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Sympathetic input to multiple cell types in mouse and human colon produces region-specific responses

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Abstract

Background & Aims—The colon is innervated by intrinsic and extrinsic neurons that coordinate functions necessary for digestive health. Sympathetic input suppresses colon motility by acting on intrinsic myenteric neurons, but the extent of sympathetic-induced changes on large-scale network activity in myenteric circuits has not been determined. Compounding the complexity of sympathetic function, there is evidence that sympathetic transmitters can regulate activity in non-neuronal cells (such as enteric glia and innate immune cells).

Methods—We performed anatomical tracing, immunohistochemistry, optogenetic (GCaMP calcium imaging, channelrhodopsin), and colon motility studies in mice and single cell RNA sequencing in human colon to investigate how sympathetic postganglionic neurons modulate colon function.

Results—Individual neurons in each sympathetic prevertebral ganglion innervated the proximal or distal colon, with processes closely opposed to multiple cell types. Calcium imaging in semi-intact mouse colon preparations revealed changes in spontaneous and evoked neural activity, as well as activation of non-neuronal cells, induced by sympathetic nerve stimulation. The overall pattern of response to sympathetic stimulation was unique to the proximal or distal colon. Region-

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specific changes in cellular activity correlated with motility patterns produced by electrical and optogenetic stimulation of sympathetic pathways. Pharmacology experiments (mouse) and RNA sequencing (human) indicated that appropriate receptors were expressed on different cell types to account for the responses to sympathetic stimulation. Regional differences in expression of alpha-1 adrenoceptors in human colon emphasize the translational relevance of our mouse findings.

Conclusions—Sympathetic neurons differentially regulate activity of neurons and non-neuronal cells in proximal and distal colon to promote distinct changes in motility patterns, likely reflecting the distinct roles played by these two regions.

Keywords

gastrointestinal; interstitial cells of Cajal; platelet-derived growth factor receptor-alpha; GCaMP

INTRODUCTION

The gastrointestinal tract executes complex functions to facilitate digestion. The colon in particular is responsible for formation, storage and expulsion of fecal matter, all while maintaining a balanced microbiome, proper epithelial barrier and appropriate immune state. To accomplish this, the enteric nervous system (ENS) initiates reflexes and coordinates activity between neuronal and non-neuronal cell types, including interstitial cells, smooth muscle cells and epithelial cells¹. For example, interstitial cells of Cajal (ICC) are electrically coupled to smooth muscle and induce pace-making waves of depolarization that contribute to motility, the overall pattern of which is influenced by neuromodulation²⁻⁴. Epithelial cells maintain a protective barrier, interact with the microbiome and innate immune cells, secrete mucins, and directly communicate with sensory neurons, including intrinsic primary afferents that initiate local motility reflexes and extrinsic primary afferents that activate spinal/vagal reflexes⁵⁻⁷.

In addition to the ENS, the colon is innervated by extrinsic sympathetic, parasympathetic and sensory neurons, allowing bidirectional communication with the central nervous system. The importance of sympathetic activity to intestinal function is most readily manifest in the key role it plays in regulating motility, secretion, and blood flow during different phases of digestion⁸⁻¹⁰. Changes in blood flow are mediated by sympathetic regulation of vascular smooth muscle, whereas regulation of motility and secretion has been attributed to sympathetic actions on myenteric¹¹ and submucosal neurons^{12,13}, respectively, through alpha-2 adrenoceptor signaling¹⁴⁻¹⁷. Sympathetic nerve stimulation inhibits spontaneous and evoked potentials in myenteric neurons¹⁸, and suppresses reflexive and rhythmic motility patterns in the guinea-pig and mouse distal colon^{11,19}. However, it is unknown whether this also applies to the proximal colon, and direct recordings of sympathetic-induced effects on large-scale, network activity in myenteric neurons are lacking.

An emerging role has also developed for sympathetic neurons as regulators of non-neuronal cells involved in colon function. For example, sympathetic transmitters activate enteric glia in isolated mouse colon²⁰, as well as ICC²¹ and epithelial cells in culture⁵. However, the sympathetic nervous system can exert its effects through epinephrine and norepinephrine

released into circulation from the adrenal gland (i.e., hormonally-mediated) or through neurotransmitters released from sympathetic postganglionic neurons (SPNs) (i.e., neutrally-mediated). Pharmacological experiments are unable to determine if the observed changes in target-cell activity are due to hormonal or neural mechanisms and cannot establish a direct relationship between activation of sympathetic neurons and real-time responses of cells receiving this input. Therefore, it is currently unknown to what extent SPNs activate non-neuronal cell types, and importantly, whether these mechanisms also exist in the human colon.

Here we used *ex vivo* preparations with semi-intact circuits to determine the impact of increasing sympathetic drive on colon motility and on the activity of myenteric neurons, colon epithelium, and interstitial cells. Using mice that genetically express GCaMP (a calcium indicator) in neurons and non-neuronal cells enabled real-time measurement of calcium transients in multiple cell types in response to SPN stimulation. SPN activation directly influenced multiple cell types to alter local and pan-colonic motility patterns in a region-specific manner, with different responses in proximal and distal colon. Remarkably, RNA sequencing provided molecular evidence that some aspects of sympathetic input to human colon are also region-specific, emphasizing the translational relevance of regional differences in sympathetic modulation of colon function.

MATERIALS AND METHODS

Animals

Male and female mice aged 3–6 months were used; information on mouse strains can be found in Supplemental Methods. Mice were housed in an AAALAC-approved facility with a 12-hour light/dark cycle and free access to water and standard chow. Animal use protocols were approved by Institutional Animal Care and Use Committees at University of Pittsburgh and University of Toledo.

Anatomical mapping of sympathetic prevertebral ganglia

NPY-GFP mice were euthanized with isoflurane and transcardially perfused with saline. A midline laparotomy was performed and organs were moved to the side to expose the abdominal aorta and sympathetic prevertebral ganglia (SPG). Anatomical mapping of SPG was performed using an Olympus MVX10 Macro Zoom microscope equipped with X-cite™ 120 Fluorescence Illumination System and Photometrics® CoolSNAP™ ES camera using appropriate filters.

Immunohistochemistry of sympathetic ganglia

See Supplemental Table S1 for all antibody information. For immunolabeling of tyrosine hydroxylase (TH), SPG from NPY-GFP mice were collected, immersion fixed in 4% paraformaldehyde for 30 min, cryoprotected in 25% sucrose in 0.01M PBS at 4°C for 24 hrs, sectioned (14µm) on a cryostat, and mounted on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Slides were washed (3 × 5 min) in 0.01M PBS and incubated in 0.01M PBS containing 10% donkey serum, 0.3% Triton, and a rabbit anti-mouse TH antibody for 24 hours at room temperature. Slides were washed in 0.01M PBS,

incubated (90 min; room temperature) with a donkey anti-rabbit CyTM3 antibody and coverslipped. Two fields per section, 5 sections, >50 μ m apart, per animal were imaged using a Leica DM4000B microscope with Leica EL6000 external light source and Leica DFC7000 T digital camera using 20x objective with appropriate filters.

Whole-mount colon immunohistochemistry

For immunolabeling, full-length colons were dissected, cut along the anti-mesenteric border, pinned flat, and fixed for 2 hours in 4% paraformaldehyde in 0.01M phosphate-buffered saline (PBS). Tissue was washed in buffer containing 0.1M Tris HCl, 1.5% NaCl, and 0.3% Triton X-100 at pH 7.4 (TNT) three times for 30 min, then incubated overnight at room temperature in TNT containing 20% normal donkey serum and primary antibodies. Colons were washed in TNT (4 hours) and incubated overnight at room temperature in TNT containing secondary antibodies. Tissue was imaged as described above.

Back-labeling of sympathetic neurons that innervate the colon

C57BL/6J mice were anesthetized with isoflurane (2%) and laparotomy was performed to access pelvic viscera. Fluorescent retrograde dyes (cholera toxin subunit beta) were injected (5–10 μ l) into the wall of the proximal (below cecum) or distal colon (base of bladder). After 3 days, SPGs were dissected and processed for immunohistochemistry, as described above. Colons were visualized to confirm successful injections.

Ex vivo colon preparations with intact extrinsic sympathetic input

NPY-GFP or NPY-ChR2 mice were euthanized with isoflurane and transcardially perfused with carbogenated (95% O₂ and 5% CO₂) artificial cerebrospinal fluid (ACSF), containing (in mM): 117.9 NaCl, 4.7 KCL, 25 NaHCO₃, 1.3 NaH₂PO₄, 1.2 MgSO₄-7H₂O, 2.5 CaCl₂, 11.1 D-glucose, 2 sodium butyrate, 20 sodium acetate. The colon and SPG were isolated, removed with nerves intact, and pinned to a Sylgard-lined dish while superfused with ACSF at 35°C (Supplemental Figure S1). Fecal contents were removed by gently flushing the lumen with ACSF. Sympathetic-induced changes in motility were measured using electrical or optogenetic stimulation using a 473 nm wavelength laser (Laserglow Technologies, Toronto, Canada) and 1.5 mm optical fiber (ThorLabs, Newton, NJ) placed on or directly above the inferior mesenteric ganglion. For local contractions, colon tissue movement was recorded before, during, and after SPN stimulation using a CMOS camera (Prime 95B Photometrics; Roper Scientific, Tuscon, AZ) under 10x objective. Images were collected with Metamorph software (Molecular Devices, San Jose, CA) at 20Hz sampling rate, 50ms exposure. Tissue movement was quantified using the Template Matching ImageJ plugin. Pan-colonic motility patterns were video recorded (Sony, HDR-CX440) for 20 minutes without stimulation and 20 minutes during SPN stimulation.

For GCaMP imaging, the *ex vivo* dissection was performed in E2aCre-GCaMP6s mice; the colon was cut adjacent to the mesentery, taking caution not to sever SPN-containing lumbar colonic nerves, and pinned flat. Nifedipine (1 μ M, Sigma), an L-type calcium channel blocker, was added to ACSF to improve stability for calcium imaging. In full thickness preparations, this concentration is sufficient to eliminate most spontaneous contractions but does not suppress calcium signaling in ICC²². Recordings of SPN responses were imaged

during electrical stimulation of nerve fibers. Because of the varying amount and location of adipose tissue surrounding nerve fibers, the electrode was placed along the mesenteric nerve in a position that activated the most cell bodies (SPNs). Calcium signals from various cell types were imaged in different colon layers before, during and after stimulation. Transients with a signal amplitude >4 standard deviations above baseline were considered to be “activity.” GCaMP imaging files were processed and analyzed as previously described²². See Supplemental Methods for additional details.

Drug preparation

Solutions containing yohimbine (100nM), guanethidine (3 μ M), or prazosin (1 μ M) (all from Sigma) were prepared with fresh ACSF on the day of the experiment.

Data analysis and statistics for mouse colon experiments

Microscopy images, video recordings of colon motility, and GCaMP imaging files were de-identified and coded for blinded analysis. Data is represented as mean \pm SEM, where n=number of mice. Statistical tests used included paired and unpaired Student’s t-tests, one-way and two-way analysis of variance (ANOVA) with post-hoc tests for multiple comparisons as indicated in the figure legends. Differences were considered significant if $p<0.05$.

For methods regarding human colon samples please see Supplemental Methods.

RESULTS

Distribution of sympathetic postganglionic neurons (SPNs) innervating proximal or distal colon

To visualize sympathetic inputs in a semi-intact model, we used an NPY-GFP reporter mouse to create a montage image of SPN innervation from sympathetic prevertebral ganglia (SPG) to the colon (Figure 1A). In the NPY-GFP reporter mouse, almost all of the TH-immunoreactive (TH+) SPG neurons were NPY-GFP+ (96%; Figure 1B) confirming the utility of this mouse line. Whole-mount immunostaining revealed dense sympathetic innervation within myenteric ganglia, with the majority of NPY-GFP+ fibers colocalized with TH (Figure 1C). NPY-GFP+ fibers in the myenteric plexus were closely associated with NOS+ and NOS- myenteric neurons (Figure 1D), as well as interstitial cells that express c-kit (Figure 1E) or platelet-derived growth factor receptor-alpha (pdgfra; Figure 1F). NPY-GFP+ terminals innervated all layers of the colon (Figure 1G), but the density of expression was region- ($p=0.0025$) and layer-dependent ($p<0.0001$, $n=5$; Figure 1H; there was no significant interaction between colon region and layer). When the hypogastric nerve was ligated and cut, allowing extrinsic fibers to degenerate, the expression of NPY-GFP+ diminished in all layers of the distal colon ($n=3$; Supplementary Figure S2), confirming that the majority of NPY-GFP+ axons in this reporter line arise from extrinsic sympathetic neurons.

To identify the source of sympathetic innervation to proximal and distal colon, two different colors of fluorescently-tagged cholera toxin subunit beta (CTB, 488nm and 555nm) were

used to back-label SPNs (n=8; Figure 1I). Tracer spread was comparable for proximal and distal injections and did not result in overlap in the colon (Supplemental Figure S3). Examination of sympathetic ganglia revealed distinct populations of SPNs that innervated either proximal or distal colon, based on green or red labeling, respectively (Figure 1G–II). Whereas most labeled neurons in the celiac ganglion (CG) projected to proximal colon, the inferior mesenteric ganglion (IMG) and pelvic hypogastric plexus (PHP) had equal proportions of neurons innervating proximal and distal regions, with no apparent topographic organization. The CG contained the greatest number of colon-projecting neurons, but the IMG contained the highest proportion of colon-projecting neurons (~60% versus 10% of TH+ neurons in IMG and CG, respectively).

Sympathetic stimulation suppresses local contractions and alters pan-colonic motility patterns

The effect of sympathetic stimulation on colon motility was examined in an *ex vivo* mouse preparation that maintains SPG continuity with the colon by preserving the lumbar colonic, mesenteric, and hypogastric nerves (LCN, MN, and HGN). Using E2aCre-GCaMP mice, which express GCaMP globally, we characterized spontaneous and electrically-evoked activity in SPNs of the IMG (Figure 2A). Without electrical stimulation, ongoing GCaMP activity was visualized in 10.5±1.9% of SPNs (n=5) and different patterns of GCaMP signals were observed (Figure 2B), reflecting different activity levels. When spontaneous smooth muscle contractions were blocked with nifedipine, the average amplitude and number of calcium transients produced by SPNs decreased (p=0.0105 and p=0.0439, respectively; n=4), suggesting some activity depended on sensory input from the colon presumably via intestinofugal neurons^{23–26}. Electrical stimulation (20Hz, 5s) of the LCN, MN, or HGN antidromically activated many SPN neurons in the IMG regardless of which nerve was stimulated, whereas activation of CG and PHP neurons was location-dependent (Figure 2C). Stimulation of the MN was most effective for activating SPNs in all ganglia and used for the remaining experiments, unless indicated. When different frequencies of stimulation were applied to the same location, the amplitude of GCaMP response from individual neurons increased with higher stimulation frequencies (p=0.0014, n=5 mice, 5–10 cells per experiment; Figure 2D), suggesting a relationship between level of activation and GCaMP signal.

We have previously shown that tissue movement within an imaging field is directly correlated to local changes in smooth muscle tension in the colon²² and used this technique to confirm that activation of SPNs suppressed colon motility. Electrical stimulation of SPNs inhibited ongoing local contractions to 30.5±10.8% of baseline (p=0.0037, n=8; Figure 2E), and after 10–20 sec spontaneous contractions resumed. Post-stimulation movement was often greater than before stimulation (9/16 trials, 56%), suggesting “rebound” contractions were produced as a result of temporary suppression of colon motility induced by sympathetic stimulation. Because electrical stimulation is non-specific and activates all fibers of passage (e.g., spinal sensory and preganglionic sympathetic neurons), we used a mouse model that expresses the blue-light activated channelrhodopsin (ChR2) in NPY neurons to selectively activate SPNs and measure local motility changes (Figure 2F). To restrict laser-stimulation to SPNs, we made a custom “shield” that prevented light from

hitting the colon directly. Similar to electrical stimulation, optogenetic activation (10s) of SPNs decreased local contractions to $36.7 \pm 2.0\%$ of baseline ($p=0.0238$, $n=4$; Figure 2G), and post-stimulation movement was greater than before stimulation in 50% of trials (8/16). The pattern of contractions produced after optogenetic stimulation were rhythmic and occurred at a similar frequency as ICC depolarization (11.4 ± 0.62 cycles/min), suggesting that SPN activation may facilitate motor patterns generated by ICC. Importantly, these data show that both electrical and optogenetic stimulation of SPNs produce comparable changes in local motility.

Colonic migrating motor complexes (CMMCs) are migrating contractions that require neurogenic (i.e., myenteric neurons) and myogenic (i.e., ICC) mechanisms for their initiation, propagation, and rhythmic nature²⁷. The presence or absence of intact SPG circuits affected CMMC characteristics (Supplemental Figure S4), suggesting that peripheral neuron connections to the colon modulate ongoing function in *ex vivo* preparations. We applied electrical (20Hz for 5s) or laser stimulation (10s) to the MN every two minutes to determine whether increasing sympathetic input disrupted CMMCs. Electrical, but not laser, stimulation decreased the frequency of CMMCs (electrical: $p=0.0450$, $n=4$; Figure 2H; laser: $p=0.7220$, $n=4$; Figure 2K), and the length of CMMC propagation was decreased by both electrical and laser (electrical: $p=0.0321$, $n=4$; Figure 2I; laser: $p=0.0088$, $n=4$; Figure 2L), such that migrating contractions rarely reached the distal colon. Ripples are myogenic motility patterns characterized by low amplitude, rapid, non-propagating contractions²⁷. Ripple-like contractions were easily visualized in the proximal colon and increased in response to electrical and laser stimulation (electrical: $p=0.0252$, $n=4$; Figure 2J; laser: $p=0.0495$, $n=4$; Figure 2M). These data suggest that sympathetic input decreases CMMC propagation to the distal colon while promoting myogenic contractions in the proximal colon.

Sympathetic input differentially influences myenteric neuron activity in proximal and distal colon

To understand the cellular mechanisms underlying sympathetic-induced motility patterns, we measured myenteric neuron activity using GCaMP imaging. SPN stimulation had biphasic effects on GCaMP activity in myenteric neurons (Figure 3A). As a population, the percentage of neurons with spontaneous GCaMP activity decreased during SPN stimulation in proximal ($p=0.0204$, $n=8$; Figure 3Bi) and distal ($p=0.0030$, $n=8$; Figure 3Bii) colon. Sympathetic-induced inhibition was often followed by a coordinated increase in GCaMP activity (“post-stimulation excitation,” Figure 3Aii). Although occasionally observed in distal colon (8/26 trials, 31%), increases in neural activity after stimulation were only significant in proximal regions (proximal: 23/28 trials, 82%, $p=0.0067$, $n=8$; distal: $p=0.8890$, $n=8$; Figure 3B). The alpha-2 adrenoceptor antagonist, yohimbine, blocked SPN-induced inhibition in proximal and distal colon (proximal: $p=0.9492$, $n=6$; distal: $p=0.9809$, $n=6$; Figure 3B); however, post-stimulation excitation still occurred ($p=0.0261$, $n=5$; Figure 3B). Interestingly, yohimbine increased the occurrence of post-stimulation excitation in distal colon from 31% (8/26 trials) to 61% of trials (11/18). Blocking norepinephrine release with guanethidine also prevented inhibition of myenteric neurons, but not post-stimulation excitation. In fact, in many instances, the post-stimulation excitatory phase in distal colon

occurred earlier in the presence of guanethidine (data not shown), suggesting that alpha-2 adrenergic signaling may suppress excitatory actions of SPN stimulation in distal colon regions. Overall, these results confirm that SPN-release of norepinephrine inhibits myenteric neuron activity via alpha-2 adrenoceptors, and experiments revealed a delayed, excitatory response in the proximal colon that was not dependent on norepinephrine-mediated inhibition.

Analysis of changes in GCaMP signals from individual myenteric neurons revealed additional insights into sympathetic-induced changes in neural activity. In control experiments (no stimulation), the majority of myenteric neurons did not exhibit significant changes in GCaMP signal amplitude over two or even three consecutive 40-s recordings (Figure 3C–D). By contrast, GCaMP signals were completely blocked in significantly more neurons during SPN stimulation compared to control experiments ($p < 0.0001$, Figure 3E), and as predicted by our population analysis, SPN-induced responses in proximal and distal colon diverged after this inhibitory phase. In distal colon, more neurons were still “blocked” compared to control experiments ($p = 0.008$), whereas more “new” neurons (not active prior to stimulation) began to exhibit GCaMP signals in the proximal colon after SPN stimulation compared to time controls ($p < 0.0001$, Figure 3F). Therefore, sympathetic input to the colon reduces ongoing activity in myenteric neurons, but also recruits activity in a new population of neurons selectively in the proximal colon, presumably causing the observed sequence of changes in smooth muscle contractions.

To test whether region-specific effects observed after SPN stimulation depended on the nerve stimulated, we compared activity changes in response to LCN, MN, and HGN stimulation (Figure 3G). There were more neurons inhibited by SPN stimulation in the distal versus proximal colon, regardless of the nerve stimulated (main effect of colon region, $p = 0.0005$; no effect of stimulus location, $p = 0.1215$; Figure 3H). In proximal colon, there were more neurons excited by SPN stimulation compared to distal colon, especially when the HGN was stimulated (main effect of colon region, $p < 0.0001$; main effect of stimulus location, $p = 0.0013$; Figure 3I). Overall, these data reveal that post-stimulation excitation is specific to the proximal colon, does not depend on alpha-2 adrenoceptor activation, and may involve components unique to pelvic (HGN) sympathetic circuits.

To determine whether different myenteric neuron subtypes are targeted by SPNs in proximal versus distal colon, we repeated experiments using more specific GCaMP models (NOS- and VGLUT2-GCaMP). Inhibitory motor neurons and interneurons express NOS, whereas VGLUT2-expressing neurons are thought to be ascending interneurons or a subtype of sensory afferent. Interestingly, a higher proportion of NOS-GCaMP+ neurons in proximal colon were inhibited with SPN stimulation compared to distal colon ($p = 0.0018$, $n = 3$) whereas more VGLUT2-GCaMP+ neurons were inhibited in distal versus proximal regions ($p = 0.0004$, $n = 3$; Supplemental Figure S5). In the NOS1-GCaMP mouse model, only 34% of GCaMP+ neurons were immunoreactive for NOS1, suggesting that many GCaMP+ neurons expressed NOS1 sometime during development but not in adulthood. VGLUT2-GCaMP+ myenteric neurons represented a more homogenous population, and we verified that no VGLUT2-GCaMP+ neurons were NOS-immunoreactive. Although we cannot confirm whether NOS1-GCaMP+ and VGLUT2-GCaMP+ neurons represent nitrergic and

glutamatergic myenteric neurons, respectively, these data support the concept that sympathetic input to proximal and distal regions is functionally unique.

Sympathetic input has region-specific effects on ascending and descending myenteric circuits

To understand the effects of sympathetic input on myenteric neurons that make up descending and ascending circuits involved in reflexive peristalsis, we compared responses to direct colon stimulation before, during, and after activation of SPNs (Figure 4A). Even without SPN stimulation, evoked responses in proximal, but not distal, colon were significantly decreased in colon preparations with intact SPG compared to isolated colons (main effect of intact SPG in proximal ($p=0.0023$, $n=5$, Figure 4B), no effect in distal colon ($p=0.6914$, $n=5$, Figure 4C)), indicating that ongoing SPN activity is sufficient to inhibit evoked activity of neurons in proximal colon regions. Further increasing SPN activity with electrical stimulation produced region-specific changes. In proximal colon, myenteric neuron responses to anal stimulation (i.e., those that receive ascending input) were significantly decreased during SPN stimulation ($p=0.0070$, $n=5$; Figure 4Dii), but responses to oral stimulation (i.e., those that receive descending input) were not altered (Figure 4Di). In middle and distal colon, myenteric neuron responses to oral stimulation were significantly decreased after SPN activation (middle: $p=0.0461$, $n=5$, Figure 4Ei; distal: $p=0.0055$, Figure 4Fi). Decreases to anal stimulation did not reach significance (Figure 4Eii and 4Fii). These results suggest that sympathetic input suppresses myenteric reflexes in a region-dependent manner; descending myenteric circuits in middle and distal colon regions are targeted to prevent relaxation necessary for anterograde movement of fecal contents, whereas ascending input to the proximal colon is inhibited, likely decreasing reflex-initiated propulsive contractions.

Sympathetic input increases calcium transients in epithelial cells of proximal colon

The colon epithelium contains a heterogeneous population of cells that interact with other cell types to regulate colon functions. Sympathetic input has been reported to influence epithelial cell proliferation²⁸, and studies of intestinal organoids indicate that epithelial enterochromaffin cells have functional alpha-2 adrenoceptors that elicit calcium transients when activated by norepinephrine⁵. To investigate whether sympathetic-to-epithelial communication exists in intact colon preparations, we examined the effects of sympathetic activation on colon epithelial cells using E2a-GCaMP mice (Figure 5A). About half of the colonic crypts per imaging field contained epithelial cells with ongoing GCaMP calcium transients (1–3 cells per “active” crypt). In proximal colon, SPN stimulation increased the number of epithelial cells with GCaMP activity ($p=0.0078$, $n=5$; Figure 5B) and increased the amplitude of GCaMP signal ($p=0.0034$, $n=5$; Figure 5C). The number of crypts with active epithelial cells did not increase ($p=0.1010$, $n=5$; Figure 5D), but SPN stimulation increased the spread of calcium signals between cells within a crypt in proximal colon ($p=0.0023$, $n=5$; Figure 5E). The addition of yohimbine prevented SPN-induced responses ($p=0.0025$, $n=5$; Figure 5F), suggesting that sympathetic input activates proximal colon epithelium via alpha-2 adrenoceptors.

SPN stimulation had no effect on GCaMP activity in distal colon epithelium using the parameters above. However, norepinephrine (10 μ M) applied to isolated distal and proximal colon tissue increased the number of active cells from both colon regions (Supplemental Figure S6). Epithelial cells in distal colon, therefore, appear to express functional adrenoceptors that are not sufficiently activated with nerve stimulation, suggesting that neural and systemic release of norepinephrine produce unique effects with respect to sympathetic actions on colon epithelium via synaptic and volume transmission.

Sympathetic input alters activity of interstitial cells in proximal colon

The syncytium of smooth muscle, c-kit-expressing interstitial cells of Cajal (ICC), and cells that express platelet-derived growth factor receptor-alpha (PDGFRA+ cells), are known as the SIP syncytium, and play an important role in the production of colon motility patterns²⁹⁻³⁰. To determine whether sympathetic-induced changes in motility were modulated in part by interstitial network activity in submucosa (Figure 6A) and/or myenteric plexus (Figure 6D), calcium transients were studied in response to SPN stimulation in E2a-GCaMP mice. Once again, SPN stimulation caused region-specific changes (Figure 6B,6E). In proximal colon, the frequency of calcium transients in submucosal interstitial “pace-making” cells (ICC-SM) was significantly increased ($p=0.0048$, $n=5$; Figure 6C). Yohimbine blocked this response ($p=0.6006$; Figure 6C), but the baseline frequency was increased compared to vehicle, suggesting that ongoing signals from SPNs modulate ICC-SM in the absence of stimulation. Because of the known alpha-2 adrenergic mediated effects on myenteric neurons and the influence they have on ICC³⁻⁴, the effects of SPN stimulation on ICC-SM may be an indirect consequence of neuron activity changes.

SPN stimulation also induced activity in interstitial cells located in the myenteric plexus (IC-MY) that consisted of an increase in: 1) the number of cells with calcium transients, 2) the number of transients evoked, and 3) the amplitude of GCaMP signal (Figure 6D). The percentage of trials that successfully produced activation was higher in proximal versus distal colon ($p=0.0093$, $n=5$; Figure 6E). Responses from IC-MY induced by SPN activation were not blocked by yohimbine ($p=0.8097$; $n=3$), but were suppressed when prazosin, an alpha-1 adrenoceptor antagonist, was added ($p=0.0080$, $n=3$; Figure 6F). Although we were unable to confirm the identity of IC-MY activated by SPNs using the non-specific E2a-GCaMP model, RNA sequencing data from mouse intestine show alpha-1 adrenoceptor expression exclusively in PDGFRA+ cells³¹⁻³³, and a recent study reports norepinephrine-induced calcium transients via alpha-1 adrenoceptors in this specific cell population³⁴. Combined, these data suggest that alpha-1 adrenoceptor activation by norepinephrine released from SPNs directly increases calcium transients in PDGFRA+ cells in the myenteric plexus (IC-MY) of proximal mouse colon, but not in distal regions when all SPG input to the colon is intact.

Molecular evidence that sympathetic neurons regulate human interstitial networks in a region-dependent manner

To determine whether human interstitial cells also express receptors implicated in sympathetic neuromodulation of mouse interstitial networks, we retrieved nuclei of ICC (gene markers *KIT* and *ANO1*), smooth muscle (*MYH11* and *ACTG2*), and PDGFRA+

cells (*PDGFRA*) (Figure 7A; Supplementary Figure S7) from human colon tissue (Supplemental Table S2) and performed RNA sequencing. The gene that encodes stimulatory alpha-1 adrenoceptors, *ADRA1A*, was significantly expressed only in the PDGFRA+ population ($p < 0.0001$; Figure 7B and 7C), and no other adrenergic receptor genes were highly expressed in cells of the SIP syncytium (Supplementary Figure S8). Figure 7D shows that PDGFRA+ cells are comparable between the two colon regions, but remarkably, *ADRA1A* expression was significantly greater in right colon compared to sigmoid colon ($p < 0.0001$; Figure 7E), emphasizing the translational relevance of region-specific sympathetic input to the colon.

DISCUSSION

The ability to visualize sympathetic neuron-mediated, real-time responses across numerous cell types in the colon painted a holistic picture of the cellular mechanisms underlying the well-established effects of adrenergic or sympathetic nerve stimulation on motility. Here, through the use of GCaMP imaging in a mouse colon preparation that keeps sympathetic circuits intact, we revealed a more complex population response of myenteric neurons, the drivers of colon motility, that differed between proximal and distal colon regions. Region-specificity of responses to sympathetic neuron activation extended to epithelial and interstitial cells. Results indicated that sympathetic neurons activate alpha-2 adrenoceptors on epithelial cells and alpha-1 adrenoceptors on interstitial cells (putative PDGFR α + cells) in the myenteric plexus, specifically in proximal colon. Importantly, RNA sequencing of right and sigmoid human colon provided molecular evidence that sympathetic input affects these two regions differently, thus emphasizing the translational and clinical relevance of regional differences in cellular responses to sympathetic input.

The unique effects of sympathetic activity on proximal and distal colon support the concept that these two regions have distinct functions and characteristics. The diversity of responses and cell types receiving sympathetic input in proximal colon supports the idea of increased circuit complexity compared to distal colon³⁵. For example, in proximal colon regions, sympathetic activation transiently reduced activity in a subpopulation of myenteric neurons, but also increased activity in interstitial cells, epithelial cells, and a new population of myenteric neurons that were not previously active. These changes in cellular activity resulted in increased ripples and non-propagating contractions conducive for mixing of fecal contents. The same stimulation suppressed motility to distal colon by inhibiting ongoing and evoked activity of myenteric neurons, as previously described^{11,19}. Whereas Spencer et al. (1999) reported that sympathetic stimulation inhibited both ascending and descending reflexive pathways¹¹, the data presented here showed inhibition only of distal myenteric neurons that received descending input in response to oral stimulation. A number of differences could account for the conflicting results. For example, muscle contractions evoked by mucosal stimulation were measured in the previous study, whereas GCaMP calcium imaging was used here to measure responses in myenteric neurons, rather than muscle activity. We used a shorter electrical stimulus to activate sympathetic neurons, and it is possible that longer stimulation is required for inhibition of ascending pathways. Importantly, the preparation used here contained input from additional sympathetic prevertebral ganglia that may influence responses in the distal colon.

It is possible that some of our results were confounded by the fact that electrical stimulation of the LCN, MN, or HGN does not selectively activate sympathetic postganglionic neurons; rather, activation of pre- and postganglionic neurons, and antidromic stimulation of sensory afferents likely occurred. We reported recently that antidromic stimulation of lumbosacral extrinsic primary afferents with electrical or chemical (capsaicin) stimulation does not directly affect activity in myenteric neurons²², and we did not observe the changes in activity in non-neuronal cells as we report here. However, we cannot rule out the possibility that direct changes occur with antidromic stimulation of thoracolumbar extrinsic primary afferents, especially given the functional and molecular differences of afferents at different spinal levels^{36,37}. Importantly, most of the changes produced by SPN stimulation were blocked with adrenoceptor antagonists, and optogenetic stimulation of SPNs (using NPY-ChR2 mice) and electrical stimulation produced comparable changes in local and pan-colonic motility. Thus, we are confident that the majority of cellular responses described here are in fact due to release of norepinephrine from sympathetic neurons.

Our findings combined with previous studies on sympathetic input to glia²⁰ and immune cells³⁸ support the concept that sympathetic neurons directly impact diverse cell types to modulate functions critical for colon health. Maintenance of ongoing, integrated colon function is complicated by the need to monitor, regulate, and rid the body of an ever-changing luminal content that includes an interdependent microbiome. To do this, the colon must coordinate epithelial/endocrine secretions, a highly reactive immune system, and overall motility to ensure that luminal contents move at the appropriate rate required for waste removal and microbiome balance. Sympathetic postganglionic neurons are anatomically and functionally positioned to influence all of these processes. More generally, sympathetic input appears to act as a moderator between the proximal and distal colon to ensure timely formation, transport, storage and expulsion of fecal contents. Although speculative, one would expect that abnormal sympathetic activity, and the resulting dysmotility, would cause profound changes in the microbiome and immune status of the colon. Future preclinical studies would benefit from characterizing the dysregulation of sympathetic input to different cell types in mouse models of GI disorders (e.g., colon cancer, chemotherapy-induced neuropathy, colitis). Such detailed understanding of the impact of sympathetic signaling would improve treatment of GI disorders that have associated sympathetic dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SPN	sympathetic postganglionic neuron
ENS	enteric nervous system
ICC	interstitial cells of Cajal
TH	tyrosine hydroxylase
NPY	neuropeptide Y
GFP	green fluorescent protein
SPG	sympathetic prevertebral ganglia
CG	celiac ganglion
IMG	inferior mesenteric ganglion
PHP	pelvic hypogastric plexus
LCN	lumbar colonic nerve
MN	mesenteric nerve
HGN	hypogastric nerve
PDGFRA	platelet-derived growth factor receptor alpha
CMMC	colonic migrating motor complex
IC-MY	interstitial cells in myenteric plexus
IC-SM	interstitial cells in submucosal plexus

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BACKGROUND AND CONTEXT

Sympathetic input is thought to regulate colon function by influencing enteric neurons and non-neuronal cells (e.g., enteric glia, epithelium, immune cells), but real-time recordings of cellular responses to direct sympathetic nerve stimulation are lacking. It is also unknown whether sympathetic neurons utilize the same mechanisms to modulate proximal and distal colon function.

NEW FINDINGS

Sympathetic-induced responses in diverse cell types and changes in motility patterns are unique to proximal and distal colon in mice. Molecular evidence from human colon samples highlights the translational relevance of region-specific sympathetic input to the colon.

LIMITATIONS

Clinical studies are required to confirm that sympathetic activity produces unique functional responses in different regions of human colon. Additional studies should also investigate how input from the central nervous system via sympathetic preganglionic neurons influence colon responses.

IMPACT

Sympathetic input differentially regulates activity in proximal and distal colon to maintain ongoing, integrated functions critical for colon health. Dysregulation of sympathetic activity (either pathological or medication-induced) should be considered as a source of dysmotility, microbiome or immune changes associated with gastrointestinal disorders.

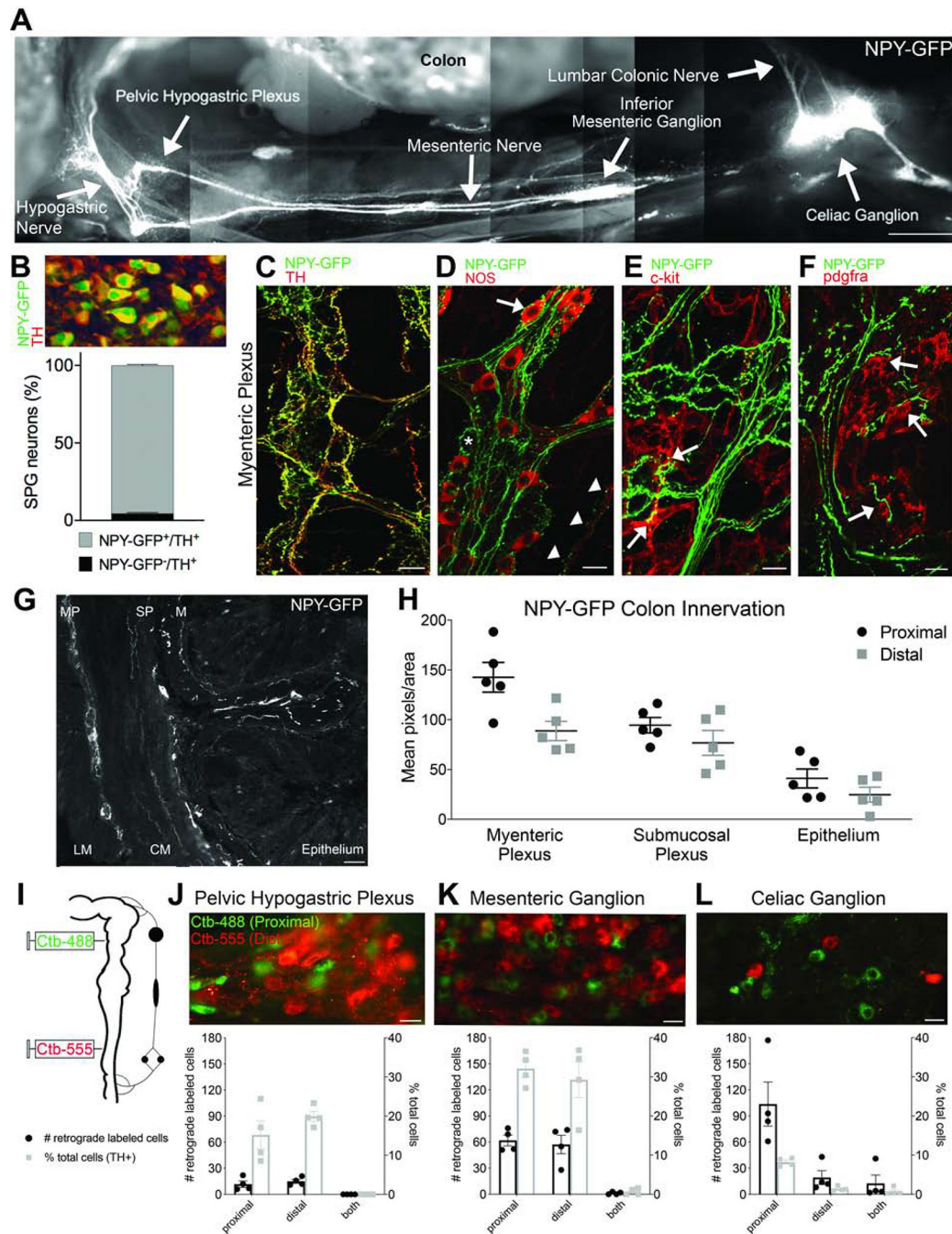


Figure 1. Individual sympathetic postganglionic neurons (SPNs) innervate proximal or distal colon.

(A) Montage shows innervation from sympathetic prevertebral ganglia to colon in NPY-GFP reporter mouse. (B) Quantification of sympathetic ganglion neurons that express NPY-GFP and/or TH (all NPY-GFP⁺ cells expressed TH). (C) Whole-mount immunolabeling reveals colocalization of TH and NPY-GFP⁺ fibers. (D) NPY-GFP⁺ fibers appear to innervate NOS⁺ (arrow) and NOS⁻ myenteric neurons (asterisk); NPY-GFP⁺ varicosities are also closely opposed to NOS⁺ terminals within smooth muscle (arrowheads). (E-F) NPY-GFP⁺ fibers make close contacts to c-kit⁺ interstitial cells of Cajal (E) and PDGFRA⁺ cells (F) in the

myenteric plexus. (G) Cross section of NPY-GFP mouse colon shows innervation to all layers; LM, longitudinal muscle, MP, myenteric plexus, CM, circular muscle, SP, submucosal plexus, M, mucosa, E, epithelium. (H) Comparison of NPY-GFP fiber density in proximal and distal colon. (I) Colon back-labeling experimental design. (J-L) Individual sympathetic postganglionic neurons in the pelvic hypogastric plexus (J), inferior mesenteric ganglion (K), and celiac ganglion (L) project to proximal (green) or distal (red) colon regions. Bar graphs show total number (left axis, black) and percentage (right axis, gray) of labeled cells. Scale bars, 1mm (A), 100 μ m (G), 20 μ m (C-F,J-L).

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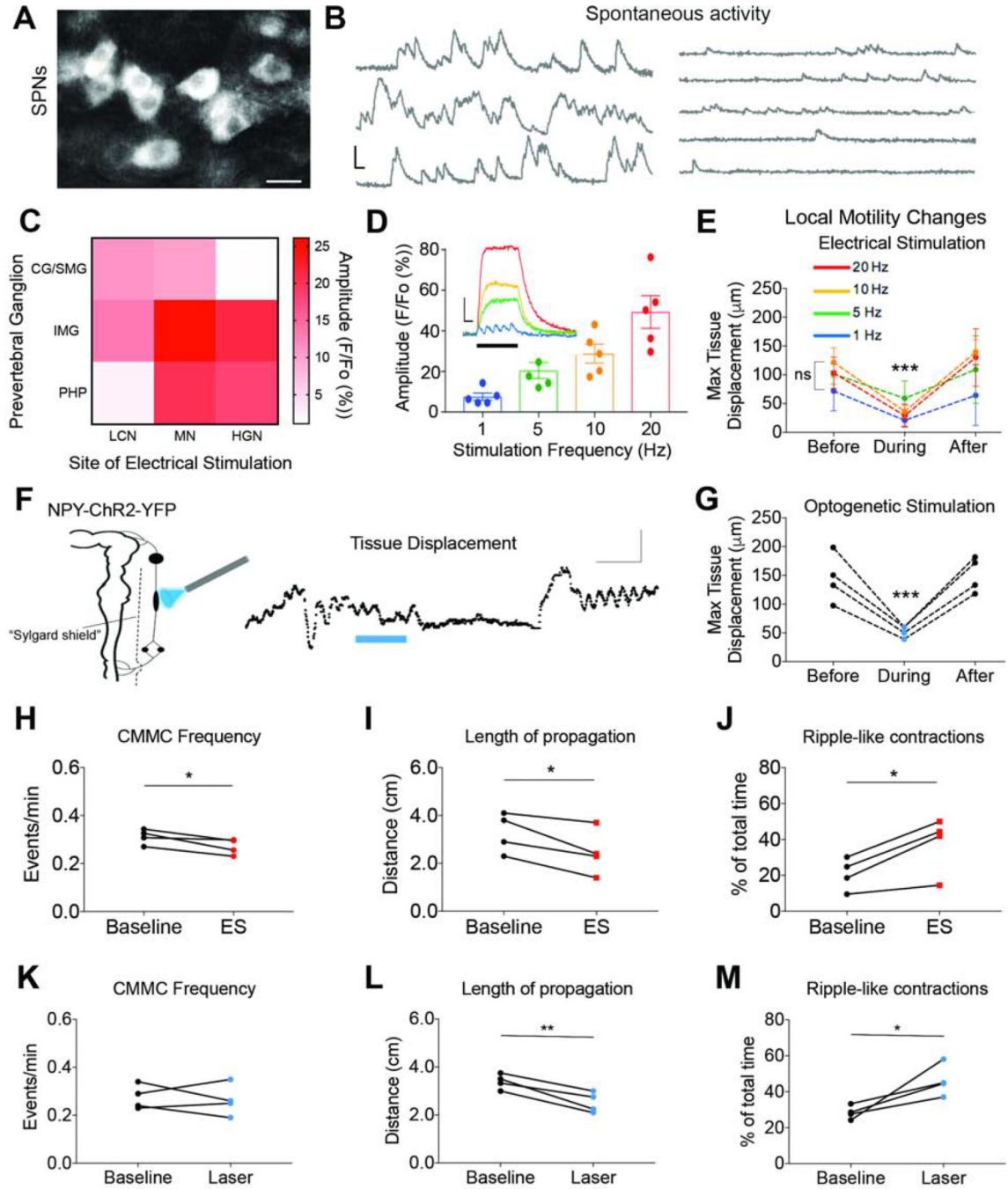


Figure 2. Electrical and optogenetic stimulation of SPNs produce local and pan-colonic motility changes.

(A) Maximum intensity projection of GCaMP fluorescence of SPNs. (B) Example traces of spontaneous activity in SPNs. (C) Electrical stimulation (20Hz) of LCN, MN, and HGN differentially activated neurons in sympathetic prevertebral ganglia; note that only MN stimulation was effective at producing responses (F) in all three ganglia. (D) Amplitude of SPN responses increased as stimulation frequency increased. *Inset*, example GCaMP traces from an individual SPN to electrical stimulation of the MN at 1–20 Hz for 5 s (black bar); (E) Average tissue movement before, during, and after nerve stimulation at 1–20 Hz.

Movement was significantly decreased during electrical stimulation (ES). (F) Experimental design to measure motility changes produced by optogenetic (blue laser) stimulation of SPNs using NPY-ChR2 mice. (G) Laser also significantly decreased local motility during stimulation. (H-J) Effects of ES on pan-colonic motility patterns. (K-M) Effects of laser on pan-colonic motility patterns. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, repeated measures (rm) two-way ANOVA, Tukey's post-hoc correction for multiple comparisons (D,E,G); paired Student's t-test (H-M). Scale bars, 20 μ m, 1s (B,D).

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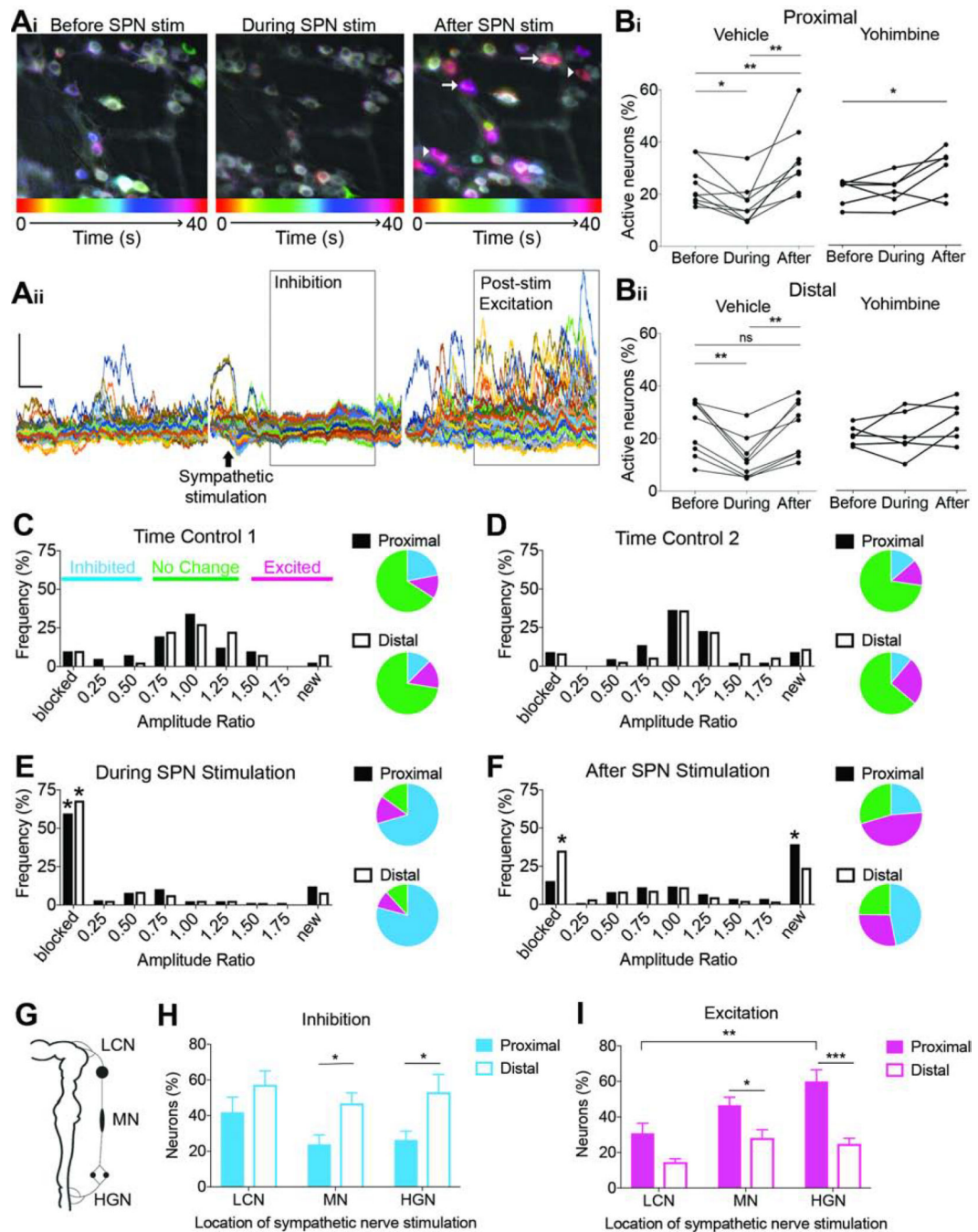


Figure 3. Sympathetic input differentially influences myenteric neuron activity in proximal and distal colon partially mediated via alpha-2 adrenergic receptors.

(A) Time-lapse color-coded images (Ai) and F/F traces (Aii) of GCaMP activity in myenteric neurons before, during, and after SPN stimulation (black arrow). Gray boxes highlight two phases of response (inhibition and post-stimulation excitation) to sympathetic activation. White arrows indicate neurons with increased spontaneous activity and arrowheads indicate neurons with “new” activity. (B) Percentage of myenteric neurons in proximal (Bi) and distal (Bii) colon with GCaMP activity before, during, and after SPN stimulation in the presence of vehicle (left) or yohimbine (right). (C-F) Histograms and

corresponding pie charts showing the relative frequency of neurons in proximal (black) and distal (white) colon exhibiting changes in amplitude of GCaMP signals over time (C and D), during SPN stimulation (E), and after SPN stimulation (F); frequency of blocked neurons during stimulation was significantly higher compared to time-controls in both regions, but after stimulation, proximal colon had more “new” active neurons, whereas more neurons in the distal colon had sustained inhibition compared to time-controls. (G) Schematic illustrates nerves from SPGs to the colon. (H-I) Comparison of the frequency of neurons in the proximal and distal colon that are inhibited (H) or excited (I) after LCN, MN, and HGN stimulation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; rm one-way ANOVA (B) and rm two-way ANOVA (H,I), Tukey’s post-hoc correction for multiple comparisons.

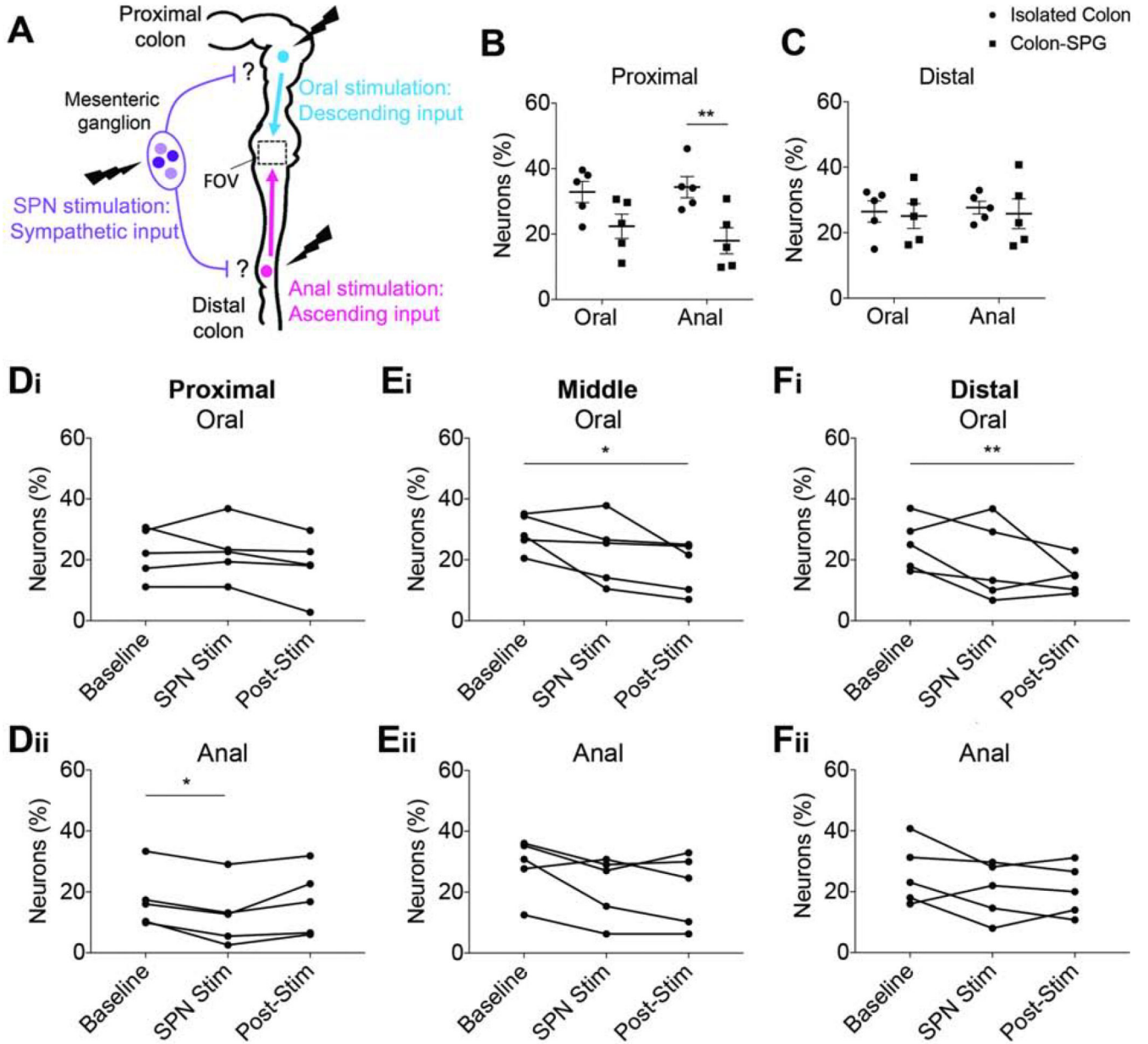


Figure 4. Sympathetic input has region-specific effects on ascending and descending myenteric neuron circuits.

(A) Diagram illustrating experimental setup. (B-C) Percentage of myenteric neurons in proximal (B) and distal (C) colon that responded to colon stimulation in isolated colon preparations (circles) and colon preparations with SPG intact (without external SPN activation; squares). (D-F) Percentage of neurons in proximal (D), middle (E), and distal (F) colon regions that responded to oral (i) or anal (ii) stimulation during and immediately following SPN stimulation. * $p < 0.05$, ** $p < 0.01$; two-way ANOVA (B,C) and one-way ANOVA (D-F) with Tukey's post hoc test for multiple comparisons.

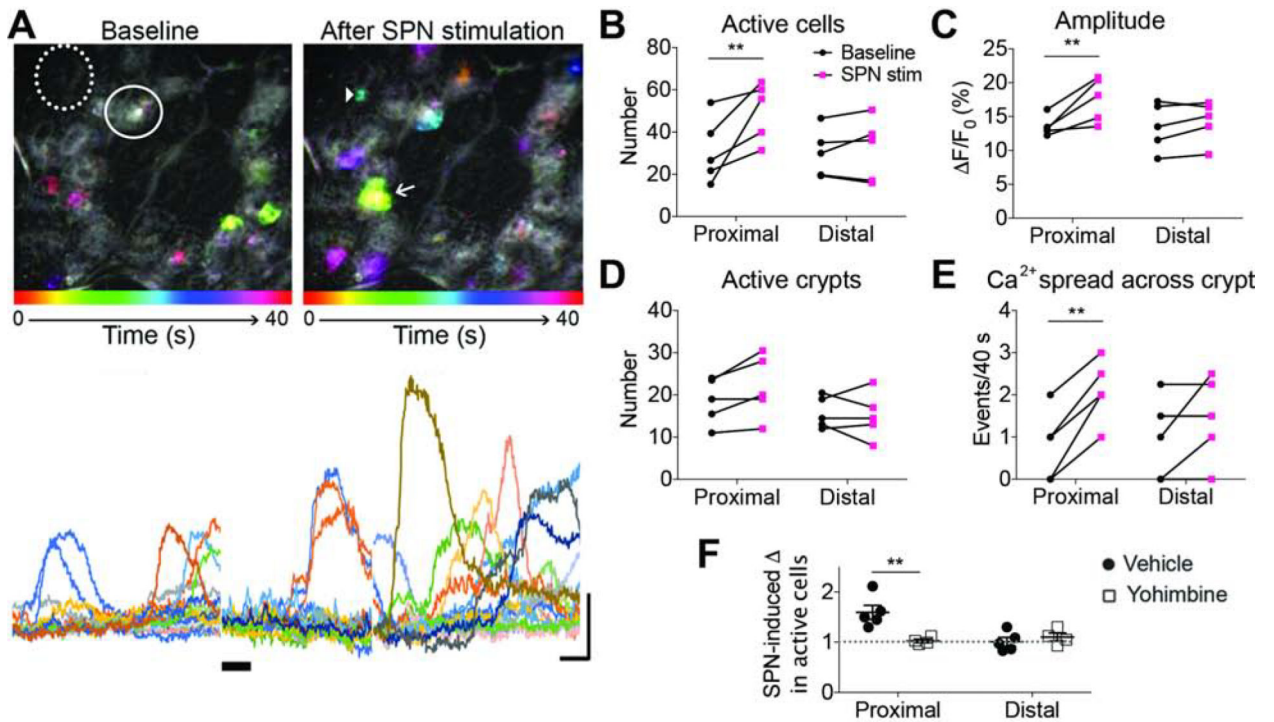


Figure 5. Sympathetic input selectively increases GCaMP activity in proximal colon epithelium. (A) Time-lapse color-coded images (top) and traces (bottom) of GCaMP activity in epithelial cells before (left) and after (right) SPN stimulation. Solid circle, active crypt; dotted circle, inactive crypt; arrowhead, active epithelial cell; arrow, “calcium spread” across >2 cells. (B-E) The number of active epithelial cells (B), amplitude of GCaMP signal (C), number of active crypts (D), and number of “calcium spread” events were measured before and after SPN stimulation and compared between the proximal and distal colon. (F) Effects of yohimbine on SPN-induced changes in active epithelial cells in proximal and distal colon; dotted at $y=1$ represents no change in activity. ** $p<0.01$; rm two-way ANOVA (B,C,E), two-way ANOVA (F) with Tukey’s post hoc test for multiple comparisons.

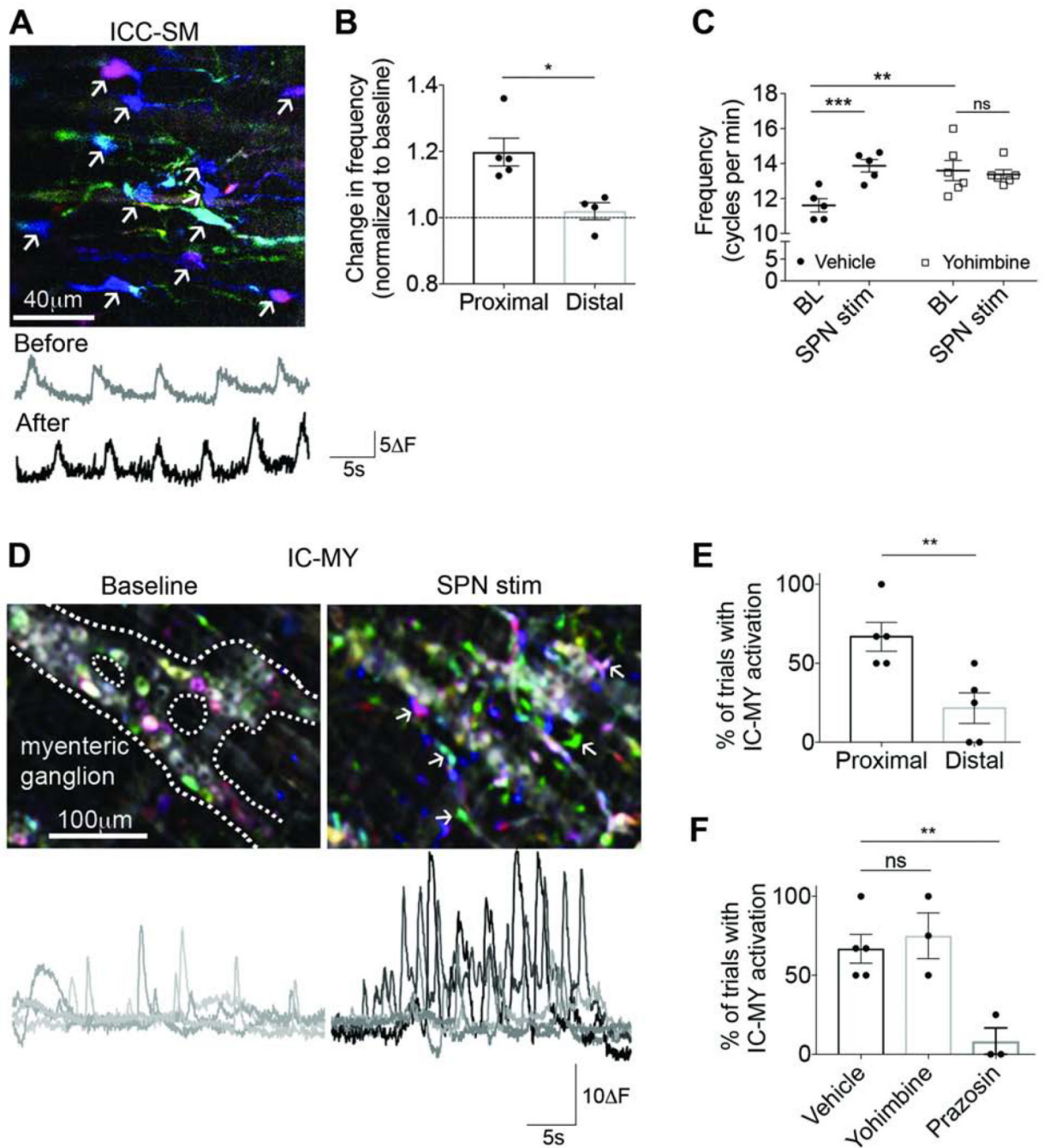


Figure 6. Sympathetic input influences interstitial cells in proximal colon.

(A) Time-lapse color-coded images (left) and traces (right) of GCaMP activity from interstitial cells of Cajal in the submucosal plexus (ICC-SM) (arrows) before and after SPN stimulation. (B) SPN-induced changes in ICC-SM frequency were significantly different in proximal and distal colon. (C) In proximal colon regions, SPN stimulation significantly increased ICC-SM frequency (filled circles, vehicle); yohimbine increased baseline frequency but blocked the response to SPN stimulation (open squares). (D) Time-lapse color-coded images (top) and traces (bottom) of GCaMP activity from interstitial cells in the

myenteric plexus (IC-MY) (arrows) at baseline and with SPN stimulation. (E) Percentage of trials with SPN-induced activation of IC-MY in proximal colon was significantly greater than distal colon. (F) Yohimbine did not prevent IC-MY activation, but alpha-1 receptor antagonist, prazosin, significantly decreased IC-MY activation due to SPN stimulation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Student's unpaired t-test (B,E), two-way (C) and one-way (F) ANOVA with Tukey's post hoc test for multiple comparisons.

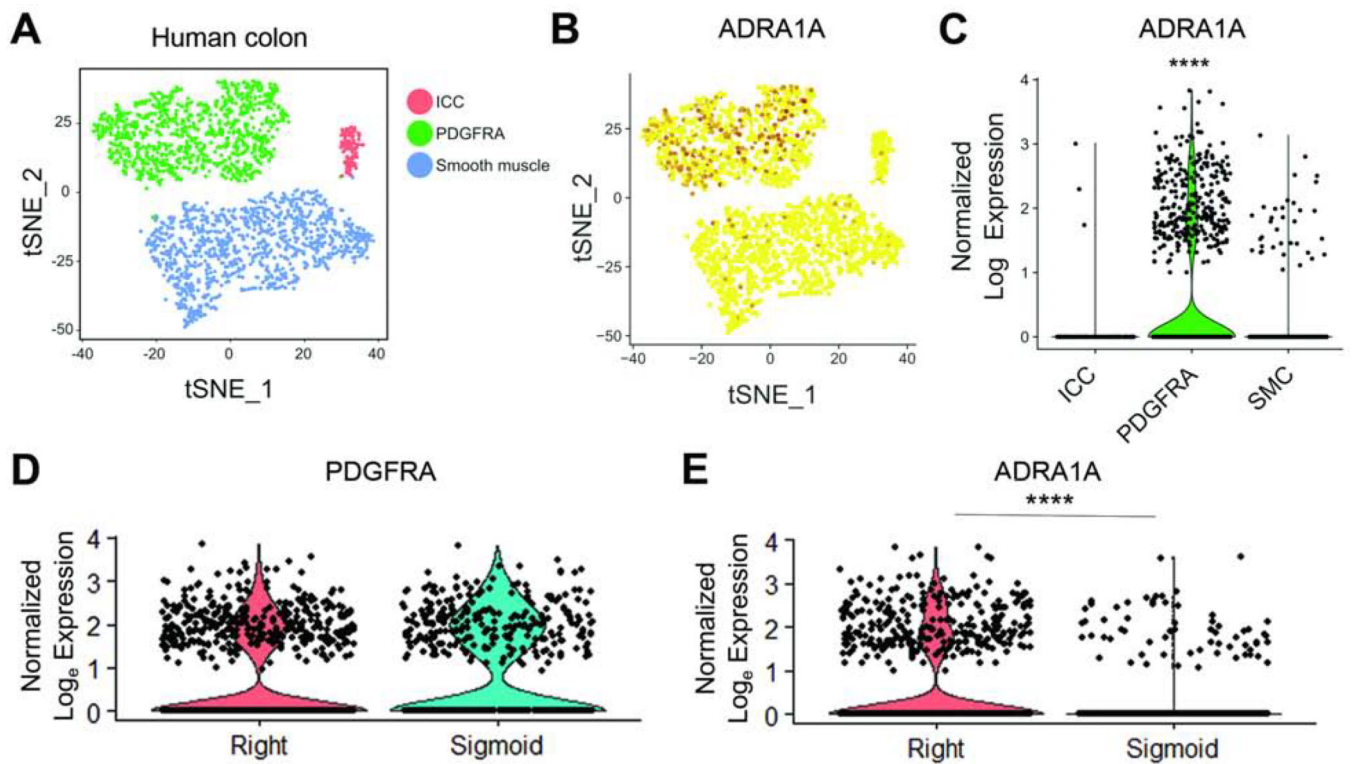


Figure 7. RNA sequencing of human interstitial cells provides molecular evidence that region-specific responses to sympathetic input exist in human colon.

(A) T-SNE plot showing 1684 SIP syncytium nuclei from human colon; ICC, PDGFRA+, and smooth muscle cells (SMC) formed distinct clusters, identified using canonical markers (Supplementary Figure S7). (B-C) T-SNE and violin plots showing the adrenoceptor, *ADRA1A*, was highly expressed in PDGFRA+ cells, but not in other groups. (D-E) *ADRA1A* expression was significantly higher in right colon compared to sigmoid colon, despite no difference in *PDGFRA*. *** $p < 0.0001$; Wilcoxon rank sum test with Bonferroni correction (C,E).