus, data were combined and indicated as WT. The efficacy of NO-GC deletion in the SMC- and ICC-GCKO has been shown previously.[8,28,31,32] Animals of either sex were sacrificed at an age of 8-16 weeks by cervical dislocation, and colons were isolated. A total of 60 animals were used (22 WT/heterozygous controls, 14 GCKO, 9 SMC-GCKO, 9 ICC-GCKO, 6 SMC/ICC-GCKO).

2.3 | Whole colon preparation

Animals were killed by cervical dislocation. The abdomen was opened, and the whole intestine was quickly removed and transferred to 4°C cold Krebs-Henseleit solution (118 mmol L^{-1} NaCl, 4.7 mmol L^{-1}, KCl, 2.5 mmol L^{-1} $CaCl_2$, 1.2 mmol L^{-1} KH_2PO_4, 1.2 mmol L^{-1} $MgSO_4$, 25 mmol L^{-1} $NaHCO_3$, pH 7.4, 7.5 mmol L^{-1} glucose) bubbled with 95% O_2/5% CO_2. The mesentery was completely removed, and fecal content was gently flushed out. To enable perfusion of the colon, small tubes were fixed at the proximal and distal part of the colon.

Isolated colon was mounted in a chamber filled with 300 mL Krebs-Henseleit buffer which was kept at 37°C and bubbled with 95% O_2/5% CO_2 (Figure 1). Movements of the intestine were recorded using a webcam (Logitech® HD Webcam C525). During the entire experiment, the colon was perfused with PBS (flowrate 30 µL min^{-1}). The outflow tube was fixed 1 cm above colon level. Outflow drops were monitored using a light barrier system. Time points of the outflow drops were documented by a single board computer (Raspberry Pi).

During the equilibration phase (60 minutes), spontaneous contractions developed which started in the proximal part and propagated distally along the whole colon. Videos were then recorded for 30 minutes under control conditions or in the presence of L-NAME (200 µmol L^{-1}), ODQ (10 µmol L^{-1}), TTX (1 µmol L^{-1}) or hexamethonium (100 µmol L^{-1}).

Video recordings were transferred into spatiotemporal maps using a Plugin (gMapsON) for ImageJ written by SP Parsons (http://www.scepticalphysiologist.com/code/code.html). After map generation, time points of outflow drops were marked in the map to correlate them with motility patterns.

2.4 | Materials

ODQ and TTX were purchased from Axxora (Lörrach, Germany). L-NAME, hexamethonium, and tamoxifen were purchased from Sigma (Taufkirchen, Germany).

2.5 | Statistical analysis

For all statistical tests, GraphPad Prism 5.0 for Windows was used. Data are expressed as mean ± SEM. n values represent the number of animals used for the respective analysis. For comparison of independent variables (WT, GCKO, SMC-GCKO, ICC-GCKO, and SMC/ICC-GCKO), Kruskal-Wallis was performed. If P was ≤0.05 for the global test, post hoc Mann-Whitney U test was performed to compare effects between WT and all other knockout mice.

3 | RESULTS

Several types of spontaneous contractions have been described to occur in the colon.[15,33,34] In this study, spatiotemporal maps were generated from video recordings to analyze the contraction patterns of whole murine colon preparations. Figure 2 shows a representative spatiotemporal map from WT colon under control conditions. Colon from WT mice developed three different types of contraction: (1) long distance contractions (LDCs) which initiate at the proximal part of the colon and propagate in anal direction. LDCs are composed of an initial relaxation phase followed by the propagating contraction (see Figure 2A-1); (2) slow phasic contractions which are generated in both the longitudinal and circular muscle layer, thus, appear as V-shaped oscillations in the spatiotemporal maps due to longitudinal muscle movement (see Figure 2A-2); (3) continuous high-frequency ripples (see Figure 2A-3). As previously reported, all three types of contraction can also be recorded with colonic rings in a myography setup (Figure 2B).[8]

In WT colon, LDCs occurred with a frequency of 0.43 ± 0.03 cpm (Figure 3A, Table 1); 94.1 ± 4.0% of the contractions evoked an outflow indicating functionality of the contractions (see Figure 3A; black/white arrowheads). In contrast, the LDC frequency of GCKO colon was increased (to 0.69 ± 0.04 cpm; Figure 3B and Table 1) and the efficiency of LDCs was decreased (59.0 ± 7.5%; Figure 3B; black/

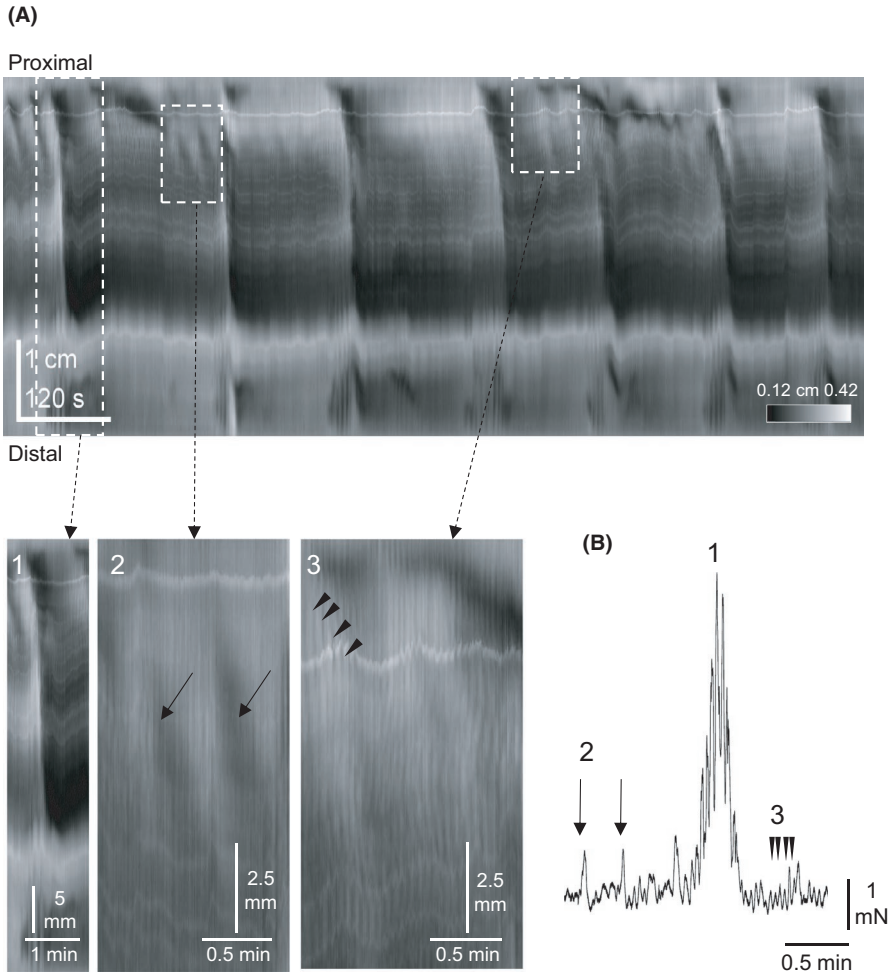

FIGURE 2 Spontaneous contractions of murine colon. A, Spatiotemporal map of WT colon showing three specific contraction types: Enlargements show long distance contractions (1), slow phasic contractions (2; arrows), and high-frequency ripples (3; arrowheads). B, The three contraction types are similarly to those seen in myography studies using proximal colon rings of 2.5 mm length. Myographical recording was performed as previously described in Beck et al[8]

white arrowheads). To evaluate the decrease in efficiency, the topographies of LDCs were analyzed in detail (Figure 3C,D). LDCs of WT colon showed the typical initial relaxation followed by a strong contraction (Figure 3C; black arrow head). In contrast, this pattern was not observed in GCKO colon. Compared to WT, the proximal part of the GCKO colon was more distended, the distal part showed stronger contraction, and the typical relaxation phase was absent. Typical LDCs rarely developed, the LDC-like contractions were frequently interrupted (Figure 3C; dotted oval); consequently, contractions propagated only over short distances (Figure 3C; white arrows) with some contractions showing retrograde direction. These data reveal that the absence of NO-GC disturbs the regular progression of LDC and that NO made a critical contribution to the normal pattern of peristalsis.

Slow phasic contractions were analyzed by generating plot profiles of a specific area (Figure 3D) by plotting colon diameter over time. In GCKO colon, slow phasic contractions were prolonged and their frequency decreased compared to WT colon (duration: 17.5 ± 1.3 vs 7.4 ± 1.0 seconds (P = 0.002); frequency: 2.9 ± 0.2 cpm vs 4.8 ± 1.0 cpm (P = 0.024) for GCKO vs WT, respectively). These data were consistent with measurements performed on colon rings.[8]

Next, colons from cell-specific knockout mice were analyzed (Figure 4). In SMC-GCKO colons, the frequency of LDCs was similar to WT colon (0.43 ± 0.01 cpm; Figure 4A). However, efficiency of the contractions was impaired as only 68.5 ± 4.9% of the contractions led to outflow drops. In contrast to GCKO colon, the outflow was concentrated on individual LDCs only (Figure 4A; asterisks). In fact, clusters of droplets resulted from regular LDCs that showed defined relaxation/contraction phases and propagated from the proximal to the distal part of the colon. Contractions not causing outflow showed topographies similar to those seen in GCKO colon.

The absence of NO-GC in colonic ICC (ICC-GCKO) led to a slightly reduced LDC frequency when compared with WT (0.36 ± 0.02 cpm, Figure 4B; Table 1). As seen in WT, most LDCs evoked an outflow drop (92.8 ± 4.0%).

Spatiotemporal maps of colons lacking NO-GC in both SMC and ICC (SMC/ICC-GCKO) resembled those of GCKO colon

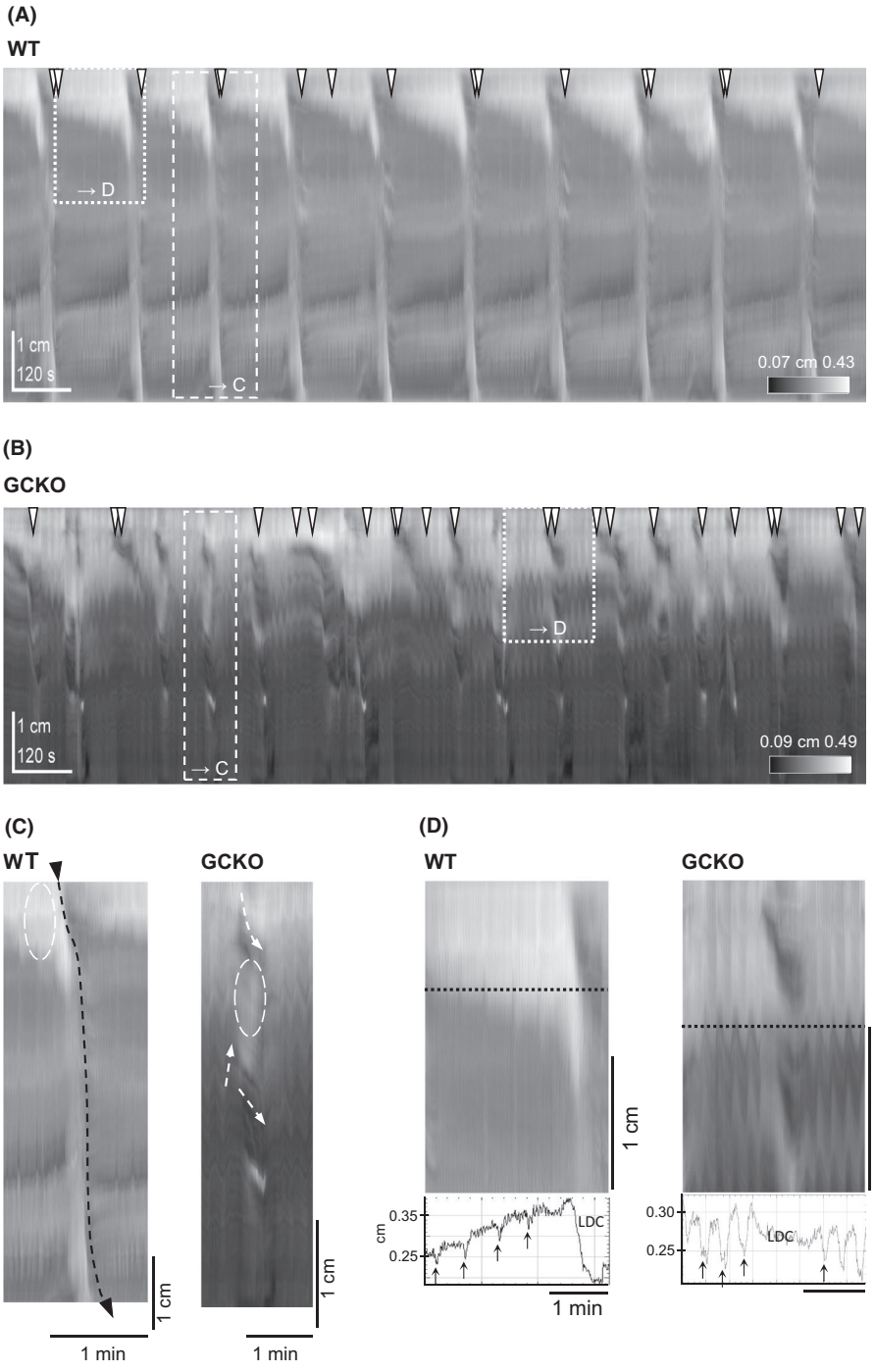

FIGURE 3 Spatiotemporal maps of colons from WT and GCKO mice. Representative maps from WT (A) and GCKO (B) colons. Areas marked by dotted lines are enlarged in (C) and (D). Arrowheads above maps indicate the time point of outflow drops, black/white arrows indicate direction of contractions; dotted oval indicates a region of strong relaxation. Note the retrograde contraction in GCKO colon in (C). D, Enlarged view of slow phasic contraction of WT and GCKO colon. Dotted lines indicate the plane of the plot profile below the spatiotemporal maps. Black arrows indicate slow phasic contractions

(Figure 4C): Frequency of LDCs was increased (0.53 ± 0.03 cpm) compared with WT, and efficiency was significantly decreased as only 41.2 ± 11.6% of the LDCs led to outflow drops. The topography of individual LDC was also altered by the double deletion as defined relaxation and contraction phases were not visible. As seen in GCKO mice, genuine LDCs did not develop in colons of SMC/ICC-GCKO; LDC-like contractions were characterized by discontinuous contractions which were limited by strong relaxations. Moreover, long-lasting contractions were observed in the distal part of the colon which exceeded the duration of a single LDC (see Figure 4C; dotted ovals).

To evaluate the impact of neuronal NO on colon contractility, the NOS inhibitor L-NAME was added (Figure 5 and Table 1). L-NAME increased the frequency of LDCs in all genotypes except for GCKO (WT 0.73 ± 0.04 cpm; SMC-GCKO 0.61 ± 0.02 cpm; ICC-GCKO 0.60 ± 0.05 cpm; SMC/ICC-GCKO 0.67 ± 0.05 cpm); these values resembled the frequency of GCKO colon under control conditions (0.69 ± 0.04 cpm). L-NAME led to a slight decrease in the frequency of the LDC-like contractions in GCKO colon (0.57 ± 0.02 cpm). Moreover, L-NAME decreased the efficiency of the contractions in WT (56.9 ± 4.7%) and ICC-GCKO colon (69.8 ± 7.5%), whereas the abnormal efficiencies of GCKO (65.7 ± 11.2%), SMC-GCKO

TABLE 1 Frequency and efficiency of long distance contractions

	Frequency (cpm)		Efficiency (%)	
	Control	+ L-NAME (200 µmol L^{-1})	Control	+ L-NAME (200 µmol L^{-1})
WT	0.43 ± 0.03	0.73 ± 0.04	94.1 ± 4.0	56.9 ± 4.7
GCKO	0.69 ± 0.04**	0.57 ± 0.02	59.0 ± 7.5**	65.7 ± 11.2
SMC-GCKO	0.43 ± 0.01	0.61 ± 0.02	68.5 ± 4.9**	58.0 ± 6.1
ICC-GCKO	0.36 ± 0.02*	0.60 ± 0.05	92.8 ± 4.0	69.8 ± 7.5
SMC/ICC-GCKO	0.53 ± 0.03	0.67 ± 0.05	41.2 ± 11.6**	54.8 ± 10.2

Data shows mean ± SEM of n = 5-6.
*$P < 0.05$;
**$P < 0.01$ compared to WT.

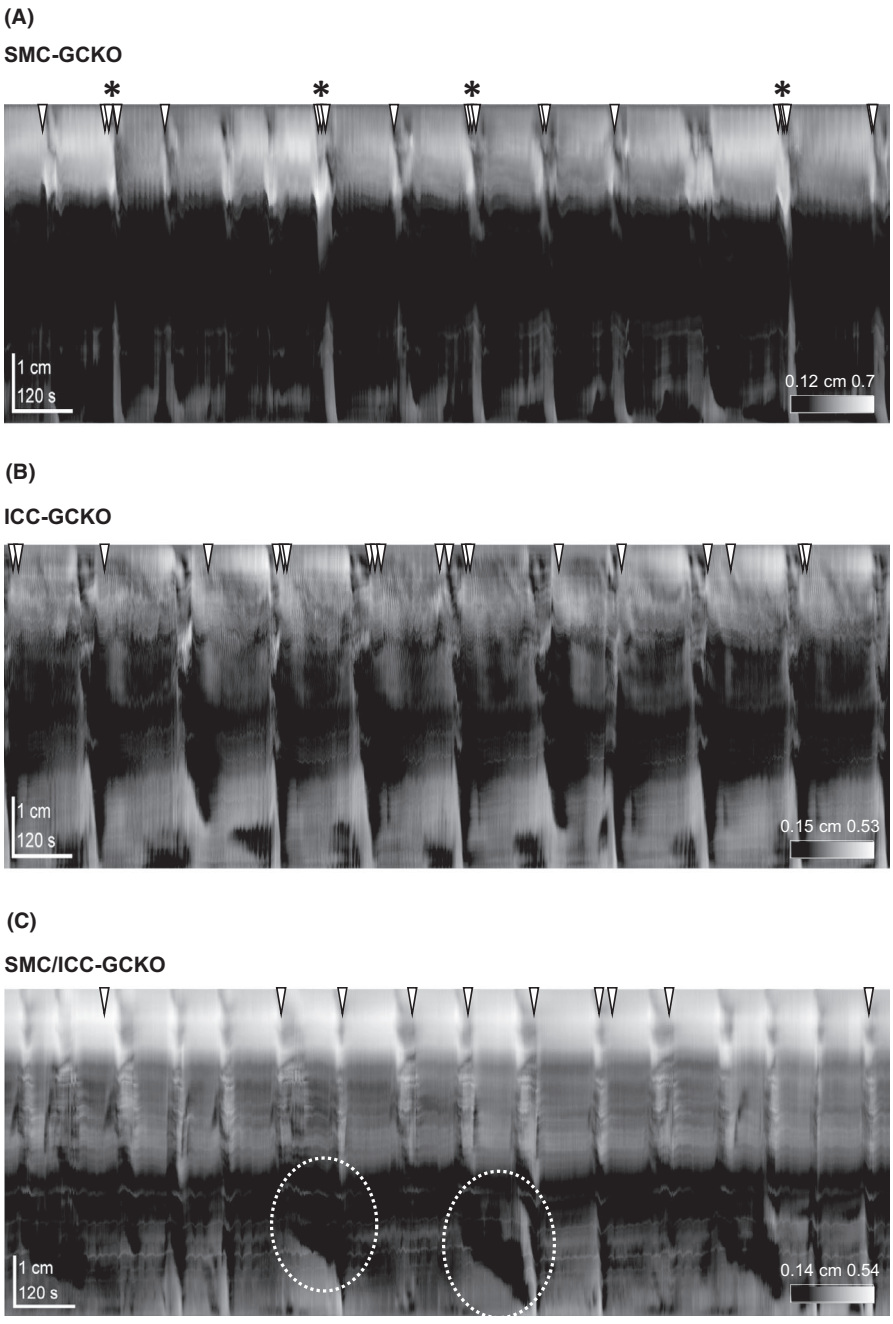

FIGURE 4 Spatiotemporal maps of colons from cell-specific knockout mice. Representative maps of colons from SMC-GCKO (A), ICC-GCKO (B), and SMC/ICC-GCKO (C) mice. White arrowheads indicate time points of outflow drops; asterisks indicate LDC in SMC-GCKO colon which produced clusters of droplets; dotted ovals indicate contractions which exceed the duration of a single LDC

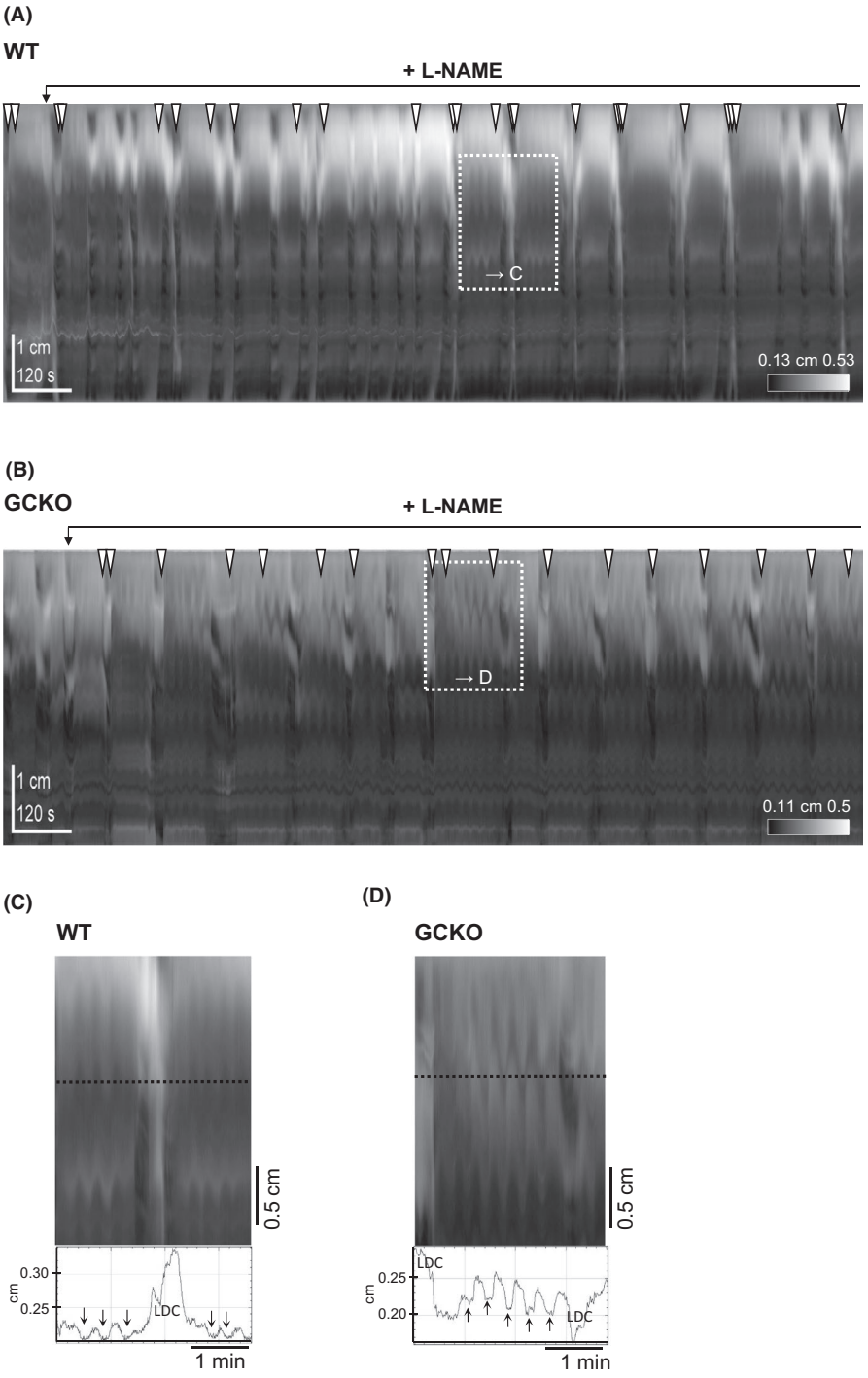

FIGURE 5 Effect of NOS inhibition on colonic contractions. Representative spatiotemporal maps of WT (A) and GCKO (B) colon in the presence of L-NAME (200 µmol L^{-1}). White arrowheads indicate time point of outflow drops; marked squares indicate slow phasic contractions enlarged in (C) and (D). Dotted lines indicate the plane of the plot profile below the spatiotemporal maps. Black arrows indicate slow phasic contractions

(58.0 ± 6.1%), and SMC/ICC-GCKO (54.8 ± 10.2%) were not further affected. The topography of a single LDC-like contraction resembled that from GCKO colon under control conditions; this included a lack of defined relaxation/contraction phases and interruption of contractions by strong relaxation phases (see Figure 5) which frequently led to short distance contractions and occasional retrograde movements. All changes by NOS inhibition were mimicked when ODQ was used to inhibit NO-GC (data not shown). These data reveal that LDC frequency and its normal peristaltic characteristics (propagation velocity, precontraction relaxation phase, and sustained proximal contraction) is NO-dependently modulated via NO-GC in both ICC and SMC, whereas LDC efficiency is regulated mainly via NO-GC in SMC.

As seen previously in colon rings,[8] inhibition of NO generation by L-NAME prolonged the duration of slow phasic contractions while decreasing their frequency in WT colon (duration 14.2 ± 0.6 seconds; frequency 3.5 ± 0.1 cpm); in contrast, slow phasic contractions in GCKO colon in the presence of L-NAME were unchanged compared to control conditions (Figure 5C/D).

To evaluate the neuronal impact, hexamethonium and tetrodotoxin (TTX) were used (Figure 6). In the presence of either compound,

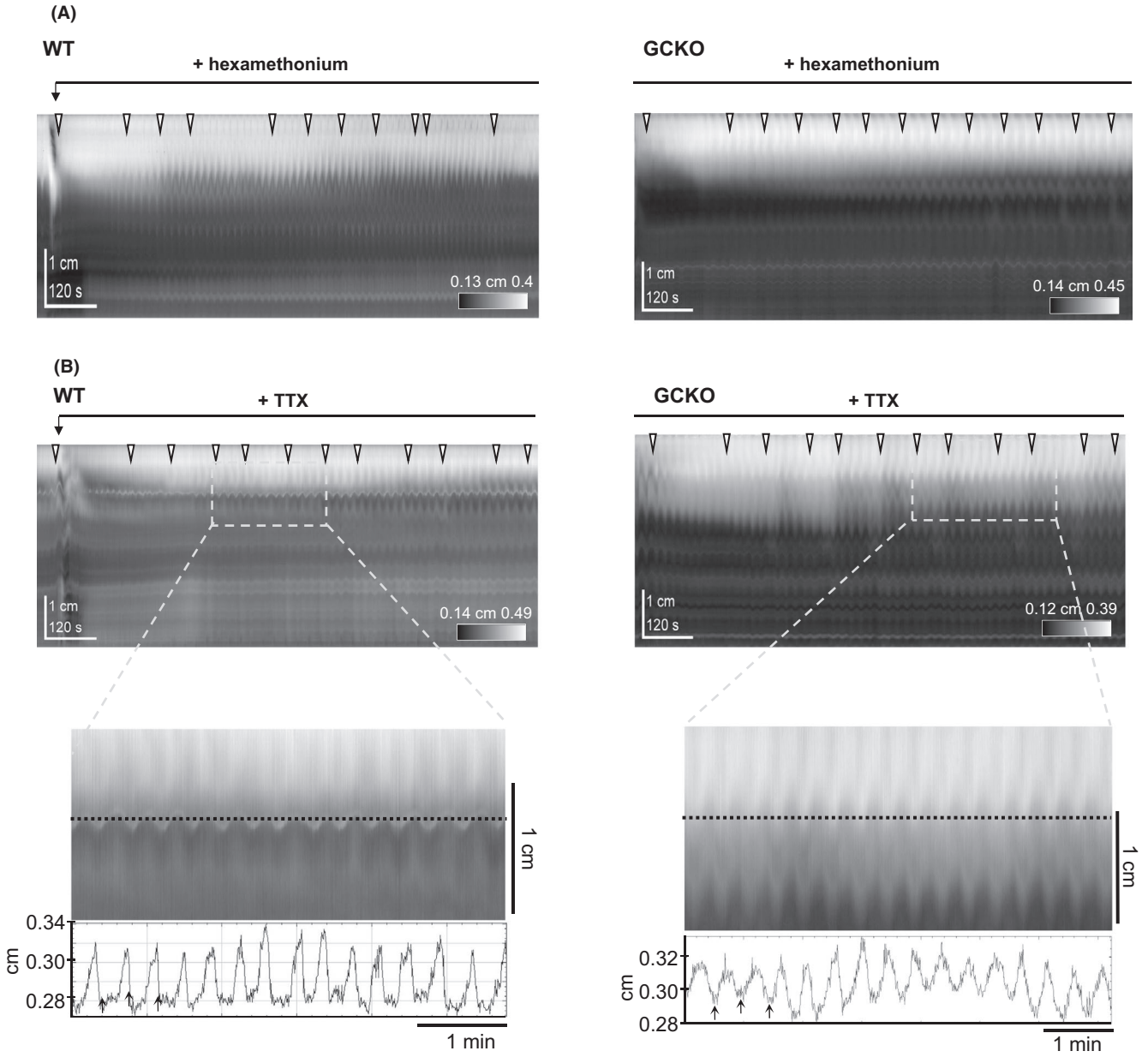

FIGURE 6 Effect of hexamethonium and TTX on colonic contractions. Representative spatiotemporal maps of WT and GCKO colon in the presence of 100 µmol L^{-1} hexamethonium (A) or 1 µmol L^{-1} TTX (B). Dotted lines indicate the plane of the plot profile below the spatiotemporal maps. Black arrows indicate slow phasic contractions

LDCs were abolished. Initially, the proximal part of the colon dilated without producing outflow drops. However, after 5 minutes, outflow occurred in regular intervals approx. every 100 seconds which corresponds to the perfusion rate.

In contrast to LDCs, slow phasic contractions still occurred in the presence of hexamethonium or TTX (Figure 6). Duration and frequency were similar to those in the presence of L-NAME (WT: duration 17.0 ± 0.6 seconds; frequency 2.7 ± 0.1 cpm; GCKO: duration 18.5 ± 1.6 seconds; frequency 2.6 ± 0.2 cpm). Thus, in contrast to slow phasic contractions, LDCs are of neurogenic origin and fluid transport is no longer actively coordinated in the presence of TTX or hexamethonium.

4 | DISCUSSION

Daily life depends on a reliable functioning of the GI tract. This involves well-regulated motility which requires both excitatory and inhibitory neurotransmission as part of intricate neuronal programs. Nitric oxide (NO) plays a role as the dominant inhibitory neurotransmitter in addition to its role in the overall programing of peristaltic contractions.[15,24,25] Nitric oxide was also shown to be essential for rhythmic electrical depolarizations in the canine colon likely involving both enteric neurons and ICC.[35] NO acts via its main receptor NO-sensitive guanylyl cyclase (NO-GC). However, the detailed regulation of nitrergic neurotransmission is still incompletely

understood. Previous studies already showed an intricate modulation of spontaneous contractions via NO-GC in smooth muscle cells (SMC), interstitial cells of Cajal (ICC) as well as fibroblast-like cells (FLC).[8,28,31,32,36,37] The nitrergic impact on long distance contractions (LDCs), the main propulsive motor pattern in mice, was still unclear. Studies with nNOS knockout mice showed controversial results including an increased LDC frequency[22] vs unaltered contraction patterns[23] after deletion of nNOS.

Using cell-specific knockout mice for NO-GC, our group has recently shown three types of contractions to occur in isolated colon rings. Based on their frequency, these contractions appear equivalent to ripples, slow phasic contractions, and LDCs shown by others in whole colon preparations. To corroborate our myography data, we here measured whole colon motility.

Using video-imaging and creating spatiotemporal maps, we were able to monitor all characteristic contractions of the colon, that is, long distance contractions (LDCs), slow phasic contractions as well as high-frequency ripples. As ripples occur in a NO-independent way in colon rings as well as in whole colon, we did not further investigate them in our cell-specific KO animals. Similarly, slow phasic contractions were not further characterized here as results were consistent with previous isometric force studies; thus, we focussed on LDCs being the motility pattern most relevant for colonic transit.

Detailed analysis of LDCs revealed NO-dependent modulation of their overall characteristics (loss of sustained proximal contraction, loss of sustained inhibitory phase in front of the propulsive contraction), their frequency, force of contraction, and propulsive efficiency. Lack of the NO receptor in both SMC and ICC (ie, GCKO and SMC/ICC-GCKO) led to an increased LDC frequency and loss of overall peristaltic appearance, which was not observed in either single KO strain. An elevated frequency was also seen after NOS inhibition. This indicates that NO, through NO-GC in both SMC and ICC, is an integral part of the generation of normal peristalsis, not just a force for inhibition. ICC and smooth muscle cells form an electrically connected system and it appears that the NO-GC system of both are needed to help orchestrate the peristaltic motor pattern in concert with the enteric nervous system. The situation appears similar to the esophagus, where loss of intramural nitrergic nerves leads to inefficient, rhythmic simultaneous contractions.[24,39,40] In ICC, the effect of NO-GC may depend on the ICC subtype: NO-GC in ICC-IM was shown to induce hyperpolarization of SMC,[15,22,36] whereas NO-GC in ICC-MP probably interacts with its pacemaker activity, and thus, modulates the generation of LDCs.[7,22,32] ICC-MP in concert with myenteric nerves orchestrate the peristaltic LDCs as shown in rat colon[6,41] and mouse colon.[5] In addition, NO was shown to decrease electrical activity which can be restored by inhibitors of the NO/cGMP signaling pathway such as L-NAME.[15] As a result, increased activity of ICC-MP in combination with enhanced SMC excitability might lead to an increased LDC frequency in GCKO and SMC/ICC-GCKO colon; therefore, these effects should be mimicked by inhibitors of the NO/cGMP signaling cascade. Nonetheless, frequency, general contraction propagation, and the response toward L-NAME differ between SMC/ICC-GCKO and GCKO colons. This effect may be driven by FLC, which still express NO-GC in SMC/ICC-GCKO but not in GCKO mice. Further experiments using respective knockout models are needed to unravel this intricate mechanism.

Continuous slow perfusion of the intestine allowed the analysis of LDC efficiency in our study. Time points of outflow drops were documented and correlated with LDCs. Our experiments clearly reveal a NO-dependent regulation of LDC efficiency. NO-GC in ICC appears not to contribute as nearly every LDC in WT and ICC-GCKO colon induced outflow. Rather, NO-GC in SMC is crucial for LDC efficiency since in GCKO, SMC-GCKO as well as SMC/ICC-GCKO colon outflow events were reduced. Lack of NO-GC in ICC and SMC led to discontinuous contractions, thus preventing unidirectional contractions distally, hence efficient peristaltic movement. In the presence of the NOS inhibitor L-NAME, similar characteristics were observed in WT and ICC-GCKO tissue. Interestingly, in colon from SMC-GCKO mice, the majority of the LDCs did not cause outflow drops; rather, outflow was limited to a few highly efficient contractions. In contrast to the inefficient contractions, these productive contractions showed a clear peristaltic wave, consisting of initial relaxation followed by a strong contraction which propagated along the whole colon, that is, a typical LDC. Thus, NO-GC in SMC is necessary for the formation of an efficient peristaltic wave.

Tetrodotoxin inhibits voltage-dependent Na^+ channels, and thus impedes an unselective release of neurotransmitters.[42] In contrast to the effect of L-NAME, LDCs were abolished in the presence of TTX. This indicates the involvement of neurotransmitters other than NO to participate in the generation of LDC. Nonetheless, outflow drops still occurred in the presence of TTX. The resulting outflow frequency (every ~100 seconds) can be attributed to the constant PBS perfusion through the colon (flowrate of 30 $\mu L\ min^{-1}$). Since LDCs have a strong neurogenic component, fluid transport is no longer driven by peristaltic movements in the presence of TTX or hexamethonium.

The results of this study are in accordance with previously reported data using small colon rings or longitudinal tissue strips for myography recordings.[8,32] Although narrow rings of colonic musculature cannot exhibit the full range of physiological motor patterns, our studies indicate that basic equivalents of whole colon motor patterns can be recognized in short in vitro preparations under the chosen experimental conditions. In our studies, the patterns of slow phasic contractions were similar in whole colon preparations (this study), short colon rings,[8] and longitudinal muscle strips.[32] This proves that typical contraction forms develop not only in whole colon preparations but also in ring/strip preparations from murine colon.

However, we observed differences between LDCs of whole colon and "large" contractions, the equivalent to LDCs, in ring preparations. In whole colon from WT mice, LDCs occurred periodically (frequency: 0.43 ± 0.03 cpm), whereas large contractions in small colon rings occurred only sporadically.[8] Without the influence of NO on NO-GC in SMC and ICC (ie, GCKO, SMC/ICC-GCKO, or addition of ODQ), we observed periodical contractions

in colonic rings (frequency approx. 0.4 cpm under all conditions[8]). Thus, the sporadic motor patterns seen by us and others might be due to physiological NO release; large contractions in these rings appear to become periodic only when the effect of NO is blocked. In general, the frequencies of whole colon preparations were higher than those of small ring preparations. This might be explained by different experimental conditions: In contrast to colonic ring preparations, whole colons were continuously perfused; thus, liquid-induced dilation with subsequent influence on LDC frequency is conceivable. Won et al[43] showed an NO-dependent stretch response in proximal colon. In this study, stretch in the proximal colon induced hyperpolarization of the muscle cells which was inhibited in the presence of TTX and L-NNA. In addition, tissue dissection and shortening of colon tissue as reported by Hibberd et al[44] can cause excessive NO release and might also underlie the differences in frequency. Nevertheless, both experimental conditions (colon rings and whole colon) revealed NO-mediated regulation of LDC frequency.

5 | CONCLUSION

This study confirms that NO/cGMP signaling is critical for normal peristaltic movements and that NO-GC in both SMC and ICC are essential, they appear to work in synchrony. The efficiency of contractions to expel fluid is particularly influenced by NO-GC in SMC.

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DISCLOSURES

No competing interests declared.

AUTHOR CONTRIBUTIONS

KB and BV designed the study; KB and AR collected data; KB analyzed the data; KB, BV, AV, SPP, JDH, and AF and interpreted the data; KB, BV, and AF wrote the manuscript; all authors critically revised the manuscript.

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