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Neuropilin 2 Is a Novel Regulator of Distal Colon Contractility

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Appropriate coordination of smooth muscle contraction and relaxation is essential for normal colonic motility. The impact of perturbed motility ranges from moderate, in conditions such as colitis, to potentially fatal in the case of pseudo-obstruction. The mechanisms underlying aberrant motility and the extent to which they can be targeted pharmacologically are incompletely understood. This study identified colonic smooth muscle as a major site of expression of neuropilin 2 (Nrp2) in mice and humans. Mice with inducible smooth muscle–specific knockout of *Nrp2* had an increase in evoked contraction of colonic rings in response to carbachol at 1 and 4 weeks following initiation of deletion. KCl-induced contractions were also increased at 4 weeks. Colonic motility was similarly enhanced, as evidenced by faster bead expulsion in Nrp2-deleted mice versus Nrp2-intact controls. In length-tension analysis of the distal colon, passive tension was similar in Nrp2-deficient and Nrp2-intact mice, but at low strains, active stiffness was greater in Nrp2-deficient animals. Consistent with the findings in conditional Nrp2 mice, *Nrp2*-null mice showed increased contractility in response to carbachol and KCl. Evaluation of selected proteins implicated in smooth muscle contraction revealed no significant differences in the level of α-smooth muscle actin, myosin light chain, calponin, or RhoA. Together, these findings identify Nrp2 as a novel regulator of colonic contractility that may be targetable in conditions characterized by dysmotility. *(Am J Pathol 2022, 192: 1592–1603; https://doi.org/10.1016/j.ajpath.2022.07.013)*

Motility of the gastrointestinal (GI) tract is regulated through coordinated activity of circular and longitudinal smooth muscles. Modulation of this activity occurs by interstitial cells of Cajal (ICCs), enteric neurons, platelet-derived growth factor receptor-α–positive (PDGFRα$^+$) fibroblast-like cells, and various paracrine-acting factors.[1] Alterations in colonic motility can result from a variety of insults, including congenital anomalies, such as Hirschsprung disease, diabetes, inflammation, infection, gut dysbiosis, and loss of appropriate innervation secondary to spinal injury.[2] Furthermore, changes in the magnitude and/or coordination of contractile activity throughout the GI

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tract can lead to dysfunctional motility with ensuing disturbances in intestinal flora, inflammation, and nutrient absorption, often with serious health consequences.[3,4]

External longitudinal and internal circular smooth muscles coordinate and maintain diverse regional patterns of motility that depend in part on the local assortment of membrane receptors, ion channels, and second messenger pathways.[5] Neuropilin 2 (Nrp2) and the related molecule neuropilin 1 (Nrp1) are 130-kDa transmembrane receptors expressed on a range of cell types that mediate the effects of two independent ligand families: class 3 semaphorins and members of the vascular endothelial growth factor (VEGF) family.[6] Interaction of neuropilins with class 3 semaphorin family members regulates axonal guidance in the central and peripheral nervous systems during development.[7–9] Manipulation of neuropilin expression *in vivo* also evokes profound alterations in vascular development, with overexpression or gene deletion yielding deleterious cardiovascular phenotypes.[10,11] Neuropilins have been shown to enhance the activity of VEGF receptor 2 following binding of VEGF family proteins to promote angiogenesis,[12] and have also been implicated in regulation of smooth muscle.[13–19] The first study implicating Nrp2 in regulation of smooth muscle found that exposure of primary smooth muscle cells to the canonical Nrp2 ligand, class 3F semaphorin, led to reduced myosin light chain phosphorylation and a dose-dependent decrease in cytoskeletal tension *in vitro*. However, genetic deletion of Nrp2 *in vivo* led to increased contractility in bladder smooth muscle in unperturbed mice.[17] The study further showed that smooth muscle–specific deletion of Nrp2 could sustain bladder contractility in mice with partial bladder outlet obstruction, and that low Nrp2 expression was associated with enhanced bladder function in men with bladder outlet obstruction.[20]

A recent study by Yamaji et al[21] reported altered GI motility in mice with smooth muscle–specific deletion of Nrp1. In that study, heterozygous deletion of Nrp1 in smooth muscle led to reduced contractility and the emergence of an obstructive phenotype in older mice. However, the role of Nrp2 in GI smooth muscle has not been explored. The current study described a novel role for Nrp2 in regulating colonic smooth muscle contractility. Studies in either mice with constitutive, ubiquitous knockout of Nrp2 or those with inducible, smooth muscle–specific Nrp2 deletion showed that contraction of the distal colon was higher in mice with Nrp2 deletion compared with Nrp2-intact controls.

Materials and Methods

Ethics Statement

All animal studies were performed with approval from the Boston Children's Hospital (Boston, MA) Animal Care and Use Committee and with strict adherence to US Public Health Service and Office of Laboratory Animal Welfare guidelines.

Nrp2 Transgenic Mice

Mice with either constitutive, ubiquitous or smooth muscle–specific deletion of Nrp2 have been described previously.[17,20] Briefly, smooth muscle–specific deletion was accomplished by breeding mice harboring an inducible Cre recombinase ($CreER^{T2}$) under control of the smooth muscle protein 22-alpha (SM22α) promoter (hereafter referred to as *SM22α-CreER*^{T2}),[22] obtained from the laboratory of Dr. Robert Feil (University of Tübingen, Tübingen, Germany), to those expressing floxed alleles of Nrp2 (*Nrp2*$^{tm1.1Mom}$*/MomJ*; stock number 006697; hereafter referred to as *Nrp2*$^{f/f}$[23]), purchased from The Jackson Laboratory (Bar Harbor, ME). Compound *SM22α-CreER*^{T2}*;Nrp2*$^{f/f}$ mice were administered 0.5 mg 4-hydroxytamoxifen (4-OHT; MilliporeSigma, Burlington, MA) per day or a corresponding volume of vehicle (1:19 v/v ethanol/peanut oil) via oral gavage for 3 consecutive days to activate Cre recombinase and induce deletion of *Nrp2*. Mice were tested 1 week ($n = 12$) or 4 weeks ($n = 8$) after initiation of 4-OHT treatment. In early experiments, *Nrp2*$^{f/f}$ mice treated with 4-OHT were also used as controls. Global *Nrp2* knockout reporter mice with green fluorescent protein (*Nrp2*$^{tm1.2Mom}$*/MomJ*[23]; stock number 006700; The Jackson Laboratory) or β-galactosidase (*Nrp2*$^{+/LacZ}$[17,20]) were also used in some experiments.

Immunostaining

Mouse (8 to 12 weeks old) tissues were fixed in 10% buffered formalin for 18 hours, processed for normal histology, and embedded in paraffin. Formalin-fixed, paraffin-embedded sections of normal human colon (T2234090; normal male; 30 years old) were purchased from BioChain Institute, Inc (Newark, CA). Paraffin sections (4 to 6 μm thick) were dewaxed in xylene and rehydrated through a graded series of alcohols to water. For staining of human tissues with anti-human NRP2 antibody, antigen retrieval was achieved using proteinase K (10 μg/mL) for 15 minutes at room temperature; staining of mouse tissues with anti-mouse Nrp2 antibody required heat-induced epitope retrieval in citrate buffer (10 mmol/L; pH 6). Next, tissues were incubated sequentially in 3% H_2O_2 in methanol to block endogenous peroxidase, and in protein blocking buffer [0.5% (w/v) blocking reagent (Perkin Elmer, Hopkinton, MA) in 0.1 mol/L Tris-HCl, pH 7.5, and 0.15 mol/L NaCl] to block endogenous proteins. Sections were stained with rabbit Prestige anti-human NRP2 polyclonal antibody (HPA039980; 1:200 dilution; MilliporeSigma) or rabbit anti-mouse Nrp2 monoclonal antibody (D39A5; 1:100 dilution; number 3366; Cell Signaling Technology, Danvers, MA), followed by biotinylated goat anti-rabbit IgG (BA-1000; 1:200 dilution; Vector Laboratories, Newark, CA) and horseradish peroxidase–conjugated avidin (Vectastain Elite ABC kit; diluted according to manufacturer's instructions; PK-6100; Vector

Laboratories). In some analyses, Alexa Fluor 488–conjugated anti-rabbit secondary antibodies (1:200 dilution; Thermo Fisher Scientific, Waltham, MA) were used. Antibody staining was visualized either with a 3,3′-diaminobenzidine peroxidase chromogenic substrate kit (SK-4100; Vector Laboratories) or by fluorescence imaging. Tissues were counterstained with Mayer hematoxylin (51275; MilliporeSigma) or Hoechst 33258 (B2883; MilliporeSigma). Specimens exposed to secondary antibody alone were included as controls and showed no specific staining. X-gal staining to detect β-galactosidase activity in *Nrp2*$^{+/LacZ}$ cryosections was performed, as described previously[17]; these sections were used as positive controls for detection of Nrp2 because the endothelium of lymphatic vessels is known to express Nrp2.

To examine the distribution of Nrp2 using confocal imaging, the distal colon was embedded in OCT medium and stored at −80°C. Tissue sections were cut (12 μm thick) using a cryostat and fixed with acetone. Slides were washed for an hour in phosphate-buffered saline (PBS) supplemented with 5% donkey serum and 0.05% Triton X-100 and then incubated overnight with Nrp2 monoclonal antibody (Cell Signaling Technology). After washing in PBS, sections were incubated with donkey–anti-rabbit AlexaFluor 488–conjugated secondary antibody (2 hours, room temperature). Sections were double labeled with goat anti-cKit antibody (1:200; sc-1493; Santa Cruz Biotechnology, Dallas, TX), goat anti-PDGFRα antibody (10 μg/mL; AF1062; R&D Systems, Minneapolis, MN), or mouse anti-synaptophysin antibody (1:10; ab8049; Abcam, Cambridge, MA). After extensive washing in PBS, sections were incubated with either anti-goat or anti-mouse AlexaFluor 568 secondary antibodies (1:2000). Sections were examined by confocal microscopy (LSM 710; Zeiss, Oberkochen, Germany). For image acquisition of double-labeled tissue, optical sections (step size = 0.546 μm) were sequentially acquired at each wavelength separately to minimize emission cross talk, and z-projections were generated from six to nine focal planes.

Immunoblot Analysis

Total protein was extracted from frozen colonic tissue using Fast-Prep matrix beads (MPBiomedicals, Solon, OH) in 300 to 500 μL cell lysis buffer [20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 2.5 mmol/L NaPPi, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, and 1 μg/mL leupeptin]. Protein concentrations were determined using the MicroBCA protein assay (Thermo Fisher Scientific). Equal amounts of total protein (5 to 40 μg) were resolved on 10% SDS-PAGE gels, and electrotransferred to nitrocellulose membrane, and protein transfer was verified by Ponceau S stain (MilliporeSigma). After rinsing in PBS and 0.05% Tween-20, then blocking with 10% fat-free milk in PBS and 0.05% Tween-20 for 1 hour, membranes were incubated with primary antibodies overnight at 4°C, including rabbit monoclonal anti-Nrp2 antibody (D39A5; 1:1000 dilution; number 3366; Cell Signaling Technology), mouse anti-RhoA (1:500 dilution; number ARH04; Cytoskeleton, Inc., Denver, CO), rabbit anti–myosin light chain (1:1000 dilution; number 3672; Cell Signaling Technology), mouse anti-calponin (1:5000 dilution; clone hCP; number C2687; MilliporeSigma), mouse anti–smooth muscle actin (1:5000 dilution; clone 1A4; number A2547; MilliporeSigma), or mouse monoclonal anti–β-actin antibody (AC-15; 1:10,000 dilution; number A5441; MilliporeSigma). Membranes were washed 3 × 15 minutes in PBS and 0.05% Tween-20 before incubation with horseradish peroxidase–conjugated species-specific secondary antibodies [horseradish peroxidase–anti-rabbit IgG (number 31460) and horseradish peroxidase–anti-mouse IgG (number 31430); Thermo Fisher Scientific] for 1 hour at room temperature. After membranes were washed in PBS and 0.05% Tween-20, target proteins were detected using enhanced chemiluminescence (Pierce SuperSignal West Pico chemiluminescent substrate; Thermo Fisher Scientific), and signals were visualized after exposure of membranes to X-ray film or quantified using a Chemi-Doc system (Bio-Rad, Hercules, CA).

Isometric Tension Testing

Colonic segments between the anal canal and cecocolic junction were removed from vehicle- and 4-OHT–treated mice. Rings were cut from the distal colons at 0.5-cm intervals. Each colonic ring (three from each mouse) was attached to a force transducer (Grass Technologies, Quincy, MA), and suspended in Krebs solution in an organ bath that was bubbled with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C. Rings were stretched to reach a force of 0.5 g and equilibrated for 1 hour. Following equilibration, spontaneous activity was quantified by calculating the area under the curve. The force-time signal above the baseline tone was integrated over a 4-minute period. Contractile responses to carbachol (1 nmol/L to 10 μmol/L), 60 mmol/L KCl, and electrical field stimulation (EFS; 30 V; 1 to 64 Hz; 0.5-millisecond pulse width; 10-second duration) were measured. To evaluate responses to EFS, the minimum or maximum force produced during the on-stimulation phase was determined relative to the baseline tone (averaged over 30 seconds) preceding stimulation. During the post-stimulation phase, the amplitude of force above the baseline level was measured. To assess the potential contribution of neurotransmitter release to contractions induced by tissue depolarization, we measured the response to KCl in the presence of tetrodotoxin (1 μmol/L). For all analyses, data were calculated as force (millinewtons) normalized by tissue cross-sectional area and are expressed as mean tension ± SEM.

Length-Tension Measurements

Colonic rings were stretched to a length at which force begins to be exerted (L_{ref}) and equilibrated for an hour. Rings were subsequently stretched by 10% of L_{ref} using a computer-controlled motorized lever arm (Aurora Scientific, Aurora, ON, Canada), and passive force was measured after equilibration at the new length. Step changes in length were repeated, and at each length, tissue was stimulated electrically (8 Hz) to measure active tension (total force − passive force). Active and passive force-length curves were generated after force measurements at any length were normalized by maximum active force, and changes in length were normalized to L_{ref}.

Colonic Motility Assay

Mice were fasted overnight in metabolic cages with free access to water. Following anesthesia with isoflurane, a glass bead (2 mm in diameter) was inserted gently into the distal colon to a distance of 2 cm from the anus. The latency of bead expulsion was measured in minutes, essentially as described.[24]

Statistical Analysis

Differences in contractile responses or protein expression between Nrp2-intact and Nrp2-deleted mice were determined by *t*-test for data that were normally distributed or by *U*-test rank-sum test for data that failed normality testing (Shapiro-Wilk). $P < 0.05$ was considered significant.

Results

A previous study showed Nrp2 to be highly enriched in visceral smooth muscle−rich organs, including the bladder and colon.[17] Herein, immunohistochemical analysis was used to demonstrate staining for Nrp2 along the length of the intestine (data not shown). Predominant signal was seen in the circular and longitudinal smooth muscle of the muscularis externa, as well as the muscularis mucosa (Figure 1, A−D), particularly in the colon. In the mucosa, Nrp2 was detected in the lacteals, consistent with expression of Nrp2 in lymphatic endothelial cells.[25] Staining of Nrp2 in human colon (Figure 1, E and F) was verified, and a strong expression was seen in the smooth muscle, consistent with findings in the mouse. Although Nrp2 expression was widespread in smooth muscle cells, other cell types,

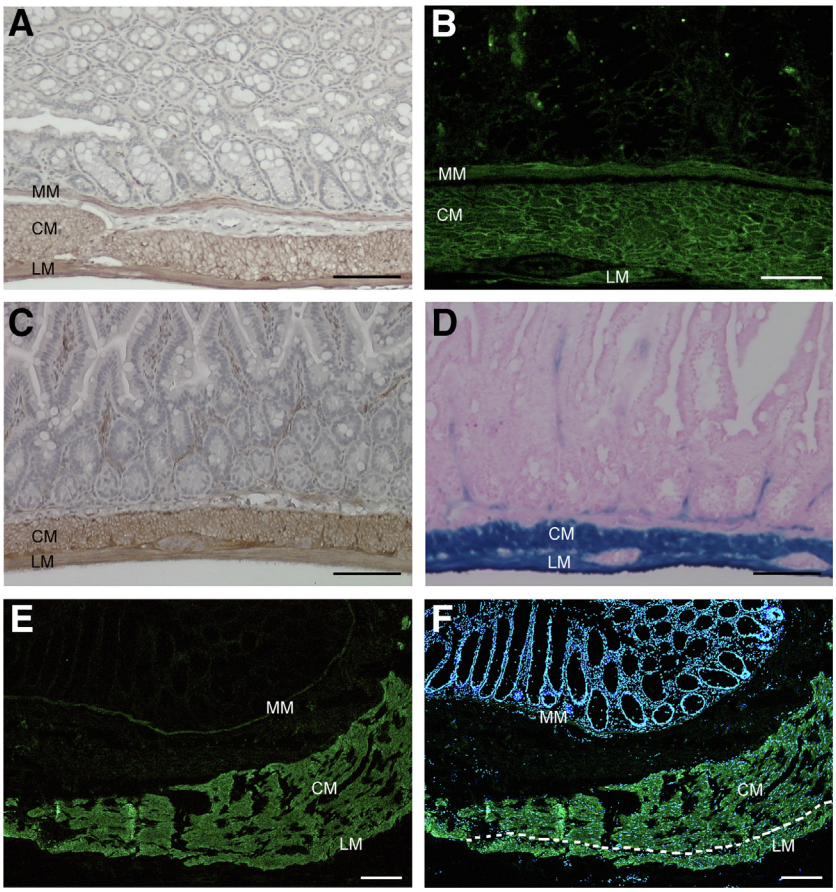

Figure 1 Neuropilin 2 (Nrp2) expression is enriched in mouse and human colonic smooth muscle. **A**−**C:** The 8-week−old C57BL/6 mouse colon (**A** and **B**) or small intestine (**C**) expressing strong Nrp2 signal (brown or green) in visceral smooth muscle layers and in the endothelium of lacteals. **A** and **C:** Nuclei counterstained with hematoxylin (light blue). **B:** Immunofluorescence imaging demonstrating membranous Nrp2 staining (green) in muscle cells. **D:** As a positive control, *Nrp2*$^{+/LacZ}$ mouse small intestine (aged 8 weeks) stained with X-gal reagent showing Nrp2 expression (blue; β-galactosidase) in smooth muscle and lymphatic endothelium. **E** and **F:** Normal human colon sections showing strong NRP2 expression (green) in all visceral smooth muscle layers. **F:** Nuclei counterstained with Hoechst (blue). **White dotted line** added to differentiate circular muscle (CM) from longitudinal muscle (LM). Scale bar = 100 μm (**A**−**F**). MM, muscularis mucosa.

particularly ICCs, PDGFRα-positive cells, neurons, and the myenteric plexus, also expressed Nrp2 (Figure 2).

Published findings demonstrated a role for Nrp2 as a regulator of evoked smooth muscle contraction in the bladder.[17,20] Compound mice in which Nrp2 deletion could be induced selectively in smooth muscle in adult mice by treatment with 4-OHT for 3 days to activate Cre recombinase were generated to determine the impact of Nrp2 deletion on evoked contraction of colonic smooth muscle. Histologic examination by hematoxylin and eosin staining showed no significant morphologic differences between the groups (Figure 3, A and B), whereas immunostaining confirmed Nrp2 knockout in smooth muscle cells in the experimental group compared with control (Figure 3, C and D). Notably, Nrp2 signal was retained in the myenteric plexus in 4-OHT−treated mice, indicating the specificity of the Cre driver. Immunoblot analysis of colonic tissues from *SM22α-CreER^{T2};Nrp2$^{f/f}$* mice following 4-OHT treatment revealed a significant reduction in Nrp2 protein levels compared with that observed in *Nrp2$^{f/f}$* mice also treated with 4-OHT (Figure 3E). In addition, visualization of green fluorescent protein in experimental tissues served as an indicator of successful Cre-mediated recombination (Figure 3F). A significant reduction in Nrp2 was also noted in the colons of *SM22α-CreER^{T2};Nrp2$^{f/f}$* mice treated with 4-OHT versus those treated with vehicle (Figure 3G). To minimize variation in subsequent analyses, *SM22α-CreER^{T2};Nrp2$^{f/f}$* mice treated with vehicle or 4-OHT for functional evaluation were used.

Contractility in the distal colon of Nrp2-deficient mice assayed 1 week following gene deletion was explored and compared with nondeleted controls. Body weight (27.43 ± 3.3 g in vehicle-treated mice, 28.20 ± 2.4 g in 4-OHT−treated mice) and colon length (6.9 ± 0.2 cm in vehicle-treated mice, 7.2 ± 0.2 cm in 4-OHT−treated mice) were not different between treatment groups. The distal colon from Nrp2-deficient mice demonstrated increased contractility in response to carbachol (Figure 4, A and B). However, EC_{50} was not different between groups in the distal colon (vehicle-treated group, $3.42 \times 10^{-7} \pm 1.3 \times 10^{-7}$; 4-OHT−treated group, $2.78 \times 10^{-7} \pm 6.8 \times 10^{-8}$). Contractions generated in response to KCl in the distal colon were not different between groups (Figure 4C). In a separate analysis, colonic rings from 4-OHT−treated *SM22α-CreER^{T2};Nrp2$^{f/f}$* mice were compared with those from 4-OHT−treated *Nrp2$^{f/f}$*

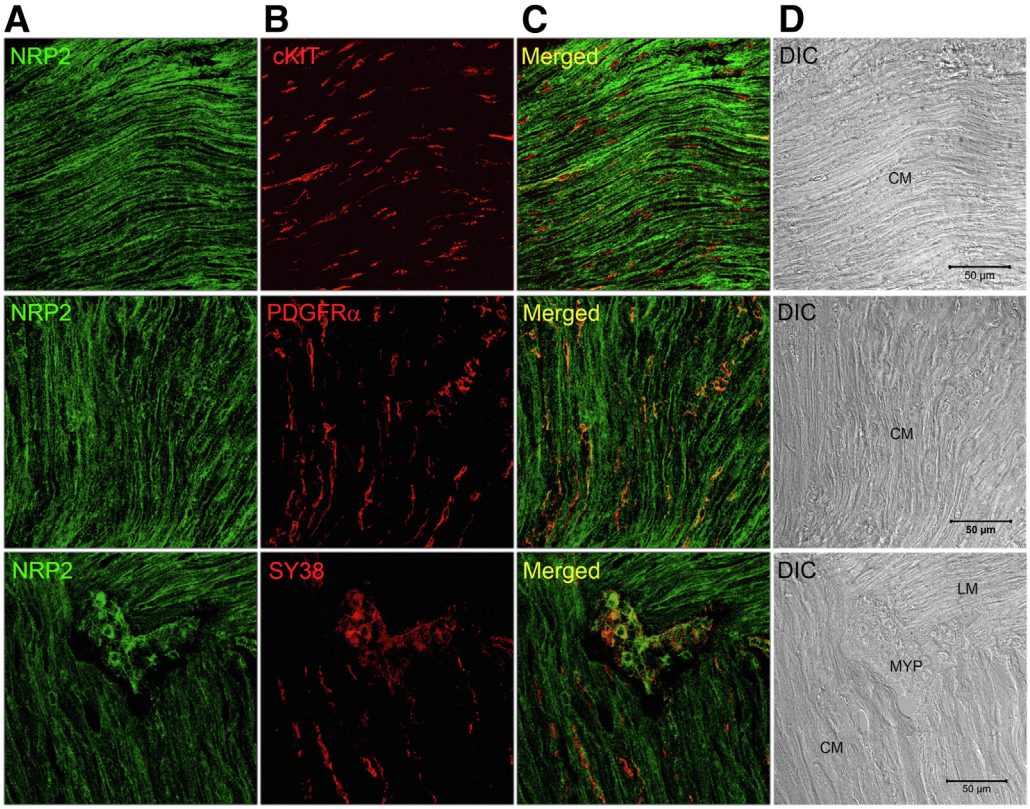

Figure 2 Distribution of neuropilin 2 (Nrp2) in distal colon. **A:** Confocal laser scanning microscopy of distal colon tissue sections from Nrp2-intact mouse. Abundant immunoreactivity for Nrp2 detected throughout the tissue with localization predominantly distributed in the smooth muscle cell membrane (green staining). **B:** Colonic tissue sections double labeled with c-Kit (**top panel**), platelet-derived growth factor receptor-α (PDGFRα; **middle panel**), or synaptophysin (SY38; **bottom panel**). **C:** Colocalized signals in the merged images (yellow) confirmed Nrp2 expression in interstitial cells of Cajal and PDGFRα-positive cells within smooth muscle layers, and in neural structures within the myenteric plexus. **A**−**C:** Images are z-projections of six to nine optical sections. **D:** Differential interference contrast (DIC) images showing tissue topography in each section [circular muscle (CM), longitudinal muscle (LM), and myenteric plexus (MYP)]. Scale bars = 50 μm (**A**−**D**).

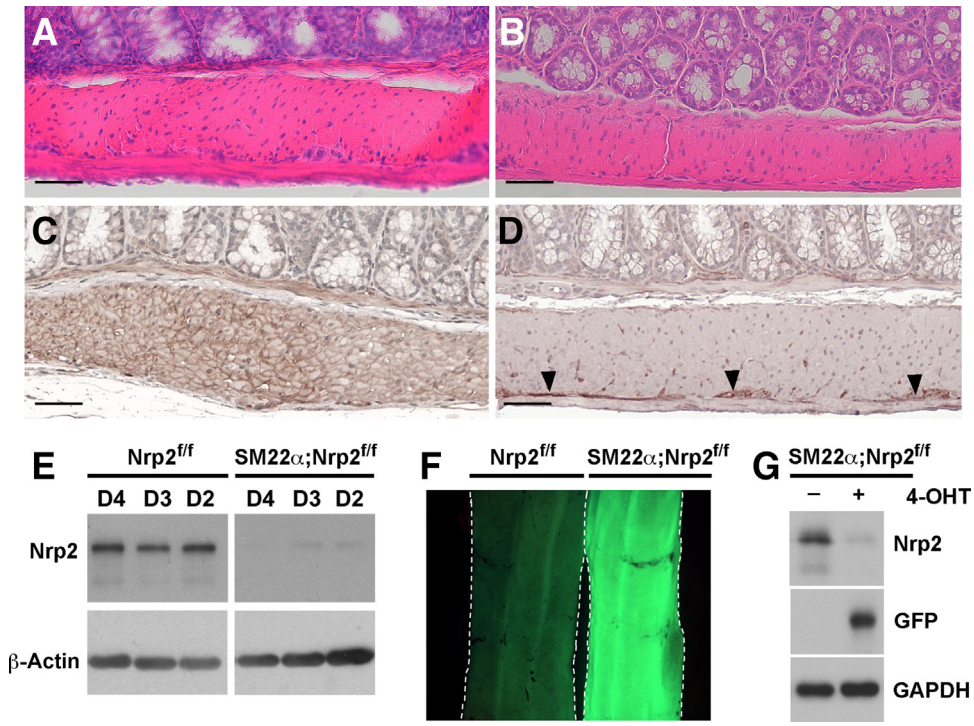

Figure 3 Conditional deletion of neuropilin 2 (Nrp2) in colonic smooth muscle. **A** and **B:** Hematoxylin and eosin images of colon in *Nrp2$^{f/f}$* (**A**) and *SM22αCre;Nrp2$^{f/f}$* mice (**B**). **C** and **D:** Nrp2 immunostaining (brown) illustrating strong expression in control mice (**C**) and tissue-specific loss of Nrp2 from smooth muscle in experimental animals (**D**). Note retention of Nrp2 staining in non–smooth muscle cell types (**arrowheads**). **C** and **D:** Nuclei counterstained with hematoxylin (light blue). **E: Right panels:** Immunoblotting indicating that treatment of *SM22αCre;Nrp2$^{f/f}$* mice with 4-hydroxytamoxifen (4-OHT) leads to efficient deletion of Nrp2 protein from colonic smooth muscle. **Left panels:** Abundant Nrp2 signal detected in *Nrp2$^{f/f}$* mice treated with 4-OHT, used as the control; β-actin used as a loading control. Colon tissue from distal colon (D2, D3, and D4, labeled from anus, with D2 most proximal to anus). **F:** Gross fluorescent images of colons from control (**left side**) and *SM22αCre;Nrp2$^{f/f}$* mice after 4-OHT (**right side**) showing strong green signal in knockout mice, indicating excision of the *Nrp2* allele and expression of green fluorescent protein (GFP). **White dotted lines** were added to outline the edges of the tissues. **G:** *SM22αCre;Nrp2$^{f/f}$* mice were treated with vehicle (−4-OHT) or with 4-OHT (+4-OHT). Distal colonic tissues were lysed and analyzed by immunoblot for protein expression. 4-OHT treatment led to robust deletion of Nrp2 and induced expression of GFP. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a loading control. Scale bar = 50 μm (**A**–**D**).

mice, with the latter serving as an independent control. In agreement with the findings above, contractility was higher in Cre-positive mice versus those without Cre (Supplemental Figure S1A). Contractility of colonic rings from 4-OHT–treated *Nrp2$^{f/f}$* mice was not different from that of vehicle-treated *SM22α-CreER^{T2};Nrp2$^{f/f}$* mice (Supplemental Figure S1B). KCl-evoked contractions in the distal colon were not different between 4-OHT–treated *Nrp2$^{f/f}$* mice and 4-OHT–treated *SM22α-CreER^{T2};Nrp2$^{f/f}$* mice (Supplemental Figure S1C).

To determine the potential contribution of neurotransmitter release to the potassium-induced contractions, responses were repeated after exposure to tetrodotoxin. In the absence of nerve depolarization, contractions evoked by KCl were not different (Supplemental Figure S2A), verifying that KCl-induced contractions predominantly reflect colonic smooth muscle properties.

As an independent test of the impact of Nrp2 deletion on colonic activity in intact mice, expulsion of a glass bead from the colon of Nrp2-expressing mice was assayed and compared to those with smooth muscle–specific Nrp2 deletion. Deletion of Nrp2 was associated with significantly faster bead expulsion, with a mean bead latency of 275 ± 59 seconds compared with 760 ± 187 seconds for Nrp2-intact controls (Figure 4D).

To determine whether evoked colonic contractility changed over time after Nrp2 deletion, contraction in mice 4 weeks following Cre-mediated Nrp2 deletion was assayed. Under resting conditions, the amount of spontaneous mechanical activity generated by the colonic rings was not different between groups (Figure 5A). However, the increased contraction of the distal colon evoked by carbachol, evident at 1 week after Nrp2 deletion, persisted at 4 weeks after deletion (Figure 5, B and C). In contrast to the similar contractile responses generated by KCl after 1 week of Nrp2 deletion, the longer duration without Nrp2 resulted in an increased response to KCl in the distal colon (Figure 5, D and E). The relaxation response to EFS at low frequencies (≤16 Hz) was not different between tissue from Nrp2-intact and Nrp2-deficient mice; contraction induced by high frequencies of stimulation was greater in Nrp2-deficient tissues at 64 Hz only (Figure 5G). In contrast, the contraction following cessation of high-frequency stimulation (16 and 32 Hz) was higher in Nrp2-deficient mice (Figure 5, F and

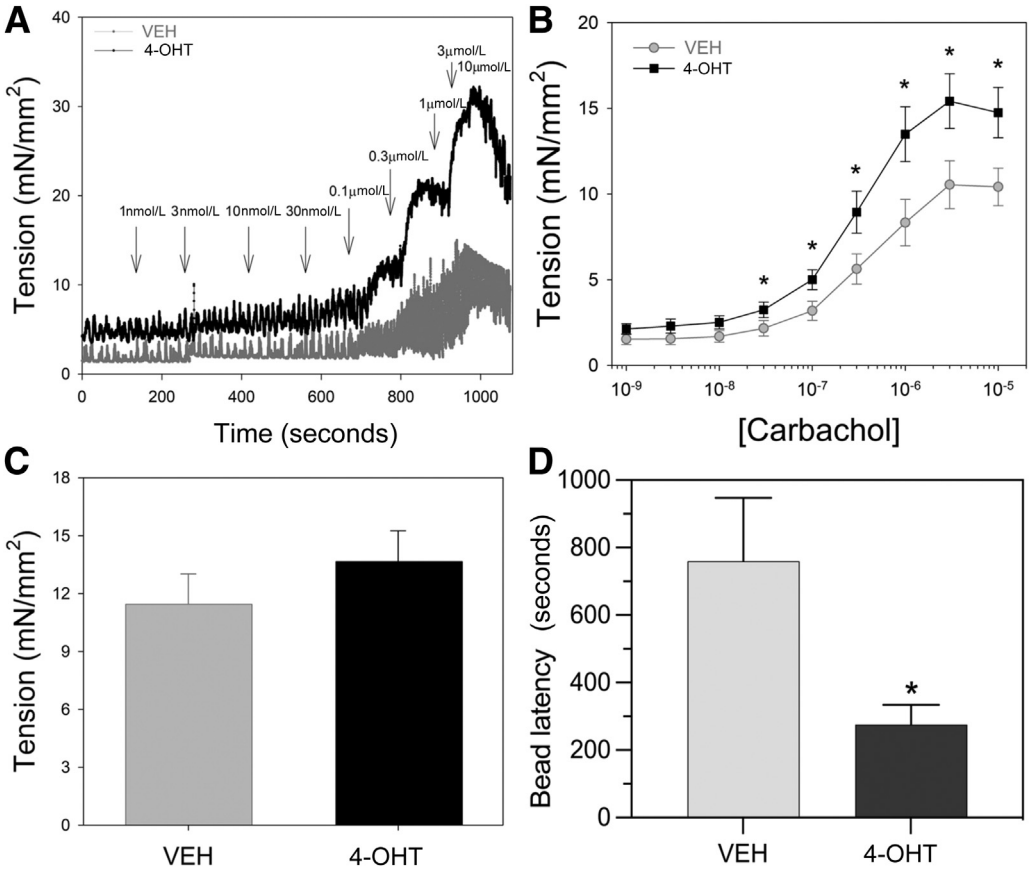

Figure 4 Colonic contractility is enhanced by conditional deletion of neuropilin 2 (Nrp2) in smooth muscle. **A:** Representative tracings of dose-dependent contractile responses to carbachol in colonic rings from Nrp2-deficient (black) and Nrp2-intact (gray) mice. **B:** Contractile response to carbachol in the distal colon 1 week after smooth muscle Nrp2 deletion (black squares) compared with the distal colon of Nrp2 intact mice (gray circles). **C:** Contractions evoked by 60 mmol/L KCl were not different between treated (black bar) and untreated mice (gray bar) in the distal colon. **D:** Time required for glass bead expulsion from the distal colon was determined and found to be significantly less (increased motility) in Nrp2-deficient [4-hydroxytamoxifen (4-OHT)] versus Nrp2-intact [vehicle (VEH)] mice. *P* value is versus mice evaluated 1 week after 4-OHT or VEH treatment. $n = 12$ vehicle- and 4-OHT–treated mice examined after 1 week (**B**); $n = 9$ Nrp2-deficient (4-OHT) mice (**D**); $n = 10$ Nrp2-intact (VEH) mice (**D**). $^*P < 0.05$.

H). Parameters used to generate EFS resulted in contractions that were tetrodotoxin sensitive (Supplemental Figure S2B).

Mechanical properties of the distal colon were explored. The passive force-length relationship of circular smooth muscle in the distal colon of Nrp2-null mice was not different than that in Nrp2-intact mice. Although the length at which maximum active force developed was similar between groups, at short lengths (<1.3 L/L_{ref}), active force was generated at shorter length in Nrp2-deficient mice (Figure 5I).

Because Nrp2 is known to be expressed in cell types other than smooth muscle in the colon, such as interstitial cells of Cajal and PDGFRα-positive cells, evoked contraction in colonic tissue was measured from mice with global deletion of Nrp2. The body weight of mice with knockout of Nrp2 was significantly lower (21.39 ± 0.75 g) than in Nrp2-intact littermates (26.21 ± 0.77 g), although the length of the colon was not different (6.4 ± 0.3 cm in Nrp2-null mice versus 6.6 ± 0.2 cm in Nrp2-intact mice). In the distal colon, Nrp2-null mice showed increased contractions in response to carbachol and KCl when compared with tissue from Nrp2-intact littermates (Figure 6, A–D). No significant difference between Nrp2-intact and Nrp2-null mice was observed in response to EFS in the distal colon (Figure 6, E and F).

To determine the potential molecular basis of differences in contractile activity of the colon following deletion of Nrp2, protein lysates from the distal colon of Nrp2-intact or Nrp2-deficient mice were analyzed 4 weeks following gene deletion for proteins previously linked to smooth muscle contraction regulation (α-smooth muscle actin, myosin light chain, or calponin) and/or implicated downstream of Nrp2 (RhoA).[26,27] As shown in Figure 7, no significant differences were noted in expression of key smooth muscle marker proteins, including α-smooth muscle actin, myosin light chain, or calponin.

Discussion

This study demonstrates a novel role for Nrp2 in regulation of contractility and motility in the GI tract. Previous published and unpublished findings indicate robust expression

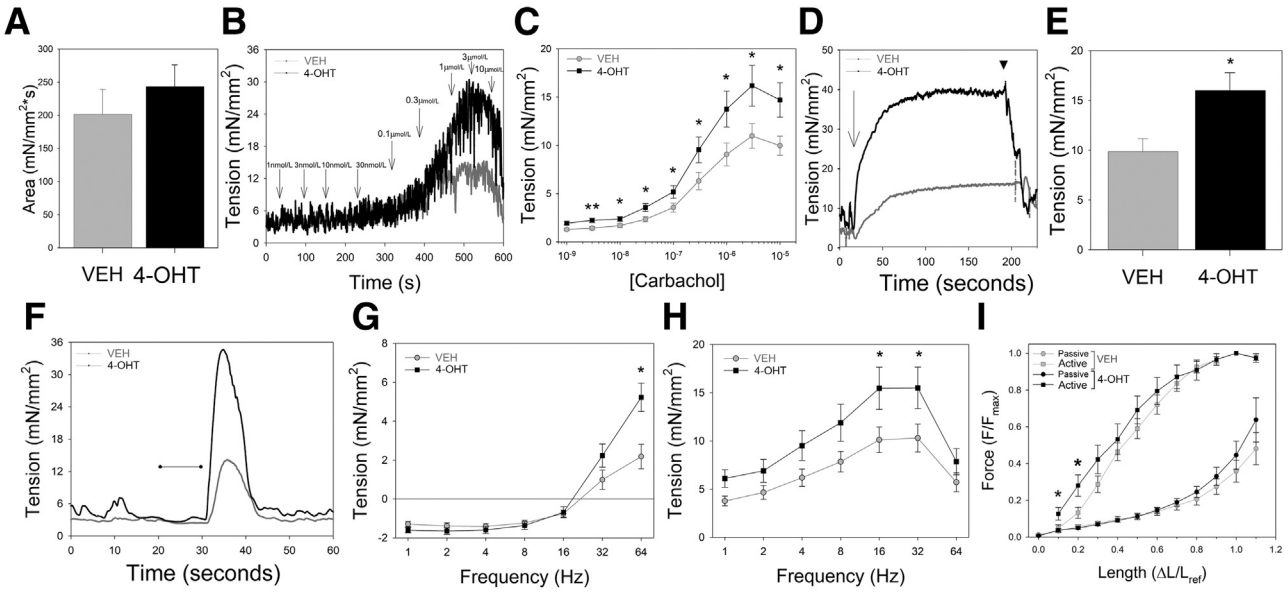

Figure 5 Contractile responses and biomechanical properties after 4 weeks of neuropilin 2 (Nrp2) deletion. **A:** Mechanical activity under unstimulated conditions, measured as the force-time integral, was similar in Nrp2-intact and Nrp2-deficient mice. **B:** Original tracings of responses to increasing concentrations of carbachol (added at **arrows**) in Nrp2-intact (gray) and Nrp2-deficient (black) mice. **C:** Contractions in the distal colon evoked by carbachol were augmented in 4-hydroxytamoxifen (4-OHT)—treated (black symbols) compared with vehicle (VEH)—treated mice (gray symbols). **D:** Representative recordings of contractile responses to KCl. Kreb solution was changed to high potassium at **arrow**, and tissues were washed with normal Kreb solution at **arrowhead**. **E:** Maximum tension generated in colonic rings by KCl was significantly higher in 4-OHT—treated (black bar) compared with VEH-treated (gray bar) mice. **F:** Original recording of mechanical response to electrical field stimulation (EFS) at 8 Hz. Line segment represents delivery of stimulation. **G:** During EFS, relaxation and contractions are measured relative to baseline tone and appear below or above the **solid horizontal line**, respectively. Relaxation responses during low-frequency ($\leq$16-Hz) stimulation were not different; contractions induced by high-frequency stimulation were greater in 4-OHT—treated mice only at 64 Hz (black squares). **H:** After cessation of stimulation, contractions following high-frequency stimulation (16 and 32 Hz) were higher in 4-OHT—treated mice (black squares). **I:** Force-length relationship of distal colon circular muscle in Nrp2-intact (gray symbols) and Nrp2-deficient mice (black symbols). Passive force (circles) and active force (squares) at each length were normalized to the maximum active force (F_{max}) and plotted as a function of the change in length relative to unloaded length. $n = 8$ to 12 vehicle- and 4-OHT—treated mice examined after 4 weeks. $^{*}P < 0.05$, $^{**}P < 0.01$.

of Nrp2 in smooth muscle cells of the bladder, ureter, and airway, but not in vascular smooth muscle, nor in cardiac or skeletal muscle.[17,20] Herein, the study found extensive Nrp2 expression in the colon that was especially prominent in circular and longitudinal smooth muscles. In addition, deletion of Nrp2, either globally or selectively in smooth muscle, led to a phenotype characterized by altered motility and by increased evoked contractions. Enhanced responsiveness of the distal colon early after smooth muscle—specific deletion of Nrp2 was particularly evident in contractions induced by cholinergic stimulation. However, longer-term deficiency in Nrp2 resulted in augmented excitatory responses not only to carbachol, but also to KCl-mediated depolarization and EFS. We speculate that the increases in these widespread excitatory responses likely indicate post-receptor signaling pathways unrestrained by loss of Nrp2, rather than altered muscarinic receptor expression. The post-stimulus contractile response to EFS is preceded by a relaxation response at lower stimulation frequencies that is mediated predominantly by the inhibitory neurotransmitters, nitric oxide, and ATP.[28] Because the relaxation response was unaffected by loss of Nrp2, a reduction in inhibitory neurotransmission can reasonably be excluded as a mechanism underlying our findings of enhanced colonic smooth muscle contractility.

Similar to the findings from mice with conditional deletion of Nrp2, the distal colon from mice with constitutive Nrp2 loss showed an increased contractile response to cholinergic and KCl stimulation. However, an enhanced off contraction in response to EFS was not evident in tissues from these mice. Thus, although deletion of Nrp2 in all cell types for the lifetime of the mouse, versus acute Nrp2 loss specifically in smooth muscle, yielded similar phenotypes, but these were not identical. The divergent contractile response to EFS may reflect differences in the role of Nrp2 in neurons, smooth muscle, and other cells within the colon and at presynaptic and post-synaptic sites.

Smooth muscle—specific loss of Nrp2 enhanced evoked contraction in the colon, suggesting an important role for Nrp2 in coordinated activity of GI smooth muscle. One notable difference between the motility and contractility assays is that motility is assessed in awake mice, whereas contractility is determined in tissues *ex vivo* following equilibration of baseline tension and subsequent stimulation. The enhanced contractile responses in Nrp2-deficient mice were detected despite subjecting each group to an equivalent basal force. Because the length at which maximum active force occurred was similar between groups, differences in evoked contractile responses are unlikely attributable to long-term length adaptation

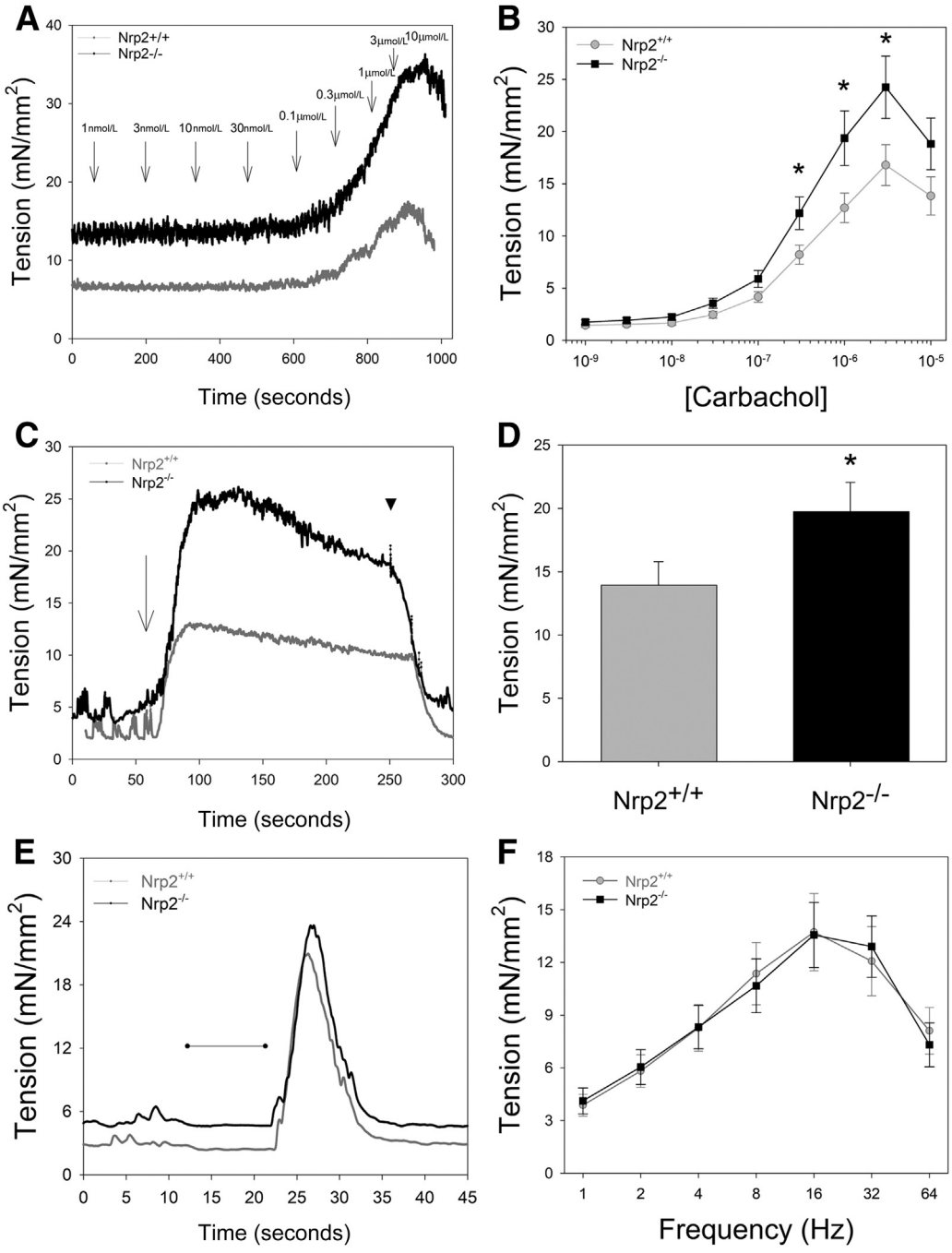

Figure 6 Constitutive deletion of neuropilin 2 (Nrp2) enhances colonic contractile responses. **A:** Representative tracings of responses to increasing concentrations of carbachol (added at **arrows**) in Nrp2-intact (gray tracing) mice and mice with constitutive deletion of Nrp2 (black tracing). **B:** The contractile response to carbachol in distal colonic rings was significantly higher in Nrp2-null mice (squares) compared with Nrp2-intact mice (circles). **C:** Example traces of contractile responses to KCl. **Arrow** indicates replacement of Kreb solution with high potassium. Tissues were washed with normal Kreb solution at **arrowhead**. **D:** Tension generation in response to KCl (60 mmol/L) was greater in the distal colon of Nrp2-null mice. **E:** Original recordings of mechanical response to electrical field stimulation at 8 Hz. Line segment represents duration of stimulation. **F:** Post-stimulation contractions were not different in colonic rings from Nrp2-intact (gray circles) and Nrp2-null (black squares) mice. $n = 14$ $Nrp2^{+/+}$ mice (**D**); $n = 9$ $Nrp2^{-/-}$ mice (**D**). $^*P < 0.05$ compared with wild-type control (littermates) (**B** and **D**).

shifting the force-length relationship. Augmented force generation in Nrp2-deficient mice was accompanied by an enhanced sensitivity to small levels of distension, findings that corroborate the *in vivo* correlate of accelerated motility in the distal colon.

The demonstration of an increase in evoked colonic smooth muscle contraction in Nrp2-deficient mice is in marked contrast to that reported recently in mice with smooth muscle–specific deletion of Nrp1.[21] In that study, loss of Nrp1 in smooth muscle cells led to reduced colonic

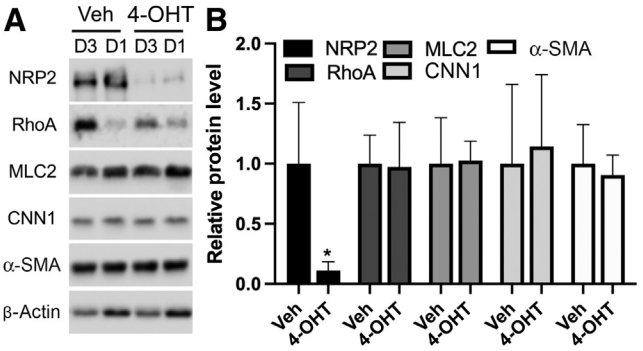

Figure 7 Deletion of neuropilin 2 (Nrp2) does not alter expression of smooth muscle contractile proteins. **A:** Levels of proteins linked to contractility were evaluated in two segments of distal colon (D1 and D3) by immunoblot analysis for the indicated antigens. D1 is closest to anus, and D3 is 1 cm rostral. The immunoblot is representative of three per segment per condition. **B:** Quantification of pooled immunoblot signals for D1 and D3 (**A**) for the indicated proteins. $^*P < 0.05$ compared with tissues from Nrp2-intact mice. CNN1, calponin; MLC2, myosin light chain; 4-OHT, 4-hydroxytamoxifen; α-SMA, α-smooth muscle actin; Veh, vehicle.

contractility in response to KCl or carbachol treatment in mice aged >18 months. Mice also displayed deficits in intestinal motility, as well as structural alterations in colonic smooth muscle. Neuropilin proteins share extensive homology at the protein level, but display largely nonoverlapping patterns of expression in many tissues.[25] At the cellular level, both Nrp1 and Nrp2 form coreceptor complexes with VEGF receptor tyrosine kinases to mediate the actions of VEGF family members and bind class 3 semaphorins in complex with plexins to inhibit RhoA−Rho-associated protein kinase (ROCK) signaling and modulate the actin cytoskeleton.[29] As a result, the opposite phenotypes with smooth muscle−specific loss of Nrp2 versus Nrp1 are somewhat surprising. However, there are several potential explanations as to why the phenotypes may differ. First, unlike Nrp2, Nrp1 is expressed in vascular smooth muscle cells in addition to visceral smooth muscle cells. Although no gross vascular defects were noted in the study by Yamaji et al,[21] the loss of Nrp1 from both visceral and vascular smooth muscle cells may influence the contractile state of the tissue. Second, the conditional deletion strategy in our study involved inducible loss of Nrp2 in smooth muscle cells in young adult mice using inducible *SM22α-Cre^{ERT2}*, compared with smooth muscle cell−specific deletion of Nrp1 using constitutively active *SMMHC-Cre* during development and for >18 months postnatally in the study by Yamaji et al.[21] Thus, the loss of neuropilin expression occurred over vastly different time frames and is likely to have distinct consequences in the affected cells and tissues. It is also possible that the distinct phenotypes observed in mice with deletion of Nrp1 versus Nrp2 reflect differences in the functional syncytium that exists between smooth muscle cells, ICCs, and PDGFRα-positive cells within the gut,[1] all of which express Nrp1 and Nrp2. One implication of the existence of the syncytium is that altered activity of one cell type will impact activity of the other cell types. Consistent with this, perturbation of ICCs or PDGFRα-positive cells has been shown to disrupt motility mediated by smooth muscle.[30,31] Development of the Smooth Muscle Transcriptome Browser has enabled expression profiling of specific genes in smooth muscle cells, ICCs, and PDGFRα-positive cells within the intestine.[32−35] Interrogation of these data reveals differing expression levels of Nrp1 and Nrp2 in colonic smooth muscle (data not shown). Thus, the distinct phenotypes observed in mice with smooth muscle−specific deletion of Nrp1 or Nrp2 are not only a reflection of altered smooth muscle function, but may also reflect changes in ICCs and PDGFRα-positive cells as well. Although interrogation of the contribution of neuropilins expressed in ICCs and PDGFRα-positive cells to altered colonic contractility was beyond the scope of the current study, the influence of these cell types clearly warrants further investigation.

The manifestation of contractile changes within 1 week following deletion of Nrp2 in our conditional mouse model is too rapid to reflect significant tissue remodeling. In fact, the lack of significant differences between groups in passive tissue properties over the physiological lengths examined is consistent with comparable histologic features observed in both groups. Therefore, augmented contractile responses in conditional Nrp2-null mice more likely indicate altered signaling. Previous analyses by us and others have demonstrated that signals transduced by Nrp2 inhibit RhoA/ROCK.[17,26,27,36] Conversely, deletion of Nrp2 is predicted to relieve the inhibition on RhoA/ROCK and thereby promote the activity of effectors, including serum response factor, the myocardin-related transcription factors MRTF-A and MRTF-B,[37] and/or the formin mDia1/2[38] by influencing their subcellular localization. Perturbation of serum response factor signaling was found to evoke profound changes in the intestine.[39,40] In those studies, inducible smooth muscle−specific loss of serum response factor was associated with intestinal obstruction and death within approximately 2 weeks. Contractile activity of colonic tissue from serum response factor deleted mice was significantly diminished compared with that in intact mice and was associated with a marked reduction in smooth muscle−specific contractile proteins, including α-smooth muscle actin, smooth muscle−myosin heavy chain, and calponin.[39,40] In our study, however, no significant differences were noted in the level of SM contractile proteins, suggesting alternative mechanisms are driving the increase in contractile force observed in our analysis.

In summary, we identified Nrp2 as a novel regulator of colonic SM contractility. The ability of Nrp2 to regulate cytoskeletal tone and restrain the contraction of smooth muscle suggests the potential for Nrp2 targeting in the context of aberrant contractility in the gastrointestinal tract.

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Author Contributions

G.L., C.D., E.V., and K.P. performed animal husbandry, experiments, and tissue harvest; V.C. performed tension testing; A.B.-A. performed tissue harvest and immunoblot analysis; D.R.B., M.P.S., and R.M.A. designed and performed the experiments, analyzed data, wrote the manuscript, and secured funding. All authors read and approved the manuscript. D.R.B., M.P.S., and R.M.A. take responsibility for the integrity of the data and the accuracy of the data analysis.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2022.07.013*.

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