University Library

MINERVA ACCESS

A gateway to Melbourne's research publications

Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:
Hung, LY;Boonma, P;Unterweger, P;Parathan, P;Haag, A;Luna, RA;Bornstein, JC;Savidge, TC;Foong, JPP

Title:
Neonatal Antibiotics Disrupt Motility and Enteric Neural Circuits in Mouse Colon

Date:
2019-01-01

Citation:
Hung, L. Y., Boonma, P., Unterweger, P., Parathan, P., Haag, A., Luna, R. A., Bornstein, J. C., Savidge, T. C. & Foong, J. P. P. (2019). Neonatal Antibiotics Disrupt Motility and Enteric Neural Circuits in Mouse Colon. Cellular and Molecular Gastroenterology and Hepatology, 8 (2), pp.298-300.e6. https://doi.org/10.1016/j.jcmgh.2019.04.009.

Persistent Link:
https://hdl.handle.net/11343/251010

License:
CC BY-NC-ND

cmgh
CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

RESEARCH LETTER

Neonatal Antibiotics Disrupt Motility and Enteric Neural Circuits in Mouse Colon

Early life antibiotics and microbiota alterations are linked to increased susceptibility to gastrointestinal (GI) diseases commonly associated with enteric neuropathy and dysmotility.[1,2] The GI tract houses a complex microbiota ecosystem that interacts with the enteric nervous system (ENS). The early postnatal period is a critical window for functional coupling between the developing ENS and microbiota,[2,3] but little is known about the bidirectional signaling or the impact of early life antibiotics on gut pathophysiology. Although there are reports of microbiota regulating the ENS,[4–6] there are no systematic studies of the effects of antibiotics on the neonatal ENS in a more clinically relevant, non–germ-free state.

Antibiotics, including vancomycin, often are administered to preterm infants to prevent GI diseases, such as necrotizing enterocolitis. To investigate the impact of early life antibiotics on gut microbiota and ENS development, we administered vancomycin orally to neonatal mice to reduce systemic effects and assess its impact on the ENS and gut motility. Vancomycin or water was given to pups in each litter, from birth to postnatal day (P)10. By P10/P11, microbiota composition and diversity were altered significantly in the small and large intestines. This included significant increases in the relative abundance of *Firmicutes* at the expense of *Bacteroidetes* and *Proteobacteria* (Figure 1*A*), and perturbations of gram-positive and gram-negative families of bacteria (Supplementary Figure 1*G*). In vitro spatiotemporal mapping methods showed increased frequency and anal propagation speed of colonic contractions of vancomycin-treated pups compared with control littermates (Figure 1*B–D*). Increased colonic motility in vancomycin-fed pups involved significant changes in the composition and function of colonic myenteric neurons (Figure 2). The density of colonic myenteric cells

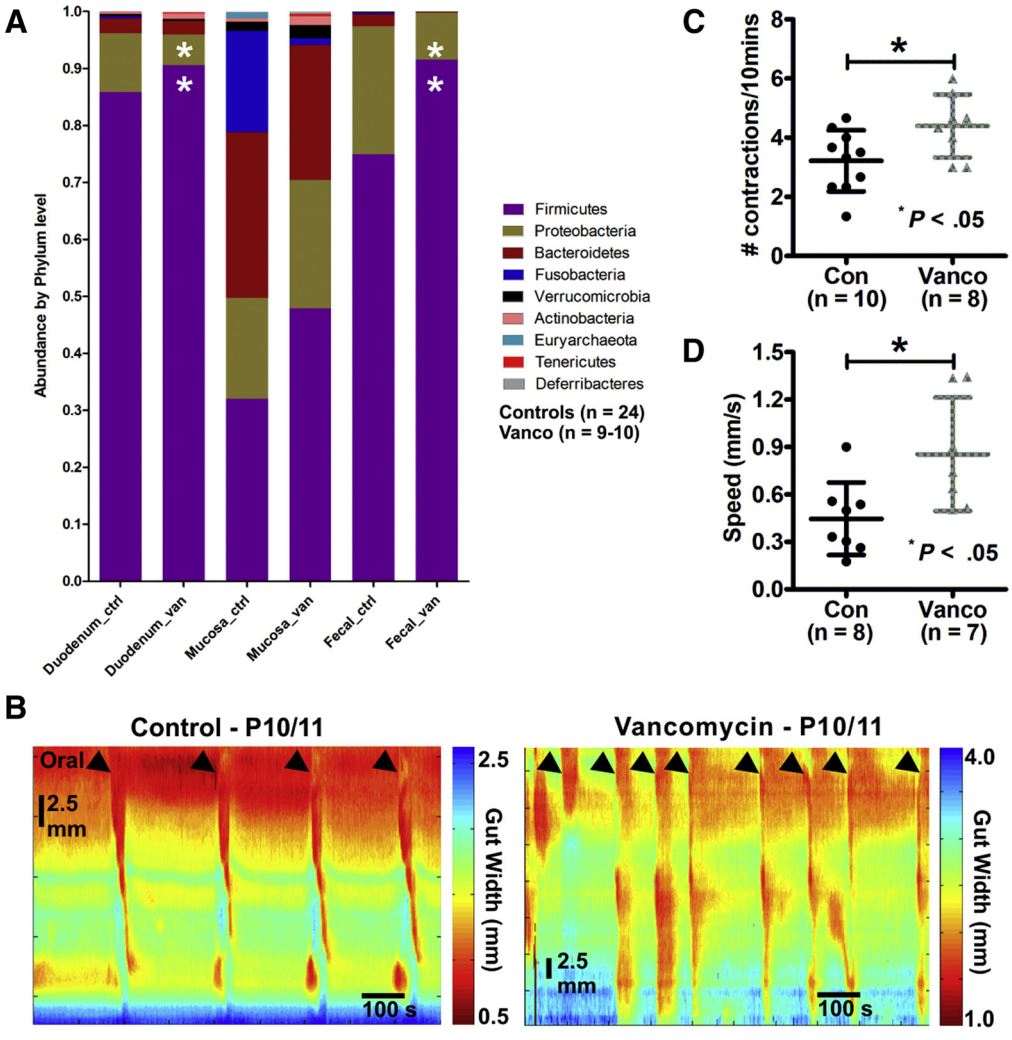


Figure 1. Neonatal vancomycin alters microbiota composition and disrupts colonic motility in P10/P11 mice. (*A*) Microbiota composition at the phylum taxonomic level. (*B*) Spatiotemporal maps of colonic motility from water- (control [Con]) and vancomycin-fed (Vanco) pups, propagating contractions indicated by *arrowheads*. (*C*) Frequency and (*D*) propagation speed represented as means ± SD. (*A* and *C*) *Mann–Whitney test, (*D*) *2-tailed unpaired *t* test.

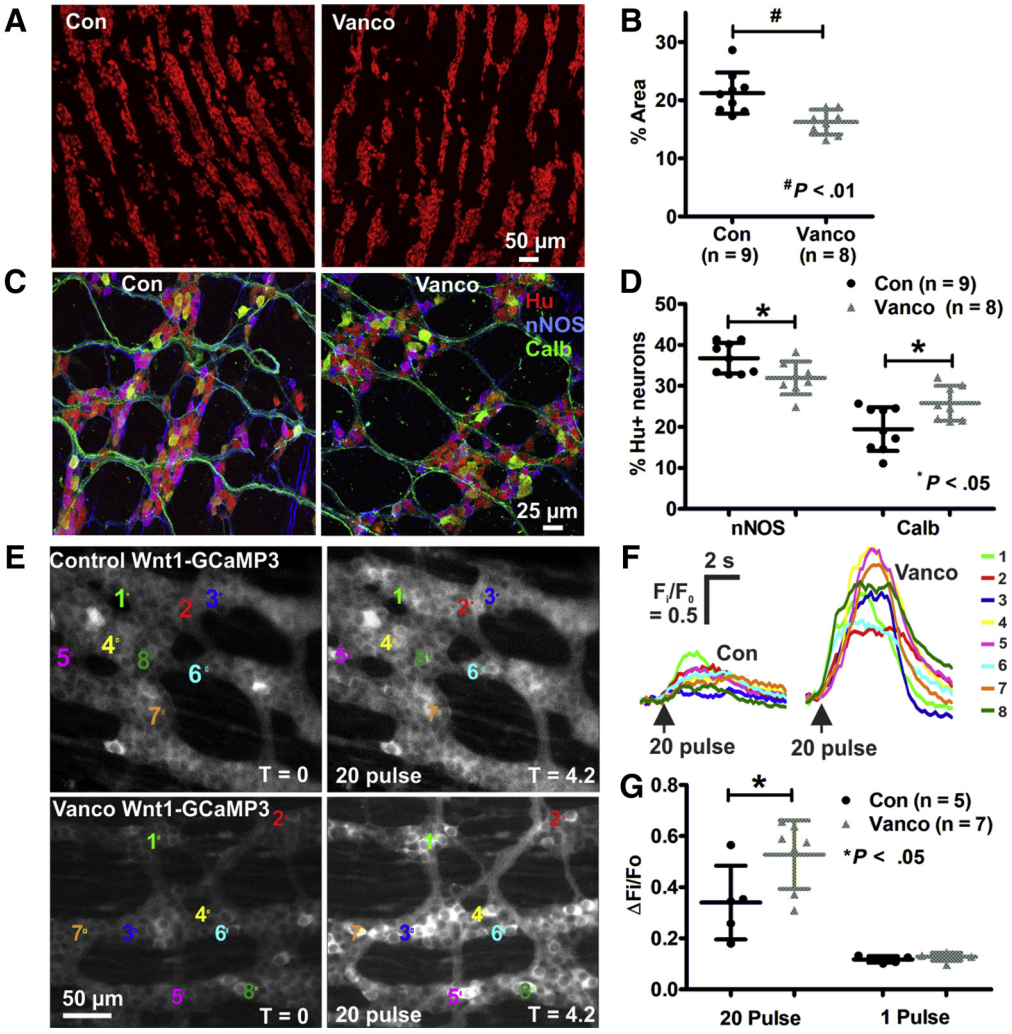


Figure 2. Neonatal vancomycin (Vanco) disrupts the structure and function of enteric neurons in P10/P11 mouse colon. (*A*) Myenteric plexus immunostained for pan-neuronal Hu (red). (*B*) Hu+ density quantification. (*C*) Myenteric plexus immunostained for Hu (red), nNOS (blue), and calbindin (green). (*D*) Proportions of nNOS+ and calbindin+ neurons relative to Hu+ neurons. (*E*) Fluorescence micrographs of 20 pulse train (20 Hz)-evoked $[Ca^{2+}]_i$ responses in myenteric neurons (GCaMP3 signal at rest [t = 0]; and after 20 pulse train stimulation [t = 4.2 s]). (*F*) Eight representative responding neurons in each condition are marked numerically (panel *E*), and their color-coded traces show 20 pulse-evoked $[Ca^{2+}]_i$ responses. (*G*) Amplitude of $[Ca^{2+}]_i$ transients ($\Delta F_i/F_0$) from all cells stimulated with a 20 pulse train vs a single pulse stimulus. Graphs represented as means ± SD. *,#Two-tailed unpaired *t* test. Con, control.

expressing the pan-neuronal marker, Hu, was notably reduced after vancomycin treatment, whereas S100β+ glial density was unaffected. Moreover, proportions of neuronal nitric oxide synthase (nNOS) neurons were reduced, but proportions of calbindin neurons, a subtype of cholinergic neurons, were increased. In contrast, no neurochemical or motility differences were found in the duodenum (Supplementary Figure 2*A*–*C*). The unaffected duodenal ENS suggests that acute drug neurotoxicity is not the major effector.

Ca^{2+}-imaging performed on colons from mice expressing the Ca^{2+}-indicator GCaMP3 in all enteric neurons and glia showed that more neurons responded to electrical stimulation of interganglionic fiber tracts after vancomycin treatment (Supplementary Table 1). This increase in transmission to myenteric neurons was mediated by a specific slow transmission 20 pulse train-evoked $[Ca^{2+}]_i$ response,[3] whereas single-pulse evoked responses were unaffected (Figure 2*E*–*G*). Post hoc immunofluorescence located these larger train-evoked $[Ca^{2+}]_i$ transients to neurons lacking nNOS (Supplementary Table 1). Thus, reduced nNOS+ neurons, including inhibitory motor neurons, and the increased proportion and synaptic excitation of calbindin+ and other excitatory neurons in the myenteric circuitry,[7] may contribute to the antibiotic-induced boost in colonic motility.

In adult mouse colon, antibiotics modulate serotonin (5-hydroxytryptamine [5-HT]) levels and gut motility via microbiota signals that promote expression of tryptophan hydroxylase 1; the rate-limiting enzyme in enteric mucosal 5-HT biosynthesis.[6,8] At P10/P11, vancomycin-fed pups had significantly fewer colonic 5-HT+ mucosal cells, which we confirmed by quantitative mass spectrometry of 5-HT levels and its biosynthetic intermediates (Supplementary Figure 3*A*–*C*). Decreased 5-HT can accelerate gut motility. Indeed, colonic migrating complexes in tryptophan hydroxylase 1 knockout mice are abnormal and propagate faster compared with wild-type mice,[9] consistent with our observations of faster colonic contractions in vancomycin-fed pups. The 5-HT transporter Serotonin-selective reuptake transporter (SERT) (*Slc6a4*) gene expression was higher in vancomycin-treated pups, but expression of

enterochromaffin cell lineage (chromogranin A) and tryptophan hydroxylase 1 was unaltered (Supplementary Figure 3*D*). Thus, we assessed the role for 5-HT signaling in vancomycin-induced colonic motility by co-treating pups with vancomycin and 5-hydroxy-L-tryptophan (5-HTP) from birth to P10/P11. 5-HTP supplementation prevented the antibiotic-induced loss of the 5-HT metabolite, 5-hydroxyindole acetic acid, loss of myenteric neuronal density, and an increase in colonic contraction frequency (Supplementary Figures 3*C* and 1*A* and *C*). However, synaptic activity in the myenteric plexus remained enhanced, as was the speed of colonic contractions in vancomycin + 5-HTP–treated animals, showing that more than one pathway mediates vancomycin effects in neonatal animals (Supplementary Figure 1*B*, *D*, and *E*, and Supplementary Table 1). Similarly, although 5-HTP supplementation prevented the effects of the antibiotic on microbial α-diversity, the microbiota composition differed significantly from controls (Supplementary Figure 1*F*–*H*). 5-HTP co-treatment also reduced SERT expression (Supplementary Figure 3*D*), which may account for protective effects on neuron density, because enteric neurogenesis is related inversely to SERT.[10]

Previous studies have identified microbial involvement in the regulation of ENS and gut motility by using germ-free mice or prolonged exposure to high doses of broad-spectrum antibiotic mixtures to abolish microbiota.[4–6] Our studies advance these key findings by providing mechanistic insight into acute antibiotic treatment-induced enteric neuropathy and dysmotility at a critical point in development, the period immediately after birth. Future studies should identify the mediators of antibiotic-induced developmental effects in the neonate, elucidate modulatory mechanisms of 5-HTP supplementation, and determine the consequences for adults of these disturbances during the immediate post-natal period.

LIN Y. HUNG,[1] *PRAPAPORN BOONMA,*[2,3,4] *PETRA UNTERWEGER,*[1] *PAVITHA PARATHAN,*[1] *ANTHONY HAAG,*[2,3] *RUTH ANN LUNA,*[2,3] *JOEL C. BORNSTEIN,*[1] *TOR C. SAVIDGE,*[2,3] *JAIME P. P. FOONG,*[1]

[1]Department of Physiology, The University of Melbourne, Parkville, Victoria, Australia

[2]Department of Pathology and Immunology, Baylor College of Medicine, Houston, Texas

[3]Texas Children's Microbiome Center, Texas Children's Hospital, Houston, Texas

[4]Faculty of Medicine, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand

Corresponding author: e-mail: j.foong@unimelb.edu.au.

References

1. Brierley SM, et al. Nat Rev Gastroenterol Hepatol 2014; 11:611–627.
2. Munyaka PM, et al. Front Pediatr 2014;2:109.
3. Foong JP, et al. J Physiol 2012; 590:2375–2390.
4. Collins J, et al. Neurogastroenterol Motil 2014;26:98–107.
5. De Vadder F, et al. Proc Natl Acad Sci U S A 2018;115:6458–6463.
6. Yano JM, et al. Cell 2015; 161:264–276.
7. Sang Q, et al. Cell Tissue Res 1996; 284:39–53.
8. Reigstad CS, et al. FASEB J 2015; 29:1395–1403.
9. Heredia DJ, et al. J Physiol 2013; 591:5939–5957.
10. Margolis KG, et al. J Clin Invest 2016;126:2221–2235.

Abbreviations used in this letter: **ENS, enteric nervous system; 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxy-L-tryptophan; GI, gastrointestinal; nNOS, neuronal nitric oxide synthase; P, postnatal day; SERT, serotonin-selective reuptake transporter.**

Most current article

© 2019 The Authors. Published by Elsevier Inc. on behalf of the AGA Institute. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

2352-345X
https://doi.org/10.1016/j.jcmgh.2019.04.009

Received January 31, 2019. Accepted April 15, 2019.

Author contributions

Joel C. Bornstein, Tor C. Savidge, and Jaime P. P. Foong designed the study and obtained funding; Lin Y. Hung, Prapaporn Boonma, Petra Unterweger, Pavitha Parathan, Anthony Haag, Ruth Ann Luna, and Jaime P. P. Foong acquired data; Lin Y. Hung, Prapaporn Boonma, Petra Unterweger, and Jaime P. P. Foong analyzed the data; and Lin Y. Hung, Joel C. Bornstein, Tor C. Savidge, and Jaime P. P. Foong wrote the paper.

Conflicts of interest

The authors disclose no conflicts.

Funding

This research was supported by a National Health and Medical Research Council of Australia Project grant APP1099016 (J.P.P.F., J.C.B., and T.C.S.); Australian Research Council grants DP130101596 (J.C.B.), P30-DK56338, U01-AI24290, and R01-AI10091401 (T.C.S.); and a University of Melbourne International Research and Fee Remission Scholarship (L.Y.H.).

Supplementary Materials and Methods

Mice

Wnt1-Cre (RRID:MGI:2386570) heterozygous mice[1] were mated with *R26R-GCaMP3* homozygous mice (RRID:IMSR_JAX:014538; Jackson Laboratory, Bar Harbor, ME) to produce progeny that expressed the genetically encoded calcium indicator, GCaMP3, in all enteric neurons and glia (*Wnt1-Cre;R26R-GCaMP3*),[2] or did not express GCaMP3. Newborn mice were designated P0 animals. Pups from each litter were given a single dose of the following: (1) sterile water or vancomycin hydrochloride (83 mg/kg^{-1}/d^{-1}; total volume, 2.0 μL/g body weight; Sigma Aldrich, Castle Hill, New South Wales, Australia)[3]; or (2) sterile water or vancomycin hydrochloride plus 5-HTP (vancomycin, 83 mg/kg^{-1}/d^{-1}; 5-HTP, 25 mg/kg^{-1}/d^{-1}; total volume, 2.0 μL/g body weight) from P0–P9/P10 by oral feeding daily using a micropipette tip. At P10/P11, within 24 hours from which animals received their last treatment, mice were killed by cervical dislocation, a procedure approved by the University of Melbourne Animal Experimental Ethics Committee. Vancomycin treatment significantly reduced the body weights of P10/P11 mice (Supplementary Figure 1*D*). The duodenum and colon were removed and immediately placed in physiological saline (118 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 11 mmol/L D-glucose, 4.8 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 1.0 mmol/L NaH_2PO_4) bubbled with carbogen gas (95% O_2, 5% CO_2) or in phosphate-buffered saline.2

Video Imaging of Gut Motility Patterns

Gut motility patterns were analyzed as previously described.[4,5] Briefly, gut segments were cannulated in an organ bath superfused with carbogenated physiological saline at 35°C–37°C and their contractile activities were recorded using a digital video camera. The recordings were converted into spatiotemporal maps using in-house software (Scribble 2.0) and a purpose-built MatLab (Mathworks, Chatswood, NSW, Australia) (2013b) plugin, which then were analyzed using Analyse2 software.

Immunohistochemistry

Segments of midcolon or duodenum were fixed overnight in 4% formaldehyde in 0.2 mol/L phosphate buffer, pH 7.2, at 4°C. After 3 × phosphate-buffered saline washes, whole-mount preparations of myenteric plexus with adhered longitudinal muscle were obtained via microdissection.[6] Midcolon sections (14 μm) were obtained via cryostat.[7] Preparations then were incubated for 30 minutes with 1% Triton X-100 (ProSciTech, Thuringowa, Queensland, Australia), followed by 3 × phosphate-buffered saline washes and 24- to 48-hour incubation with primary antibodies (rabbit anti-calbindin, 1:1600, SWANT (Swant/CB-38a, Marly 1, Switzerland)/CB-38a; sheep anti-nNOS, 1:1000 [a gift from Dr P. Emson]; human anti-Hu, 1:5000 [a gift from Dr V. Lennon]; rabbit anti-S100β, 1:1000, Z0311 [Dako, Carpinteria, CA]; rabbit anti–5-HT, 1:1000, 20080 [Immunostar, Hudson, WI]) at 4°C. After 3 phosphate-buffered saline washes and a 2.5-hour incubation with secondary antibodies all (raised in donkey) (anti-rabbit AF 647, 1:400, A31573; anti-rabbit AF 594, 1:400, A21207; anti-rabbit AF488, 1:400, A21206; anti-sheep AF 647, 1:500, A21448; and anti-sheep AF 488, 1:400, A11015; all ThermoFisher Scientific, Hudson, WI; anti-human 594, 1:750, 709-585-149; Jackson Immuno Labs, West Grove, PA) at room temperature, preparations were given another 3 phosphate-buffered saline washes, and then mounted on slides.

Imaging and data analysis. Immunostained preparations were imaged using a Zeiss (Carl Zeiss Microscopy, North Ryde, NSW, Australia) fluorescence microscope (Axio Imager M2) or a Zeiss confocal microscope (LSM800 or Pascal), and analyzed using FIJI (ImageJ version 1.51s; National Institutes of Health, Bethesda, MD). Neuron density was examined by thresholding images and measuring the coverage of Hu+ particles in a given area of the plexus (% area). The total number of each neuronal subtype is expressed as a proportion of the total number of Hu^+ neurons in each preparation. The total number of 5-HT–positive cells was counted within the mucosa of each colonic section. Six preparations were imaged and analyzed per animal.

Calcium Imaging

Tissue preparation. Midcolon longitudinal muscle myenteric plexus preparations of *Wnt1-Cre;R26R-GCaMP3* mice[2] were placed in an organ bath constantly superfused with carbogenated physiological saline at room temperature.

Imaging and data analysis. GCaMP3+ preparations were imaged using a 20× (Numerical Aperture, 1.0) water-dipping objective on an upright Zeiss Axio Examiner.Z1 microscope with a Zeiss Axiocam 702 mono camera, and images (1920 × 1216) were acquired at 7 Hz with 20-ms exposure per frame. Neurons were stimulated electrically via a focal electrode (tungsten wire; 50-μm diameter) placed on an interganglionic fiber tract leading into the imaged ganglion of choice where a single pulse and a train of 20 pulses (20 Hz) were elicited. After live-imaging experiments, preparations were fixed and immunostained for the neuronal subtype markers nNOS and calbindin.

Analyses were performed using custom-written directives in IGOR Pro (WaveMetrics, Lake Oswego, OR) as previously described.[2] The fluorescence intensity was calculated as a fraction of the baseline fluorescence: F_i/F_0. The amplitudes of calcium transients were measured as the maximum increase in $[Ca^{2+}]_i$ above ($\Delta F_i/F_0$).

Sample Collection for Microbiota Analysis, Mass Spectrometry, and Droplet Digital-Polymerase Chain Reaction

Samples of duodenal mucosa, midcolonic mucosa and submucosa, and fecal contents were obtained under sterile conditions.

Droplet Digital-Polymerase Chain Reaction

RNA was extracted from midcolonic mucosal samples using the Promega (Alexandria, NSW, Australia) ReliaPrep RNA Tissue Miniprep system Kit, and converted into complementary DNA using the SensiFast cDNA Synthesis kit (Bioline, Alexandria, NSW, Australia). Quantitative expression of tryptophan

hydroxylase 1, chromogranin A, and the serotonin transporter SERT (SLC6A4) was examined via droplet digital–polymerase chain reaction[2,8] and normalized to the *HPRT* housekeeping gene. Primers (Integrated DNA Technologies, Coralville, IA) used were as follows: *HPRT*: forward, 5′-AACAAAGTCTGGCCTGTATCC-3′, reverse, 5′- CCCCAAAATGGTTAAGGTTGC-3′; tryptophan hydroxylase 1: forward, 5′-CTCGATGTGTAACAGGCTCAC-3′, reverse, 5′-AACAAAGACCATTCCTCCGAA-3′; chromogranin A: forward, 5′-CCAAGGTGATGAAGTGCGT-3′, reverse, 5′-TGGTGTCGCAGGATAGAGA-3′; and *SLC6A4*: forward, 5′-CATCGTCTGTCATCTGCATCC-3′, reverse, 5′-CGTTGGTGTTTCAGGAGTGAT-3′.

Mass Spectrometry and Microbiota Analysis

Levels of 5-HT biosynthetic intermediates were measured using Selected Reaction Monitoring-Mass Spectrometry; microbial DNA extraction, 16S ribosomal RNA sequencing, and bioinformatics were performed as previously described.[9]

Data Presentation and Statistics

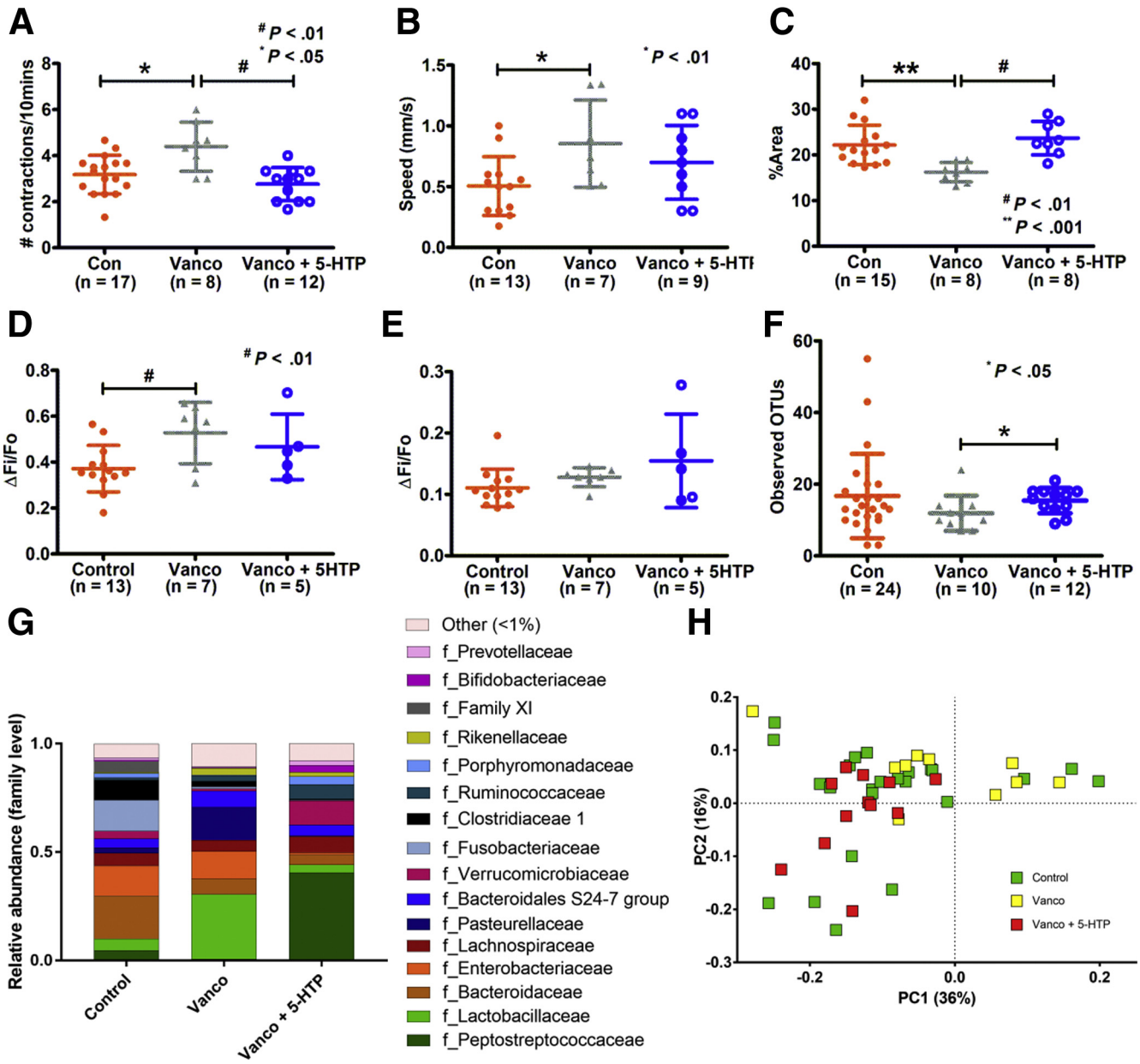
For each method, mice from at least 3 separate litters were examined per treatment condition. Data are presented as means ± SD, where n is the number of animals examined. Statistical comparisons between vancomycin- and water-fed littermates were performed using unpaired *t* tests, the Mann–Whitney test, or the chi-square test with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA), where $P < .05$ was considered statistically significant.

Statistical comparisons between controls, vancomycin-, and vancomycin + 5-HTP groups were performed using the Mann–Whitney test. In this case, controls were pooled populations of the water-fed littermates of vancomycin- and vancomycin + 5-HTP–fed pups.

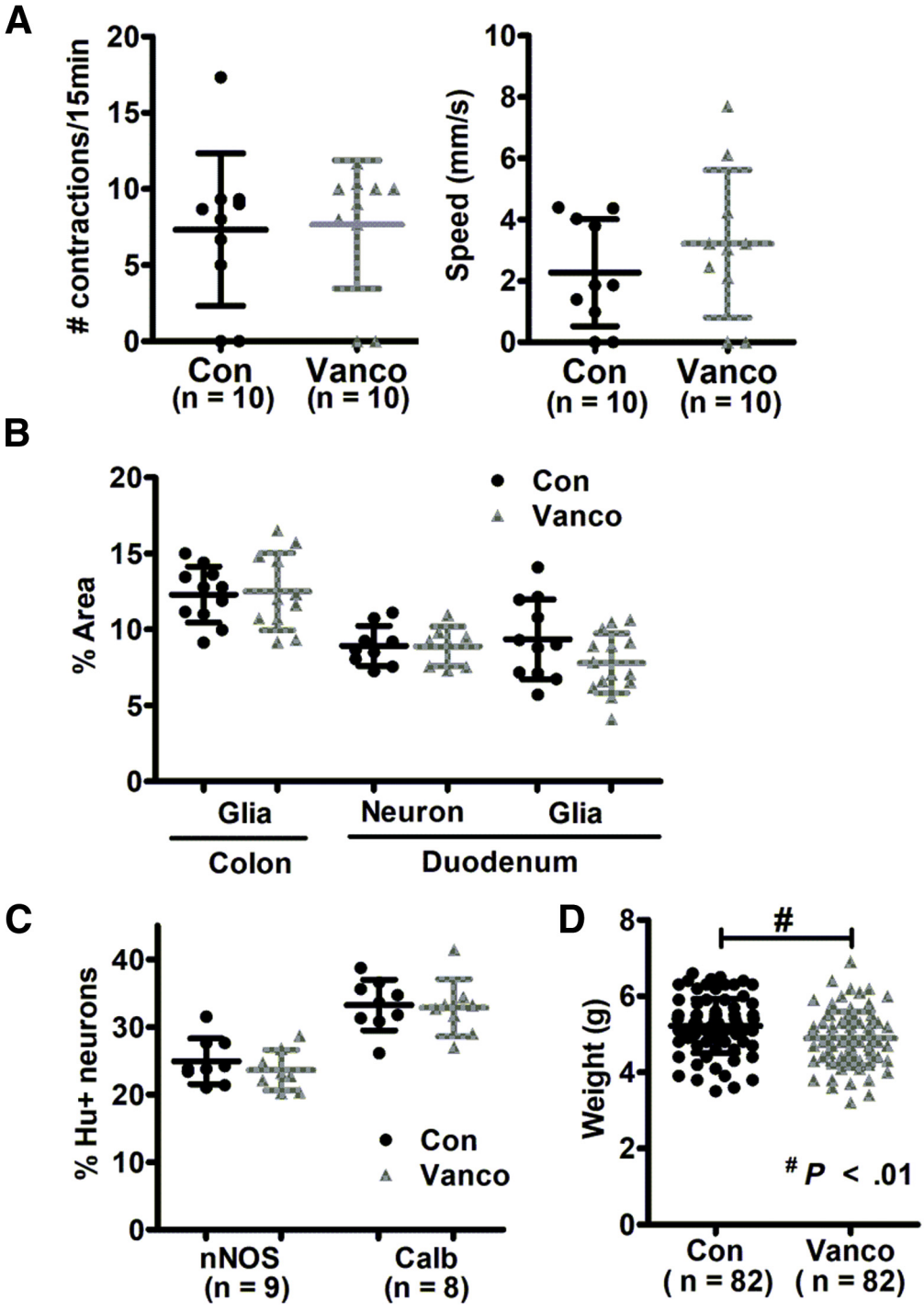
All authors had access to the study data and reviewed and approved the final manuscript.

Supplementary References

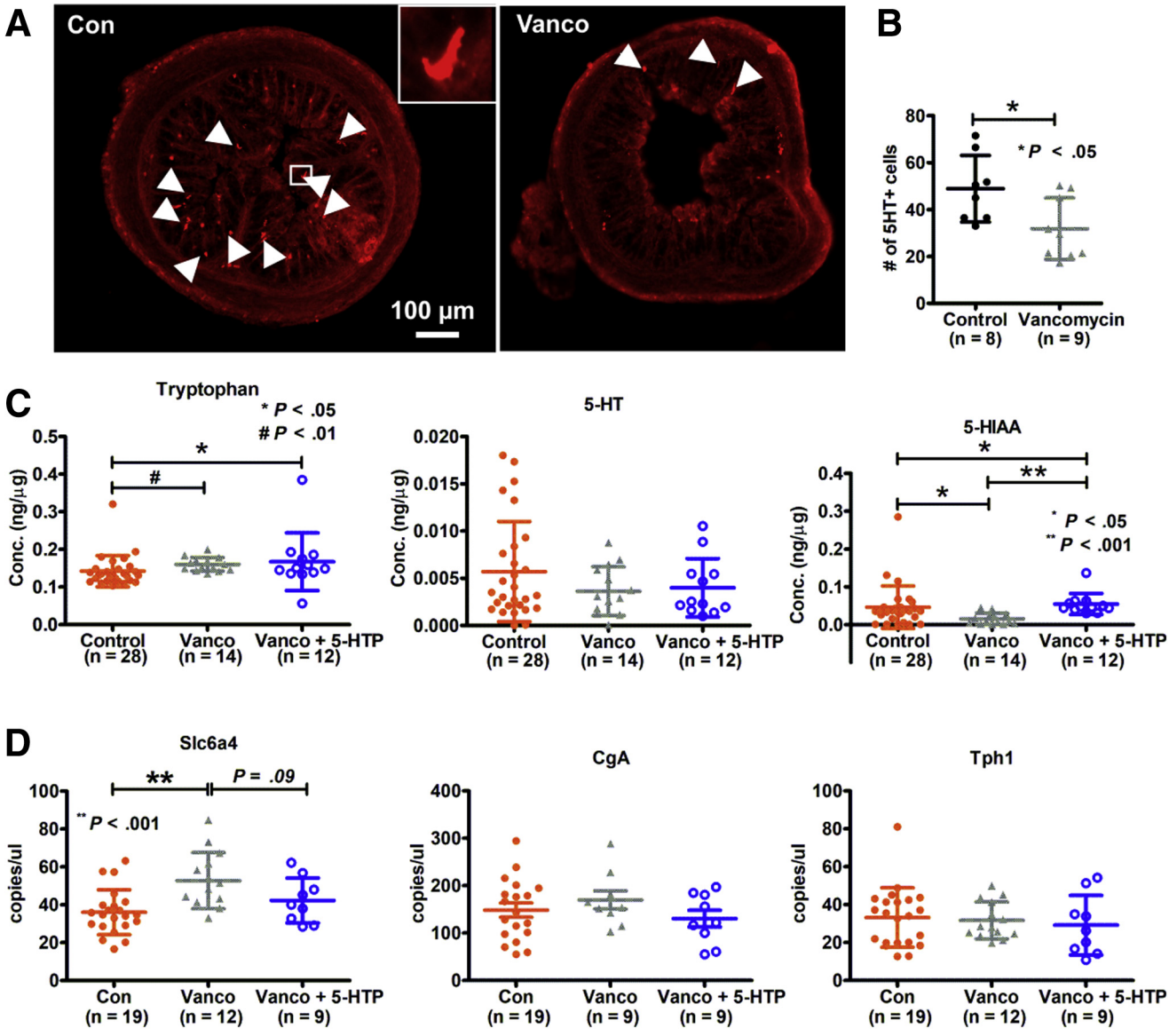
1. Danielian PS, et al. Curr Biol 1998; 8:1323–1326.
2. Foong JP, et al. J Neurosci 2015; 35:7106–7115.
3. Hansen CH, et al. Diabetologia 2012;55:2285–2294.
4. Roberts RR, et al. Am J Physiol Gastrointest Liver Physiol 2007; 292:G930–G938.
5. Swaminathan M, et al. J Vis Exp 2016:53828.
6. Foong JP, et al. J Physiol 2012; 590:2375–2390.
7. Gwynne RM, et al. Gastroenterology 2009;136:299–308 e4.
8. Hime GR, et al. Genom Data 2015; 5:106–108.
9. Luna RA, et al. Cell Mol Gastroenterol Hepatol 2017;3:218–230.
10. Kabouridis PS, et al. Neuron 2015;85:289–295.
11. Sekirov I, et al. Infect Immun 2008;76:4726–4736.
12. Tulstrup MV, et al. PLoS One 2015;10:e0144854.



Supplementary Figure 1. 5-HTP supplementation prevented some vancomycin-induced effects on the ENS and microbiota. Graphs indicate the (*A*) frequency and (*B*) speed of colonic contractions, (*C*) colonic neuronal (Hu+) density, and amplitude of $[Ca^{2+}]_i$ transients ($\Delta F_i/F_0$) from all cells stimulated with a (*D*) 20 pulse train and a (*E*) single pulse stimulus, and (*F*) α diversity. All data were examined from P10/P11 of water-, vancomycin-, and vancomycin + 5-HTP–fed pups. All graphs represent means ± SD. (*A* and *F*) Treatment effects were assessed with a Mann–Whitney or a 2-tailed unpaired *t* test. (*G*) Summary of microbiota composition at the family taxonomic level and (*H*) principal coordinate analysis of colonic mucosa samples from P10/P11 of water-, vancomycin-, and vancomycin + 5-HTP–fed pups. (*G*) Vancomycin treatment reduced the abundance of specific gram-positive families, including *peptostreptococcaceae* and *clostridiaceae 1*. However, in accordance with other studies,[11,12] a more general dysbiosis was observed, in which some gram-negative families also were affected by vancomycin. OTU, operational taxonomic unit.



Supplementary Figure 2. Neonatal exposure to vancomycin has no effect on S100β+ myenteric glia density, duodenal ENS, and motility, but significantly reduced body weight. (*A*) Frequency and speed of duodenal contractions. (*B*) Neuronal (Hu+) and glial (S100β+) density measured from the myenteric plexus of the colon and duodenum of control (Hu+, n = 9; S100β+, n = 11) and vancomycin-fed (Hu+, n = 8; S100β+, n = 11–14) mice. S100β+ glia from the mucosa, and not enteric plexi, previously were reported to be microbiota-dependent.[10] Our observations of the ineffectiveness of vancomycin treatment on S100β+ immunostaining in the myenteric plexus is in accordance with this, but we cannot exclude the possibility that the antibiotic treatment may have affected mucosal glia, which were not examined in our study. (*C*) Proportion (relative to Hu+ neurons) of nNOS+ and calbindin+ myenteric neurons in the duodenum of control and vancomycin-fed P10/P11 mouse pups. (*D*) Body weight of P10/P11 control and vancomycin-fed mice. All graphs represent means ± SD. Treatment effects were assessed with a 2-tailed unpaired *t* test. Calb, calbindin; Con, control; Vanco, vancomycin.



Supplementary Figure 3. Vancomycin-induced effects on the gut are linked to alterations in 5-HT availability. (*A*) Confocal micrograph of the midcolon cross-section of a vancomycin (Vanco)- and a water (control [Con])-fed pup immunostained for 5-HT (5-HT+ cells indicated by *arrowheads*, enlarged example in *inset*). (*B*) Number of 5-HT+ cells in the colonic mucosa of water- and vancomycin-fed pups. (*C*) Levels of tryptophan, 5-HT, and 5-hydroxyindole acetic acid (5-HIAA); and (*D*) transcripts encoding Slc6a4, chromogranin A (CgA), and tryptophan hydroxylase 1 (Tph1) were quantified in the colonic mucosa of control-, vancomycin-, and vancomycin + 5-HTP–fed mouse pups. Concentrations of transcripts are expressed as copies/μL of amplified polymerase chain reaction mixture normalized to the concentration of *Hprt*. All data were examined at P10/P11. All graphs represent means ± SD. (*B* and *C*) Treatment effects were assessed with a Mann–Whitney or a 2-tailed unpaired *t* test.

Supplementary Table 1. Electrically Evoked $[Ca^{2+}]_i$ Transients of Neurochemically Identified Neurons in P10/P11-Treated Pups

Electrical stimulation	Neurochemistry	Control, $\Delta F_i/F_0$	Vancomycin, $\Delta F_i/F_0$	Responders[a]
1 pulse	Calbindin+	0.1 ± 0.04 (6)	0.1 ± 0.02 (40)	1.4[b]
	nNOS+	0.1 ± 0.02 (23)	0.1 ± 0.01 (58)	
	Calbindin-/nNOS-	0.1 ± 0.01 (64)	0.1 ± 0.01 (122)	
20 pulse	Calbindin+	0.4 ± 0.07 (21)	0.7 ± 0.04 (113)[b]	1.9[c]
	nNOS+	0.4 ± 0.03 (55)	0.5 ± 0.02 (154)	
	Calbindin-/nNOS-	0.4 ± 0.02 (162)	0.6 ± 0.02 (366)[c]	
			Vancomycin + 5-HTP ($\Delta F_i/F_0$)	
1 pulse	Calbindin+	0.11 ± 0.01 (60)	0.13 ± 0.01 (70)	1.6[c]
	nNOS+	0.09 ± 0.00 (68)	0.11 ± 0.01 (86)[c]	
	Calbindin-/nNOS-	0.11 ± 0.01 (123)	0.13 ± 0.01 (141)[b]	
20 pulse	Calbindin+	0.35 ± 0.01 (137)	0.53 ± 0.03 (129)[c]	1.1[d]
	nNOS+	0.35 ± 0.01 (163)	0.46 ± 0.02 (145)[c]	
	Calbindin-/nNOS-	0.36 ± 0.01 (255)	0.51 ± 0.02 (208)[c]	

[a]Responders refers to the proportion of neurons responding in vancomycin or vancomycin + 5-HTP normalized to their controls, chi-square test.
[b]$P < .01$, 2-tailed unpaired *t* test, number of neurons examined written in parentheses.
[c]$P < .0001$, 2-tailed unpaired *t* test, number of neurons examined written in parentheses.
[d]$P < .05$, 2-tailed unpaired *t* test, number of neurons examined written in parentheses.