Received: 11 March 2024 | Revised: 19 June 2024 | Accepted: 29 July 2024

DOI: 10.1111/nmo.14891

ORIGINAL ARTICLE

Neurogastroenterology & Motility NGM WILEY

Decreases in mucosally-evoked tachykinin signaling pathways can explain age-related reductions in murine colonic motility patterns

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Funding information
Biotechnology and Biological Sciences Research Council

Abstract

Background: Increasing age increases the incidence of chronic constipation and fecal impaction. The contribution of the natural aging process to this phenotype is unclear. This study explored the effects of age on key motility patterns in the murine colon and determined the contribution that altered neurokinin 2 (NK_2) -mediated signaling made to the aging phenotype.

Methods: Mucosal reflexes, colonic migrating motor complexes (CMMCs) and colonic motility assays were explored in isolated ex vivo colons from 3, 12–14, 18- and 24-months old mice and the NK_2-mediated response determined. Electrical field stimulation (EFS) or exogenous drug application were used to explore the role of the mucosa in colonic segments.

Key Results: Aging reduced the force of contraction of the distal colon mucosal reflex, the frequency and force of contraction of CMMCs and the NK_2-mediated component of both motility patterns. Ondansetron, a $5\text{-}HT_3$ receptor antagonist, blocked a component of both motility patterns in full thickness but not in mucosa-free segments of the distal colon. 5, hydroxytryptamine (5-HT) and EFS-evoked NK_2-dependent contractions were reduced with increasing age. Smooth muscle sensitivity to 5-HT or neurokinin A (NKA) was not altered with age. In isolated colon motility assays application of NKA decreased transit time in 24-months colon and the NK_2 antagonist GR159897 increased transit times in both 3- and 24-months old colons.

Conclusions and Inferences: Aging impairs key motility patterns in the murine colon. These changes involve a decrease in mucosally-evoked NK_2-mediated signaling. Targeting NK_2-mediated signaling may provide a novel approach to treating age-related motility disorders in the lower bowel.

KEYWORDS
aging, colonic transit, constipation, tachykinin

1 | INTRODUCTION

Chronic constipation is a debilitating gastrointestinal disorder. The incidence of this condition increases with age affecting up to 34% of over 84-year-olds, while in the institutionalized the incidence is increased to ≈80%. The high rate of re-occurrence of bouts of constipation can dramatically affect quality of life,[1,2] be extremely costly to manage[3,4] and contribute to complications such as overflow incontinence.[5–7] The etiology of chronic constipation in older people is likely to be multifactorial and may occur because of extrinsic factors such a medication, dietary changes, or reduced mobility.[6] However, our previous work showed that intrinsic age-related changes in the functioning of the lower bowel contribute to chronic constipation.[8–10] Previous studies by others showed that decreases in the number of myenteric neurones correlated with alterations in colonic function in both humans and a range of animal models (reviewed in[11]).[12,13] However, our previous work has demonstrated no significant change in the total number of myenteric neurones or in specific neuronal nitric oxide synthase or calbindin positive populations in mice between 3 and 24 months.[14] This is despite observing significant changes in the functioning of the colon and the presence of a constipation phenotype denoted by fecal impaction, reduced pellet output and reduced pellet water content.[8] A similar lack of change in myenteric neuron number has recently been demonstrated in aged human colon, although the bowel habits of these patients was not reported.[15,16] These data suggest that more subtle alterations in signaling may be important early determinants of colonic dysfunction and an understanding of these changes may provide important insights into the etiology of this disorder and identify new targets for treatment.

Colonic migrating motor complexes (CMMCs) in mammals[17–20] and their equivalent high amplitude propagating complexes in humans[21] and the mucosal reflex[22–25] are important regulators of motor function in both animal and human colons where they are involved in driving rhythmic smooth muscle contractions. Enterochromaffin (EC) cells are present in the epithelial layer lining the lumen of the colon.[26] EC cells respond to chemical and mechanical stimuli and are important transducers for signaling the presence of fecal matter in the colon.[27] Stimulation of the EC cells causes the release of 5-HT which acts via 5-HT_3 receptors on the terminals of intrinsic primary afferent neurons. Activation of these neurons then activates excitatory motor neurons to contract the gastrointestinal smooth muscle upstream of the pellet and inhibitory motor neurons to relax smooth muscle downstream of the pellet allowing the fecal pellet to move in an oral to anal direction. In the case of the CMMCs, the mucosa/5-HT has either been shown to be essential for the generation of CMMCs or required to modulate the frequency and force of CMMCs.[28,29] An important contractile component of both the CMMC and mucosal reflex is driven by the release of tachykinins from excitatory motor neurones present in the myenteric plexus.[30–32] Tachykinins act through three main receptors in the colon namely the NK_{1-3} receptors. In rodent models, NK_2 antagonists have been shown to synergistically reduce the area under the curve of CMMCs[33] and NK_2 antagonists increase compliance in human colon[34] consistent with tachykinins regulating the tone of colonic smooth muscle. Localisation studies showed that NK_2 receptors are predominantly located on longitudinal and circular smooth muscle cells in mice,[33,35] and humans.[36,37]

Key points

- Ageing increases the incidence of chronic constipation.
- Ageing reduced the frequency and force of contraction of key motility patterns.
- Decreases in NK_2 receptor signaling could explain these changes.
- Targeting NK_2 receptors could alleviate age-related chronic constipation.

In humans, infusions of neurokinin A (NKA) an NK_2 receptor agonist increased the amplitude and frequency of migrating motor complexes MMCs[38] whereas nepadutant, an NK_2 receptor antagonist caused a transient reduction in the number of bowel movements per day[34] consistent with a prokinetic role for tachykinins. In support of these findings NK_2 antagonists had little effect on EFS-evoked contractions in colonic tissue taken from patients with slow transit constipation,[39] while NK_2 agonists were able to enhance EFS-evoked contractions[40] or increase the maximum contraction of the colon taken from patients with idiopathic chronic constipation[41] compared to healthy controls. Human chronic constipation was also associated with changes in the expression of the tachykinin, substance P (SP). In some studies SP expression was seen to decrease in patients with chronic constipation,[42–44] although no change[45] and even an increase in SP expression[46] have been observed.

In mice, studies using an ex vivo pellet motility assay, artificial pellets moved down the colon in a stepwise manner. NKA increased the step distance, step velocity and step frequency in the colon of 24-months old mice but had no effect on 3-months old colon.[8] Similarly, the NK_2 antagonist GR159897 reduced the step distance, step velocity and frequency of bowel movements in 3-months old mice but was without effect in 24-months old colon.[8] Taken together these findings strongly suggest that the constipation phenotype seen in aged mice might be due to deficits in NK_2-mediated signaling. To conclusively test this hypothesis and to understand the underlying causes of any changes, this study explored the effects of the natural aging process on NK_2-mediated signaling in the murine colon.

2 | MATERIALS AND METHODS

2.1 | Animals

All procedures were carried out according to U.K. Animals (Scientific Act), 1986 and associated guidelines and were approved by the University of Brighton Ethics Committee. Male C57BL/6J mice were obtained from Harlan UK at 8 weeks of age and housed in groups

of 3–4 until required. Animals were maintained at 19.0 ± 1°C, 55% humidity and fed on a maintenance diet (RM1 (E) 801,002 chow, Special Diet Services) and had free access to water. The animals were kept on a 12-h light/dark cycle and studied at 3–4, 12–14, 18 and 24 months of age.

2.2 | Mucosal reflex

The whole colon was harvested and placed in ice cold oxygenated (95% O_2 and 5% CO_2) Krebs buffer solution, pH 7.4. The colon was opened by cutting along the mesenteric border and the tissue pinned mucosal surface up in an organ bath and perfused at 1 mL min^{-1} with Krebs buffer solution at 37°C. Force transducers were attached to enable recordings of circular muscle contractions in the proximal and distal parts of the colon. Tissue was placed under 6 mN tension and circular muscle contractions were evoked by stroking the mucosa with a fine artist brush (Figure 1A). Contractile responses from the colons of different aged animals were recorded following 1–5 brush strokes applied at a rate of 1 stroke per second. To maintain consistency between the force applied to the different preparations the bristles of the brush were pressed against the mucosa until half the length of the bristles was bent perpendicular to the brush handle. The signal from each force transducer then passed to a preamplifier and ADI Powerlab before being stored on computer using LabChart software.

2.3 | Measurement of CMMCs

Briefly the whole colon was placed in a Sylgard-lined recording chamber and a thin glass rod (1 mm diameter) placed through the lumen and secured at each end to the Sylgard base of the perfusion chamber (Figure 2A). The preparation was perfused at 1 mL min^{-1} with Krebs buffer solution at 37°C. Recordings of circular muscle contractions were made at two locations along the whole isolated colon, one at the proximal end and one at the distal end. Fine suture silk was tied through the muscle layers at each location and connected to two separate isometric force transducers. The muscle was placed initially under a low level of tension 4 mN and then tension increased over the next 40 min until a final tension of 6 mN was reached. The signals from each force transducer were recorded as described in section 2.2.

2.4 | Electrical field stimulated, and 5-HT evoked distal colon longitudinal muscle contractions

The whole colon was removed, and 2 cm full thickness sections of distal colon were suspended in an organ bath containing Krebs buffer solution maintained at 37°C and bubbled continuously with 95%O_2 5% CO_2. Distal colon segments were chosen as this was the region previously shown to be most affected by the aging process.[8] Responses were evoked by acetylcholine, EFS or 5-HT and recorded in the presence of a range of drugs. Values given are the final bath concentrations. 100 μM acetylcholine was then added to the tissue for 1 min every 10 min, until successive applications yielded a consistent response. Following this a frequency response curve was generated by passing current pulses across the tissue (40 V, 0.3 ms pulse duration, 0.1–30 Hz). Tissues were stimulated for 30 s every 5 min. The tissues were then washed, and the frequency response curves repeated in the presence of 1 μM MRS2500, 100 μM NG-nitro-L-Arginine, 10 μM guanethidine and 1 μM scopolamine to

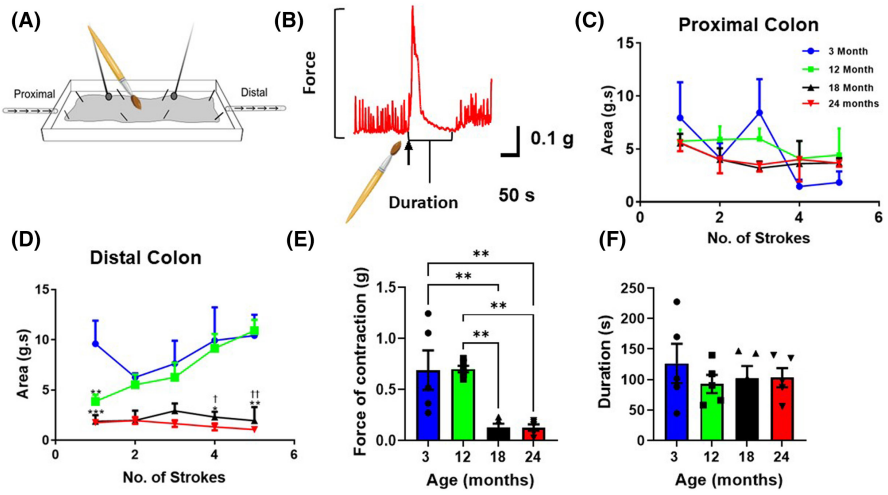

FIGURE 1 Increasing age impairs the mucosal reflex. (A) Diagrammatic representation of the setup for recording the mucosal reflex. (B) Typical distal colon response to stimulation of the mucosa with a fine artist's brush, showing key parameters to be monitored. (C) Graph showing that the integral of the mucosal reflex is not altered by age or the number of brush strokes in the proximal colon. (D) Graph showing that increased age reduces the integral of the mucosal reflex in the distal colon. (E) Bar graph showing that increased age reduces the force of contraction of the mucosal reflex. (F) Increasing age does not alter the duration of the reflex. Values represent the mean ± SEM. $N = 5$ for each age group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus 3 months, † $p < 0.05$, †† $p < 0.01$ versus 12 months. (C–F), $n = 5$ for each group.

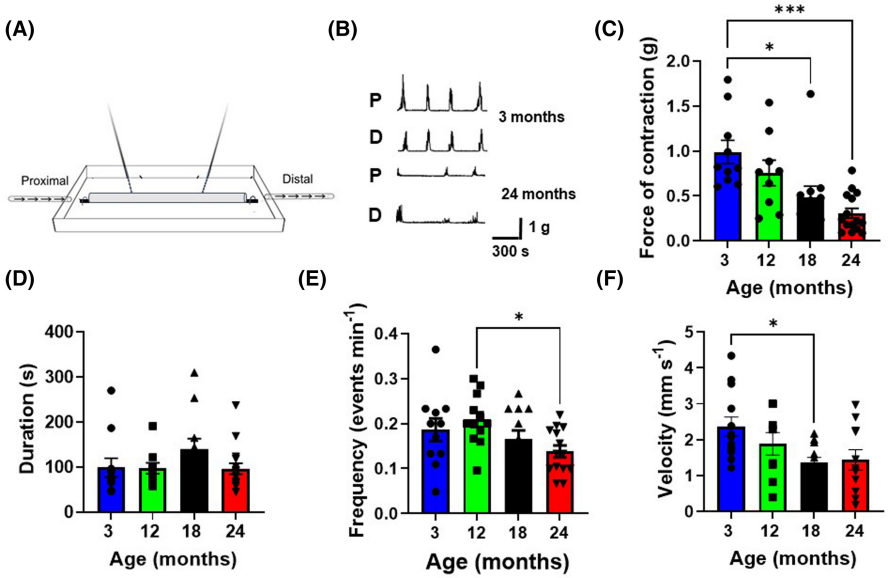

FIGURE 2 Increasing age impairs colonic migrating motor complexes (CMMCs). (A) Diagrammatic representation of the setup for recording CMMCs. (B) Samples recordings of CMMCs from 3- and 24-month colon. P, Proximal and D, distal. (C–F) Bar graphs showing the effects of increasing age on the properties of the CMMCs. Values represent the mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$ versus 3 months controls. (C) $n = 10$, 9, 11, and 15 for the 3, 12, 18, and 24-months groups. (D) $n = 11$, 10, 11 and 16 for the 3, 12, 18 and 24-months groups. (E) $n = 11$, 12, 12, and 14 for the 3, 12, 18, and 24-months groups. (F) $n = 13$, 9, 12, 11 for the 3, 12, 18 and 24-months groups.

isolate the tachykinin response and the NK_2 component identified following administration of 1 μM GR159897.

In a separate set of tissues NK_2 contractions were recorded following the application of 5-HT (100 nM—30 μM). In both cases recordings were collected on Labchart software as described previously. In several preparations, the mucosa was removed using fine forceps and micro-scissors and the ability of 5-HT to evoke contractions explored in the presence of 1 μM ondansetron a 5-HT_3 receptor antagonist.

A range of experiments were carried out to determine whether changes in smooth muscle responses to NKA or 5-HT could explain any age-related changes in the NK_2-evoked contractions. These included concentration response curves to NKA (30 nM −1 μM) and the response of the colon to 30 μM 5-HT. Both experiments were performed in the presence of 400 nM tetrodotoxin a blocker of voltage-gated Na^+ channels that inhibits enteric neuronal activity.

2.5 | Pellet motility assays

The whole colon was harvested and placed in ice cold oxygenated (95% O_2 and 5% CO_2) Krebs buffer solution, pH 7.4 containing (117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose). The mesentery was trimmed using fine scissors and the whole colon was then loosely pinned in a Sylgard-lined flow bath, allowing a lateral movement of approximately 0.5 cm about the mid-line and perfused with oxygenated Krebs buffer solution at 37 ± 1°C at a flow rate of 8 mL min^{-1}. A small (2 mm) incision was made in both ends of the colon and the openings pinned flat to facilitate pellet insertion and its expulsion at the distal end. In most preparations spontaneous evacuation of endogenous pellets occurred within a 30-min time window. In a minority of preparations where this did not occur, the fecal pellets were removed from the isolated colon by gently flushing the lumen of the colon with warmed Krebs buffer solution. The colon was then left to stabilize for 15 min, prior to recordings of pellet motility. Measurements of motility were carried out using a 2 mm diameter epoxy-coated fecal pellet. The artificial fecal pellet was inserted 3–4 mm into the proximal end of the bowel using a fire-polished glass capillary and the movement of the pellet was monitored using a video camera. Pellet motility was tracked using Ethovision tracking software. Following a successful trial, the experiment was repeated two further times and the average response utilized for statistical analysis. Tissues were then perfused with 1 μM Neurokinin A (NKA) or 1 μM GR159897 an NK_2 antagonist and pellet motility recorded for a further three trials. The maximum time that any single trial was conducted was 45 min. The total transit time of the artificial fecal pellet was determined.

2.6 | Data analysis

Comparisons between age were analyzed using a one-way ANOVA followed by a post-hoc Tukey test. Experiments where two variables were being compared for example age and number of brush strokes, or age and drug, were analyzed using a two-way ANOVA followed by a post-hoc Tukey test. Values are presented as the mean ± SEM and $p < 0.05$ was taken as being significant.

3 | RESULTS

In the first series of experiments, we explored the effects of increasing age on the mucosal reflex and on colonic migrating motor complexes two key motility patterns responsible for the movement of fecal matter down the colon.

Mucosal reflexes were recorded from both the proximal and distal regions of the colon (Figure 1A). Brush stroke-evoked reflexes consisted of a rapid contraction followed by a slower decline in tension (Figure 1B). Plotting the integral of the response versus the number of brush strokes failed to show any effect of increasing age in the proximal colon (Figure 1C). In the distal colon age had a significant effect on the integral of the evoked contraction. This was most apparent after 5 strokes where the 3- and 12-month-old responses were significantly greater than those recorded in the 18- and 24-month colons ($F_{(3,17)} = 13.96$; $p < 0.0001$; Figure 1D). There was also a significant interaction (age × brush stroke number; $F_{(12,48)} = 3.423$, $p < 0.01$). 3-, 18- and 24-month-old responses showed no significant changes in their responses to increasing numbers of brush strokes. However, increasing numbers of brush strokes caused responses in the 12-month group to increase incrementally (Figure 1D). A more detailed analysis of the distal colon evoked responses recorded following five brush strokes for the four different age groups showed that the natural aging process reduced the force of contraction ($p < 0.01$; Figure 1E) but not the duration of the evoked responses (Figure 1F).

The effects of increasing age were also explored on spontaneously evoked CMMCs. Sample traces of the CMMCs recorded from 3- and 24-months old colon are shown in Figure 2B. Analysis of the properties of the CMMCs showed that increasing age reduced the force ($p < 0.01$; Figure 2C) and frequency ($p < 0.0001$; Figure 2E) of the evoked CMMCs but not the duration (Figure 2D) or velocity (Figure 2F).

The excitatory motor neurons in the murine distal colon utilize acetylcholine and neurokinin A to drive smooth muscle contraction. This study chose to explore the role played by NK_2 receptor mediated NKA signaling in the regulation of colonic motility patterns as our previous studies had shown that exogenous NKA was able to rescue age-related deficits in ex vivo pellet motility assays.[8] Block of the NK_2 receptors using 1 μM GR159897 caused a significant reduction in the force of the evoked distal colon mucosal reflex ($F_{(1,13)} = 33$; $p < 0.0001$; Figure 3A). Post hoc analysis showed these reductions to be significant in both the 3- and 12-months groups but not in the 18- and 24-months groups consistent with a significant age x GR159897 interaction ($F_{(3,13)} = 7.477$; $p < 0.01$). Analysis of the % block of the evoked response by GR159897 showed this to also decrease with age ($p = 0.05$; Figure 3B). The mucosal reflex was also shown to be dependent on 5-HT_3 receptors as 1 μM ondansetron a 5-HT_3 receptor antagonist was able to significantly reduce the response (Figure 3C). Similar experiments were performed on the CMMCs recorded in the distal colon. GR159897 caused a significant reduction in the force of contraction of the CMMC ($p < 0.0001$;

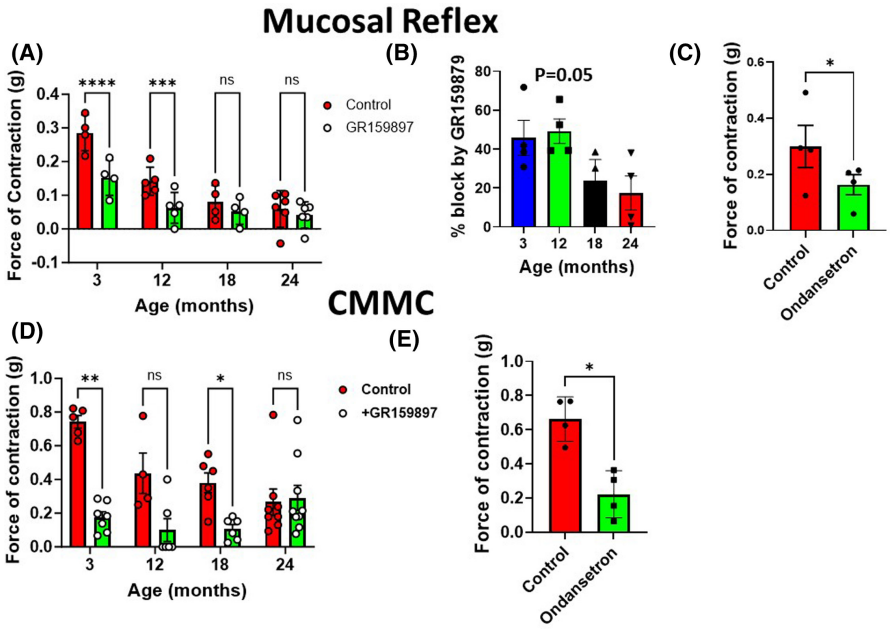

FIGURE 3 Increasing age reduces the NK_2 component of both the mucosal reflex and the CMMC. (A) Increasing age reduces the NK_2-dependent contraction of the mucosal reflex. (B) The % block by the NK_2 antagonist GR159897 is reduced with increasing age. (C) A significant proportion of the mucosal reflex is blocked by the 5-HT_3 antagonist ondansetron. (D) Increasing age reduces the NK_2-dependent contraction of the CMMC. (E) Partial block of the CMMC with ondansetron. Values represent the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ versus 3 months controls. (A), $n = 4$, 5, 4, and 6 for the 3, 12, 18, and 24-months groups respectively. (B, C), $n = 4$ for each group. (D), $n = 5$, 4, 6, and 8 for the 3, 12, 18 and 24-months controls and $n = 7$, 6, 6, and 9 for the GR159897 treated 3, 12, 18 and 24-months groups. (E), $n = 4$ per group.

Figure 3D). A post hoc analysis showed this decrease was significant in 3- and 18-months old colon, but not in 12- or 24-months colons. Although the reduction in the 12 months old group was not significant, GR159897 did decrease the force of contraction, compared to age-matched controls. Like the mucosal reflex the distal colon CMMC was also sensitive to ondansetron ($p < 0.05$; Figure 3E).

Chemical and mechanical stimuli from the fecal pellets in the colon evoke 5-HT release from murine enterochromaffin cells. This 5-HT then signals to intrinsic primary afferent neurons whose terminals lie in the mucosa via 5-HT_3 receptors. The mucosa has been shown to be important in modulating both the mucosal reflex and CMMCs.[19,28,29,47] Therefore, we were keen to see if 5-HT-evoked responses in full thickness segments of the distal colon were sensitive to blockade with ondansetron and whether 5-HT could evoke an NK_2-mediated contraction and if so, how this was affected by the natural aging process. Application of 30 μM 5-HT to full thickness section of distal colon evoked a net contraction in both 3 and 24 months old colonic muscle (Figure 4A,B). This response was significantly reduced following the application of 1 μM ondansetron. In mucosa-free preparations application of 5-HT evoked a small relaxation response which was not significantly affected by ondansetron. Isolation of the 5-HT-evoked tachykinin response using a combination of scopolamine and L-NNA to block the cholinergic and nitrergic components of the response left a sustained contraction the majority of which could be blocked by the NK_2 antagonist GR159897. 5-HT concentration response curves of the NK_2-sensitive component showed a significant effect of 5-HT ($F_{(6,114)} = 43.89$; $p < 0.001$) and age ($F_{(3,19)} = 4.570$; $p < 0.05$; Figure 4C). Post hoc analysis showed that for the three highest concentrations of 5-HT (1, 3, and 10 μM) the responses evoked in the 3 months tissue were significantly greater than those in the 18- and 24-months tissue (Figure 4C). Electrical field stimulation (EFS) was also used to evoke an NK_2-mediated responses in full thickness segments of the distal colon. Responses were typically slow to rise and fall and were only consistently evoked at frequencies ≥3 Hz. EFS-evoked NK_2-mediated contractions showed a significant effect of frequency ($F_{(5,85)} = 9.346$; $p < 0.0001$) and age ($F_{(3,17)} = 4.298$; $p < 0.05$; Figure 4D) and a significant age x frequency interaction ($F_{(15,85)} = 1.843$; $p < 0.05$). Post hoc analysis showed responses in the 3-month colon were significantly greater than those in both the 18-months and the 24-months old tissue at the three highest stimulation frequencies.

To explore whether the age-related reduction in the NK_2-evoked contractions was due to changes in the response of the colonic muscle to released tachykinins or 5-HT, the direct effects of exogenous 5-HT and NKA on colonic muscle were explored in the presence of TTx which blocks all neuronal activity (Figure 5). Administration of 10 μM 5-HT caused a contraction in distal colon muscle, however, the amplitude of the contraction was not significantly altered with increasing age (Figure 5A). Application of increasing concentrations of NKA (30 nM—1 μM) produced concentration-dependent increases in the colonic contractions in all age groups, although there was no significant difference in the response of the muscle from the different age groups (Figure 5B).

Finally, to explore whether the changes in tachykinin signaling were responsible for age related decrease in colonic motility, pellet motility assays were performed on 3- and 24-months ex vivo colon in the absence of any drugs or in the presence of NKA or GR159897 and colonic transit times determined. In 3-month, ex vivo colon addition of NKA did not significantly alter colonic transit times, however, application of GR159897 increased transit times and in most instances prevented the artificial pellet passing through the entire length of the colon in the 45-min cut off period (Figure 6). In 24-month, preparations addition of 1 μM NKA caused a significant reduction in transit time ($p < 0.01$), while application of GR159897 slowed transit times further and prevented pellet migration through the colon.

4 | DISCUSSION

In this study we explored the effects of age on key motility patterns in the murine colon (mucosal reflex and CMMCs) and whether

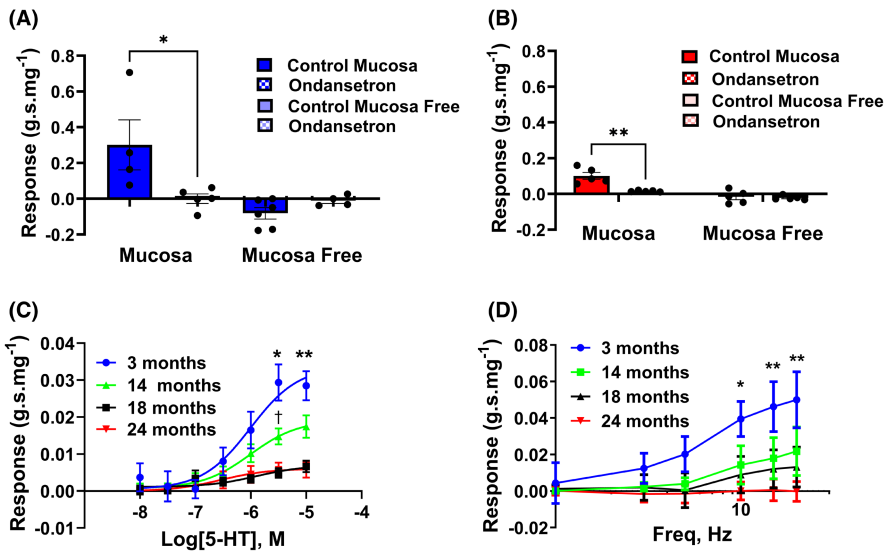

FIGURE 4 5-HT evoked NK_2-mediated contractions are reduced in the aged distal colon. 5-HT-evoked contractions require the mucosa and are blocked by ondansetron in 3-months (A) and 24-months (B) distal colon. 5-HT (C) and EFS (D) evoked NK_2-dependent contractions are reduced with increasing age. Values-represent the mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$ versus 18- and 24-months. (A) $n = 4$; (B) $n = 5$ for all data points; (C, D) $n = 6$ for all data points.

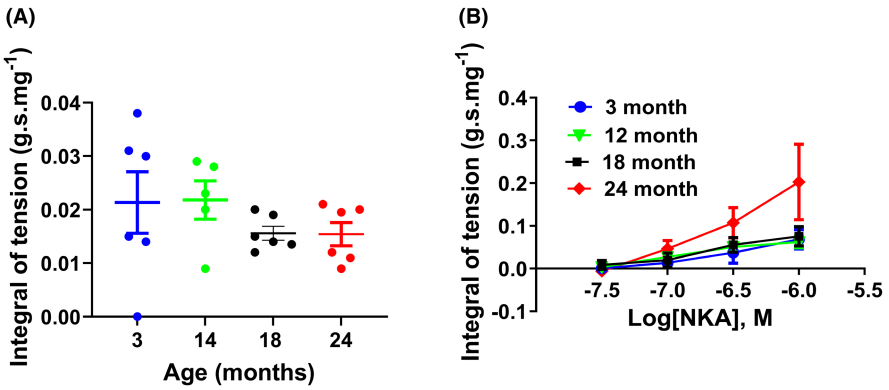

FIGURE 5 Increasing age does not alter the sensitivity of the muscle to 5-HT or Neurokinin A (NKA). Increasing age does not alter the direct effects of 5-HT (A) or NKA (B) on distal colon smooth muscle. For (A, B), $n = 5$ for 3 and 12–14 months and $n = 6$ for 18 and 24 months. N.B. All recordings were carried out in the presence of 400 nM Tetrodotoxin to block neuronal inputs to the muscle.

age-related differences in the observed motor patterns could be explained by changes in NK_2-mediated signaling. This study showed that the natural aging process reduces the force of contraction of both the mucosal reflex and CMMCs and reduced the frequency of the CMMCs. These changes could be explained by an age-related reduction in the ability of the mucosa to evoke NK_2-mediated signaling. Given the importance of both these motility patterns to the movement of fecal matter through the colon, these findings provided a basis for explaining the impaired motility previously observed in aged colon.[8]

The mucosal reflex is evoked by stroking the mucosal surface, driving the enterochromaffin cells to release 5-HT. 5-HT then acts on 5-HT_3 receptors to activate IPANs which in turn signal to ascending interneurons to excite excitatory motor neurons to drive smooth muscle contraction (Figure 7). This model is supported by our observation that ondansetron a 5-HT_3 receptor antagonist significantly reduced the area under the curve for the mucosal reflex in the distal colon. Together these data suggested an age-related deficit in the ability of the EC cells on the luminal surface of the mucosa to evoke a full mucosal reflex. Brush stroke stimulation of the 3-month-old distal colon produced a large all or nothing response that was insensitive to the number of brush strokes. However, in 12-months old colon, increasing the number of brush strokes significantly increased in the integral of the distal colon reflex. The response of the 12 months-old colon was consistent with many stimulus-evoked responses and is supported by Grider et al. who saw a similar relationship in 6-month-old murine colon.[48] The cause of the lack of a similar graded response in the 3-month colon is unclear but probably reflects the development of this reflex between 3 and 12 months. This proposal is consistent with time course of development of other reflexes in the mouse[19] and the differing rates of development in different mice most likely explains the increased variability seen in the young dataset. The observation that the mucosal reflex is then reduced and lost by 24-months could explain the reduced motility seen in the aged colon. The effects of age on the mucosal reflex were limited to the distal region of the colon a result this is consistent with our previous observations in ex vivo pellet motility assays.[8] Here the speed of pellet motility through the distal colon slowed significantly with increasing age with no observable change observed in the proximal colon. The precise reason for the region-specific effects of age on motility are unknown, although region specific effects of age have also been observed in human colon.[16,49]

Mucosal 5-HT has also been shown to modulate murine CMMCs with removal of the mucosa either stopping CMMC generation[19] or reducing the frequency of events.[29] We have shown that increasing age reduced the frequency, velocity, and force of contraction of CMMCs, results that are consistent with increasing age impairing the ability of the mucosal EC cells to signal to the myenteric neurons. Support for this hypothesis came from the observation that the force of contraction of the CMMCs is reduced following application of ondansetron a 5-HT_3 antagonist that would block EC cell derived 5-HT from stimulating the IPANs (Figure 7). Previous work in a different mouse strain has shown that increasing age reduced the velocity of CMMCs without changing the frequency or force of contraction.[50] The additional changes observed in our current study, may reflect differences in the ways that the CMMCs were recorded with the Kunze lab canulating their colons and evoking CMMCs using the pressure evoked by the luminal flow of saline, while our preparation recorded spontaneous CMMCs. Alternatively, this could be related to strain differences in the mice used, CD-1 vs C57BL/6 mice in the current study.

Fecal pellet movement through the isolated C57BL/6 colon occurred in a stepwise manner.[8] We have previously demonstrated that exogenous application of NKA to 24-months isolated colon could reverse age related decreases in the step distance, step velocity and the frequency of steps.[8] Infusion of NKA had also been shown to increase the force and frequency of migrating motor complexes in humans.[38] As tachykinin signaling is an important component of both the murine mucosal reflex[48] and the CMMC,[33] we explored whether the age-related differences in both motility patterns could be explained by decreases in NK_2-mediated signaling. Using a range of pharmacological antagonists to isolate the NK_2-mediated contraction of both motility patterns we showed that the force of contraction of both the mucosal reflex and the CMMC were reduced in the aged distal colon.

Given that blocking 5-HT$_3$ receptors impaired the generation of both the mucosal reflex and CMMCs we decided to apply exogenous 5-HT to full thickness distal colon segments to bypass activation of the EC cells and to explore whether 5-HT-evoked NK$_2$-mediated contractions were reduced with age (Figure 7). The NK$_2$-mediated component of the 5-HT-evoked contraction was reduced with increasing age to a similar degree to that observed for the mucosal reflex and CMMC experiments. This strongly suggested that decreases in the sensitivity of the EC cells to mechanical touch or their ability to release 5-HT were not major determinants of the observed age-related decrease in NK$_2$ signaling. Indeed, our previous work showed that 5-HT overflow from the EC cells was increased with increasing age (Figure 7). This was due to an increase in tumor necrosis factor α downregulating the 5-HT transporter, a protein that acts to remove 5-HT from the extracellular space (Figure 7).[9] We speculated that this increase in 5-HT overflow would desensitize the 5-HT$_3$ receptors on the IPANs as has been previously shown in the guinea pig[51] and this would reduce IPAN excitability and therefore the release of NKA (Figure 7). Despite a range of 5-HT receptor types in the colon, our observation that the 5-HT-evoked contraction was blocked by ondansetron suggests that 5-HT was mainly working by activating 5-HT$_3$ receptors on the IPANs. That said 5-HT$_3$ receptors have also been shown to be present on other enteric neurons, suggesting that exogenous 5-HT could bypass the IPANs to drive a contraction. However, the contractile response to exogenous 5-HT was lost following removal of the mucosa, along with its sensitivity to ondansetron. This strongly suggested that the 5-HT-evoked contractile effect is due mainly to the activation of 5-HT$_3$ receptors on the terminals of the IPANs[23,28,52] rather than any direct effect via 5-HT$_3$ receptors on myenteric neurons[53] or any effect on constitutively active 5-HT$_3$ receptors.[54]

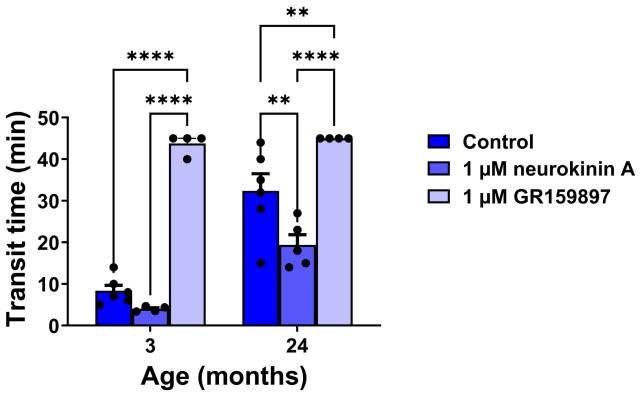

FIGURE 6 Effects of manipulating NK$_2$ signaling on colonic transit times in the isolated colon. Blocking NK$_2$ signaling using GR159897 was able to slow colonic transit times in both 3- and 24-months old colon. Application of exogenous NKA did not alter transit times in 3-months colon but was able to reduce transit times in 24-months colon. Values represent the mean ± SEM. $^{**}p < 0.01$, $^{****}p < 0.0001$ versus 3 months controls. For the 3-months data, $n = 6$, 4, and 4, and for the 24-months group $n = 6$, 5, and 4 for the control, NKA and GR159897 groups respectively.

The age-related differences in the properties of both the mucosal reflex and CMMCs could not be explained by differences in colonic smooth muscle sensitivity to 5-HT or NKA and therefore must reflect changes in the ability of the excitatory motor neurons to ultimately release NKA. The lack of an age-related change in the ability of NKA to drive smooth muscle contraction strongly suggest that NK$_2$ receptor expression and its associated downstream signaling pathways are not altered with increasing age (Figure 7).

In the murine distal colon tachykinin evoked contractions could be released following electrical field stimulation (EFS) frequencies > 1 Hz. EFS will depolarise all the neurons present within the isolated segment of colon causing them to release their neurotransmitters and in the presence of the appropriate pharmacological agents it

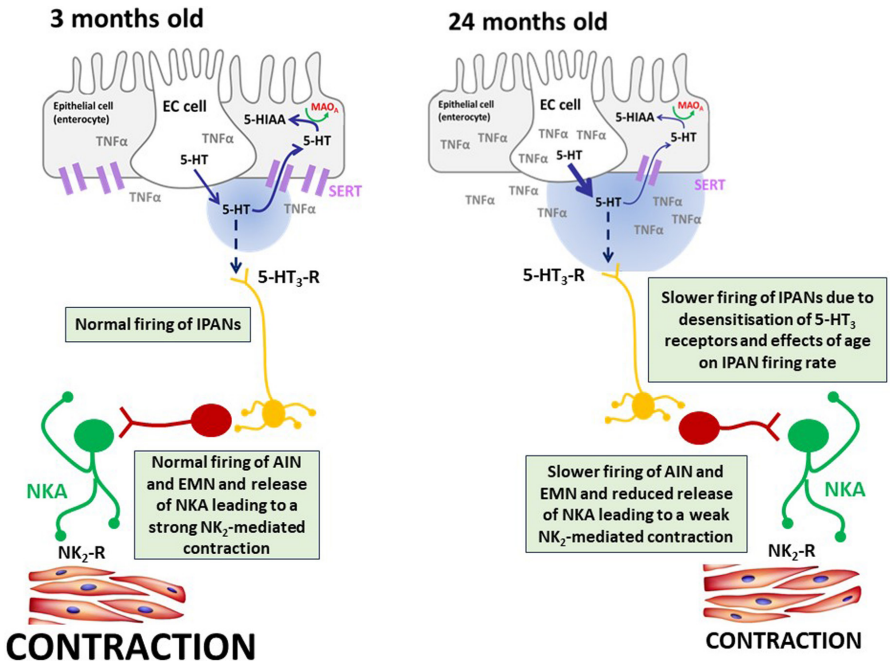

FIGURE 7 Diagram explaining the proposed mechanism by which the natural aging process impacts on colonic motility patterns driving a constipation phenotype. Data on 5-HT overflow from the enterochromaffin (EC) cell is adapted from.[9] AIN, ascending interneuron (brown neuron); EMN, excitatory motor neuron (green neuron); 5-HT, 5, hydroxytryptamine; 5-HIAA, 5, hydroxyindole acetic acid; IPAN, Intrinsic primary afferent neurones (yellow neuron); MAOA, monoamine oxidase A; NKA, Neurokinin A; NK$_2$, neurokinin 2 receptor; TNFα, Tumor necrosis factor alpha.

was possible to selectively record NK_2-mediated muscle contractions. Deficits in EFS-evoked NK_2-mediated contractions would, therefore, represent a more fundamental impairment of signaling within the distal colon. EFS-evoked NK_2-mediated contractions were also reduced with increasing age and could be explained by a decrease in neuronal excitability that has been observed in a range of organisms.[55] A recent study showed an age-related reduction in the excitability of the vagus nerve in mice and these reductions correlated with a decrease in colonic motility.[50] The authors of this study proposed that decreased IPAN excitability might underlie both the reduced motility and vagal firing rate and therefore may well be a common factor driving age-related reductions in colonic motility. Decreased IPAN excitability with old age would impair the ability of both mucosal stimulation and exogenous 5-HT to activate ascending interneurons and in turn the excitatory motor neurons. If the firing frequency of the motor neurons was reduced, this would explain the reduction in the sustained NK_2-mediated contractions, particularly given the relatively high firing frequencies required to release NKA compared to the other main excitatory transmitter, acetylcholine, that is released at much lower stimulus frequencies (≥0.1 Hz; Figure 7).

We have presented evidence supporting age-related changes in (1) the ability of 5-HT to activate the IPANs and (2) reduce in nerve cell excitability in the distal colon. However, we cannot exclude the possibility that the natural aging may also decrease the number of NKA-containing nerve cells and/or reduces the NKA content of the remaining nerve cells in the distal colon. While substance P down regulation has been linked in humans to chronic constipation,[42–44] no change[45] and even an increase in SP expression[46] have also been observed. To our knowledge the only study to have explored how tachykinin signaling is affected by the natural aging process is our own work on the anorectum. This study showed that the density of Substance P containing fibers was reduced with increasing age.[56] Whether this was due to a loss of tachykinin neurons was not explored but our previous work showed this was unlikely as there is no change in either total neuronal number or in sub populations of calbindin positive and neuronal NOS positive neurons which labeled approximately 43% and 42% of the total neuronal population respectively.

Application of NKA was able to reverse the age-related increase in ex vivo in colonic transit time and the NK_2 antagonist, GR159897, slowed transit in both 3- and 24-months old colons. These effects most likely represent modulation of NKA signaling at the smooth muscle. However, it is possible that they reflect changes in signaling within the myenteric plexus. While there is no data that has examined whether there are NK_2 receptors on IPANs, IPANs have been shown to release tachykinins and therefore the addition of NKA or the blockade of NK_2 receptors could regulate IPAN signaling to ascending interneurons.

5 | CONCLUSIONS

This study has shown that aging reduced the force of contraction of the mucosal reflex in the distal colon and the frequency and force of contraction of CMMCs. Analysis of these motor patterns showed a reduction in the NK_2-mediated contractile response of both motor patterns. A similar decrease in NK_2-mediated contractions was observed following the application of 5-HT and to a lesser extent EFS. Together these data show that mucosal 5-HT signaling via $5\text{-}HT_3$ receptors on the IPANs is impaired with aging and suggest an additional age-related decrease in neuronal excitability (Figure 7).[55] Treatments that enhance NK_2-mediated signaling in the colon may be useful for treating age-related colonic motility disorders such as chronic constipation and fecal impaction.

AUTHOR CONTRIBUTIONS

N.K., M.S.Y., A.H. and I.H. performed the experiments and analyzed the data. B.A.P. assisted with performing the experiments, analyzed the data, co-designed the experiments, and co-wrote the paper with M.S.Y. S.F. assisted with experiments and critically evaluated the manuscript. R.N.R. and M.J.S. contributed to the overall project design and critically evaluated the manuscript.

ACKNOWLEDGMENTS

This study was funded by BBSRC Grant No. (grant numbers BB/GO15147/1 and BB/GO15988/1). The funder played no role in study design, data collection, analysis and interpretation of data, or the writing of this manuscript.

FUNDING INFORMATION

This work was supported by BBSRC 'Ageing bladder and bowel' grants BB/G015147/1 awarded to MSY and BB/G015988/1 to MJS and RNR and a Royal Society award to BAP.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

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How to cite this article: Yeoman MS, Fidalgo S, Hobby I, et al. Decreases in mucosally-evoked tachykinin signaling pathways can explain age-related reductions in murine colonic motility patterns. *Neurogastroenterology & Motility.* 2024;36:e14891. doi:10.1111/nmo.14891