RESEARCH LETTER

Muscularis Propria Macrophages Alter the Proportion of Nitrergic but Not Cholinergic Gastric Myenteric Neurons

The enteric nervous system consists of more than a dozen types of neurons aggregated into networks of ganglia throughout the gastrointestinal t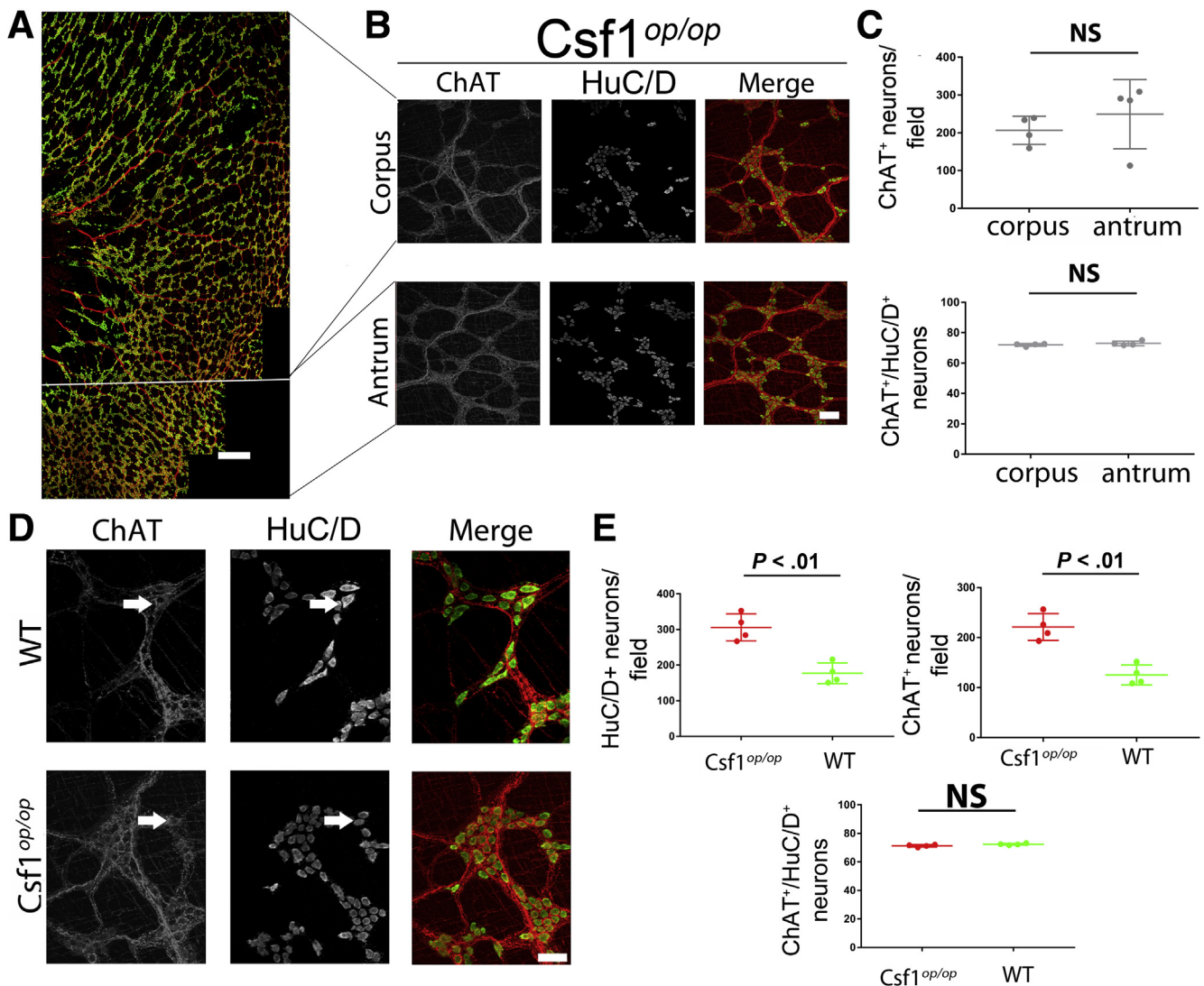ract, which regulate contractile activity, mucosal secretion, absorption, and local blood flow.[1,2] Mechanisms that contribute to remodeling of the enteric neuronal networks are of great interest. In the central nervous system, it has been suggested that microglia contribute to the fate, connectivity, and identity of neurons during development.[3] Muscularis propria macrophages (MPM) within the enteric nervous system may have similar functions to microglia. Mice homozygous for the osteopetrosis mutation (Csf1$^{op/op}$) which do not have MPM, have more neurons in the small intestine[4] and a higher proportion of gastric neurons that express nitric oxide synthase (NOS1).[5] Myenteric neurons serve diverse functions that can be indicated by their morphology, projections and the expression of marker proteins that define their "chemical code." This study finds a previously unidentified

Figure 1. (*A*) **Distribution of HuC/D$^+$ and ChAT$^+$ myenteric neurons across gastric regions.** *Scale bar*: 200 μm. (*B*) Images of HuC/D$^+$ and ChAT$^+$ neurons in the gastric regions of Csf1$^{op/op}$ mice. *Scale bar*: 50 μm. (*C*) Quantification of HuC/D$^+$ and ChAT$^+$ neurons in the gastric regions of Csf1$^{op/op}$ mice (Mann–Whitney test; P = NS). (*D*) Images of gastric HuC/D$^+$ and ChAT$^+$ neurons in WT and Csf1$^{op/op}$ mice. *Scale bar*: 60 μm. *Arrow* indicates typical HuC/D$^+$ and ChAT$^+$ co-expressing neurons. (*E*) Quantification of HuC/D$^+$ and ChAT$^+$ neurons in WT and Csf1$^{op/op}$ mice (n = 36 fields; N = 4 mice) (Mann–Whitney test; P < .01). (*C* and *E*) *Bars* and *whiskers* indicate means ± SD and *points* indicate individual fields for all panels.
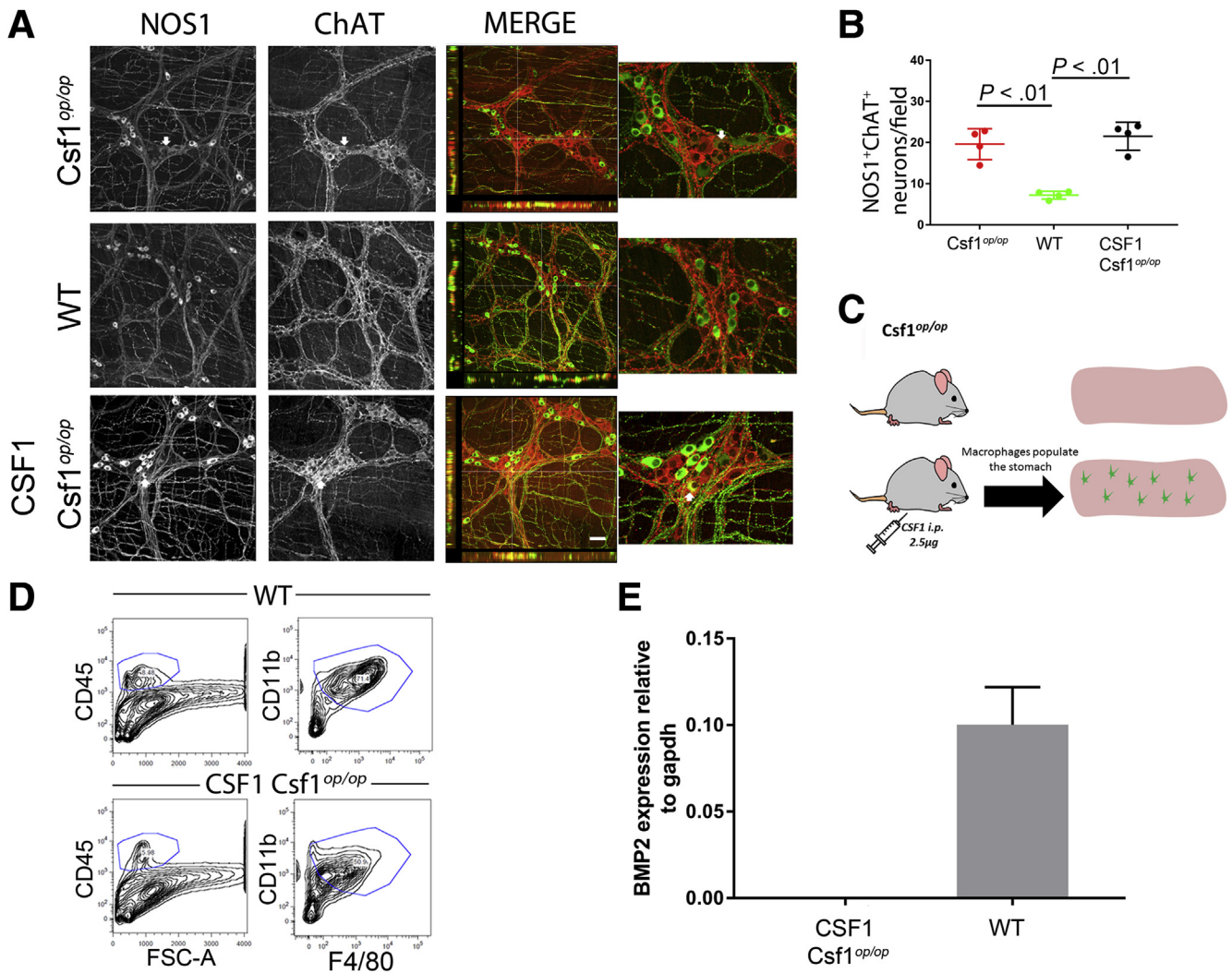
Figure 2. (A) Images of NOS1$^+$/ChAT$^+$ neurons. *Scale bar*: 50 μm. *Arrows* show NOS1$^+$ neurons that are also ChAT$^+$. (*B*) Quantification of NOS1$^+$ChAT$^+$ double-labeled neurons. *Points* represent individual fields of view. *Bars* and *whiskers* indicate means ± SD (1-way analysis of variance; $P < .01$; N = 4). (C) Experimental model for CSF1 treatment. Fluorescence-activated cell sorter (FACS) strategy to isolate CD45$^+$CD11b$^+$F4/80$^+$ macrophages from the gastric muscularis propria of WT (*top*) and CSF1-treated Csf1$^{op/op}$ mice (*bottom*). (*E*) BMP2 expression levels in macrophages isolated from Csf1$^{op/op}$ and WT mice (Mann–Whitney test; N = 3; $P < .01$).

role for MPM in altering the chemical code of myenteric neurons.

Csf1$^{op/op}$ mice were maintained on a specialized liquid diet to keep their weight comparable with age-matched-wild type (WT) mice (Supplementary Figure 1*A*). In the myenteric plexus of WT mice, populations of MPM, absent in Csf1$^{op/op}$ mice[5] (Supplementary Figure 1*B* and *C*, Supplementary Movie 1 and 2), were associated closely with neurons, suggesting functional interactions.[6] We first tested whether the number of choline acetyltransferase$^+$ (ChAT$^+$) neurons was affected by the absence of MPM in Csf1$^{op/op}$ mice (Supplementary Table 1). The density of neurons, defined by Embryonic lethal, abnormal vision, Drosophila-like protein 3/4 (HuC/D) immunoreactivity, was similar between gastric regions in both WT and Csf1$^{op/op}$ mice (Figure 1*A*–*C*, Supplementary Figure 2*A*) (Mann–Whitney test, P = NS; N = 4), yet was higher in Csf1$^{op/op}$ mice than in WT mice (Figure 1*D* and *E*) ($P < .01$, Mann–Whitney test, n = 36 fields, N = 4). Likewise, the density of ChAT$^+$ neurons was higher in Csf1$^{op/op}$ mice compared with WT mice (Figure 1*D* and *E*) ($P < .001$, Mann–Whitney test, n = 36 fields, N = 4). However, in contrast to an increase in the percentage of NOS1$^+$ neurons,[5] the percentage of ChAT$^+$ neurons did not differ between Csf1$^{op/op}$ and WT mice (Figure 1*D* and *E*) (Mann–Whitney test, n = 36 fields, N = 4). This result suggests that the presence of macrophages alters the proportion of nitrergic but not cholinergic gastric myenteric neurons.

Interestingly, in Csf1$^{op/op}$ mice, the combined percentages of NOS1$^+$ (30%) and ChAT$^+$ neurons (72%) exceeded 100% (Supplementary Figure 2*B*), indicating partial overlap between these markers. Therefore, we

investigated whether the number of NOS1$^+$ChAT$^+$ double-labeled neurons was changed in Csf1$^{op/op}$ mice. In Csf1$^{op/op}$ mice, Nitric Oxide Synthase 1 (NOS1$^+$) ChAT$^+$ neurons were more numerous than in WT mice (Figure 2A and B) (Csf1$^{op/op}$: 7.8 ± 7.1 cells/field; WT, 1.7 ± 1.6 cells/field; 1-way analysis of variance; $P < .001$; n = 24; N = 4). This result suggests the ability of macrophages to not only modulate the neuronal number but also affect myenteric neuron differentiation. Enteric neurons are not required for bowel colonization by macrophages,[7] but macrophages interact with neurons after birth, by expressing genes, such as bone morphogenetic protein 2 (BMP2), needed for macrophage-enteric neuron interaction and neuronal development.[4] To test the intrinsic ability of resident macrophages to modify the neuronal chemical code by establishing functional interaction with neurons, we treated Csf1$^{op/op}$ with CSF1 (Colony Stimulating Factor 1) for 7 weeks to populate the stomach with macrophages (Figure 2C). In CSF1-treated Csf1$^{op/op}$ mice, the proportion of NOS1$^+$ChAT$^+$ neurons remained similar to the proportion of NOS1$^+$ChAT$^+$ neurons in Csf1$^{op/op}$ mice (Figure 2A–C) (1-way analysis of variance; n = 24; N = 4). We previously showed that repopulating macrophages in CSF1-treated Csf1$^{op/op}$ mice had a different phenotype from resident macrophages.[5] Consistent with this observation, BMP2 was not expressed by macrophages isolated from CSF1-treated Csf1$^{op/op}$ mice (Antibodies and PCR primers listed in Supplementary Tables 2 and 3), whereas BMP2 was expressed by macrophages isolated from WT mice (Figure 2D and E) (Mann–Whitney test; $P < .001$; N = 4), as reported elsewhere.[4]

During development, the chemical code of myenteric neurons changes and the overlap between NOS1 and ChAT decreases as neurons mature.[8] Therefore, increased numbers of double-labeled myenteric neurons may reflect incomplete maturation of myenteric neurons in Csf1$^{op/op}$ mice. MPMs functionally interact with enteric neurons starting at 2 weeks of age,[7] therefore the role of resident MPM in promoting myenteric neuron maturation likely happens early in life. Interestingly, MPMs that populate the gastric muscularis propria did not express BMP2, a cytokine important for establishing functional interactions between MPMs and neurons during development. Therefore, as previously suggested,[4,9] BMP2 may be required for the changes in NOS1 and ChAT expression associated with neuronal maturation.

Taken together, our results show a role for MPM in enteric neuronal maturation as indicated by the changes in chemical code in gastric myenteric neurons. The mechanisms by which MPM regulate neuronal numbers and chemical codes needs further investigation because it may be significant to the development or plasticity of the adult enteric nervous system and normal gastric function.

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Supplementary Materials and Methods

Animals

These studies were approved by the Mayo Clinic Institutional Animal Care and Use Committee. Mice were humanely killed by carbon dioxide exposure followed by cervical dislocation. Mice homozygous for the $Csf1^{op}$ mutation and WT littermates were studied. These mice were bred in-house from a $Csf1^{op/+}$ colony of hemizygous breeders with founders originating from The Jackson Laboratory (Bar Harbor, ME). Wild-type $Csf1^{+/+}$ mice were identified by genotyping as previously described.[1] $Csf1^{op/op}$ mice were maintained on a specialized wet diet (Bio-serv, Frenchtown, NJ) after weaning at 3–4 weeks of age to keep their weight comparable with age-matched WT mice (Supplementary Figure 1A). After 12 weeks of age, $Csf1^{op/op}$ mice were treated with CSF1 (2.5 μg intraperitoneally once daily, recombinant mouse macrophage colony stimulating factor-1 (rmM-CSF); Pepro-tech, Rocky Hill, NJ) (Figure 2A).

Immunolabeling

The mucosa was removed and muscularis propria was fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer for 4 hours. Then, whole mounts were rinsed in 0.1 mol/L phosphate-buffered saline and blocked in the presence of 10% normal donkey serum in phosphate-buffered saline and 0.3% Triton X-100 (Thermo Fisher, Waltham, MA) overnight at 4°C and gastric muscularis propria was labeled with primary antibodies overnight at 4°C. After washing, the tissue was incubated with secondary antibodies (Jackson ImmunoResearch, West Grove, PA), washed, and then incubated with 4',6-diamidino-2-phenylindole dilactate (Invitrogen, Carlsbad, CA) for 30 minutes. Neurons were identified by HuC/D-immunoreactivity (ANNA1, a gift from Dr Vanda Lennon, Mayo Clinic, Rochester, MN), cholinergic neurons using a goat anti-ChAT antibody (EMD Millipore, Burlington, MA), and nitrergic neurons using a rabbit anti-NOS1 antibody (EMD Millipore). Muscularis macrophages were identified using the MHCII primary antibody (eBioscience, Waltham, MA).

Controls omitting the primary antibody and controls in double-labeling experiments that used the wrong secondary antibody were performed for all experiments. For quantification, 3 different fields were taken from the corpus and 3 from the antrum. The list of antibodies is shown in Supplementary Table 1.

Confocal Microscopy

A laser scanning confocal microscope using a 20×, numerical aperture, (NA), 0.95 XLUMPlanFl objective (Olympus, Tokyo, Japan) in Fluoview (Olympus), with the optimal confocal aperture to provide a resolution of 0.994 × 0.994 × 1.13 μm (X × Y × Z), was used. Stacks of confocal images of the entire muscularis propria were collected from 4 different mice (n = 4). For quantification of the labeling, all of the confocal image stacks were flattened into projections using the FV10-ASW Viewer (Olympus). The flattened images were renumbered in random order and the enteric neuronal number was determined while blinded to the source. All cells were counted from fields with dimensions of 636 × 636 μm.

Images used for reconstruction and orthogonal view were taken from a Zeiss LSM 780 microscope using either a 40× 1.2 NA water immersion objective at a resolution of 0.415 × 0.415 × 0.444, or a 100 × 1.4 NA oil immersion objective at a resolution of 0.133 × 0.133 × 0.373 μm per pixel. Images were analyzed using Imaris-Microscopy Image Software by Bitplane (Supplementary Figure 1A).

Isolation and Analysis of Gastric Muscularis Macrophages

Cell sorting was performed using a fluorescence activated cell sorting Aria Cell Sorter cytometer running fluorescence activated cell sorting Diva 6 software (Becton Dickinson, San Jose, CA), located in the Mayo Clinic Flow Cytometry Core Facility. Aliquots of cells were either unstained or stained with individual fluorescently labeled antibodies (Zurich, Switzerland, Supplementary Table 2) to establish instrument voltages, compensation, and appropriate gates. Each positive control tube was initially run without storing the data to ensure that the positive signals were on scale. Data were analyzed using FlowJo X software (Tree Star, Inc, Ashland, OR).

Gastric $CD45^+CD11b^+F4/80^+$ cells were isolated directly into the lysing buffer provided by the RNeasy micro plus kit (Qiagen, Hilden, Germany). The extraction was performed following the instructions provided and the RNA concentration was determined by using a NanoDrop spectrophotometer. The RNA extracted was used for a real-time quantitative reverse-transcription polymerase chain reaction. The SuperScript VILO complementary DNA Synthesis Kit (Invitrogen) was used to generate complementary DNA. Quantitative reverse-transcription polymerase chain reaction was performed on complementary DNA using commercial primer sets (Supplementary Table 3) and RT2SYBR Green/ROX quantitative reverse-transcription polymerase chain reaction master mix according to the manufacturer's instructions (SABiosciences, Frederick, MD). The data were normalized to the expression of the glyceraldehyde-3-phosphate dehydrogenase by transforming the difference in threshold cycle for the gene of interest and the housekeeping gene to the second power, and expressed as the means ± SEM.

Statistics

Data are expressed as scatter plots with medians and quartiles and analyzed by the Mann–Whitney test. A *P* value less than .05 was considered significant. The method used for statistical analysis of 3 different groups was 1-way analysis of variance with multiple comparisons. Normality was addressed by applying D'Agostino and Pearson normality tests. Statistical analysis was performed with GraphPad Prism (GraphPad Software, La Jolla, CA).

Supplementary References

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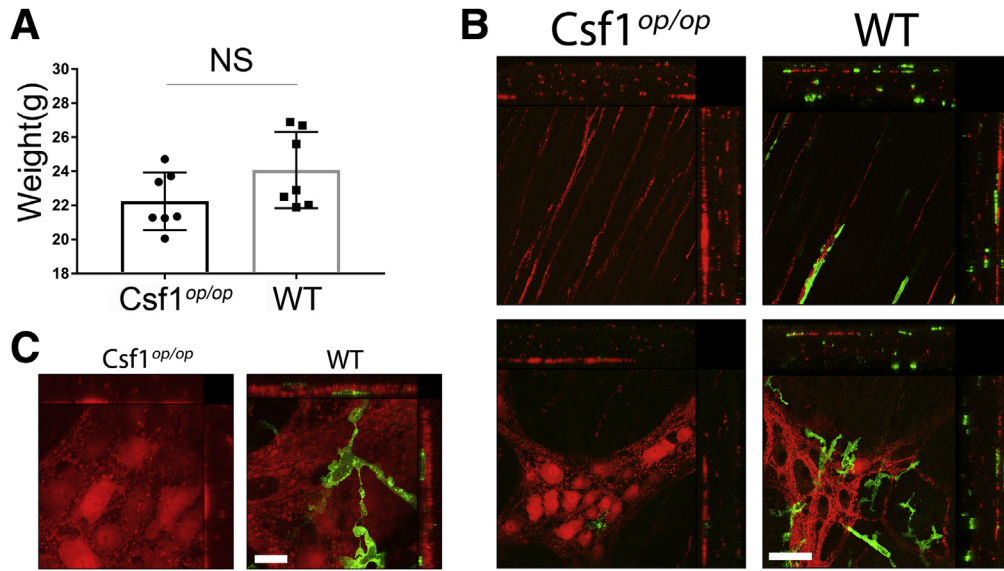
Supplementary Figure 1. (*A*) **Weight of WT and CSf1**$^{op/op}$ **mice (***P* = NS; Mann–Whitney test; N = 7 mice for each group). (*B* and *C*) Major histocompatibility complex class II (MHCII) macrophages (green) and Protein gene product 9.5 (PGP 9.5) fibers in smooth muscular layers (*upper panels*) and myenteric plexus (*lower panels*). The small panels show orthogonal views generated by projecting the z-series in the x (*right*) and on the y plane (*above*). *Arrows* point to macrophage/fiber interactions and *squares* show macrophage/fiber interactions in orthogonal views. PGP 9.5 immunoreactivity was unusually bright in the cell bodies of myenteric neurons in CSf1$^{op/op}$ mice when compared with WT tissues. Scale bars: (*B*) 20 μm, (*C*) 10 μm.

	WT		Csf1$^{op/op}$	
	Mean	**SD**	**Mean**	**SD**
HuC/D	213.6	75.05	429.5	109.5
NOS1	49.77	18.25	129.6	42.52
ChAT	124	34.87	219.7	81.36
NOS1%	23.5	4.289	30.03	5.481
ChAT%	71.32	4.712	72.62	2.83
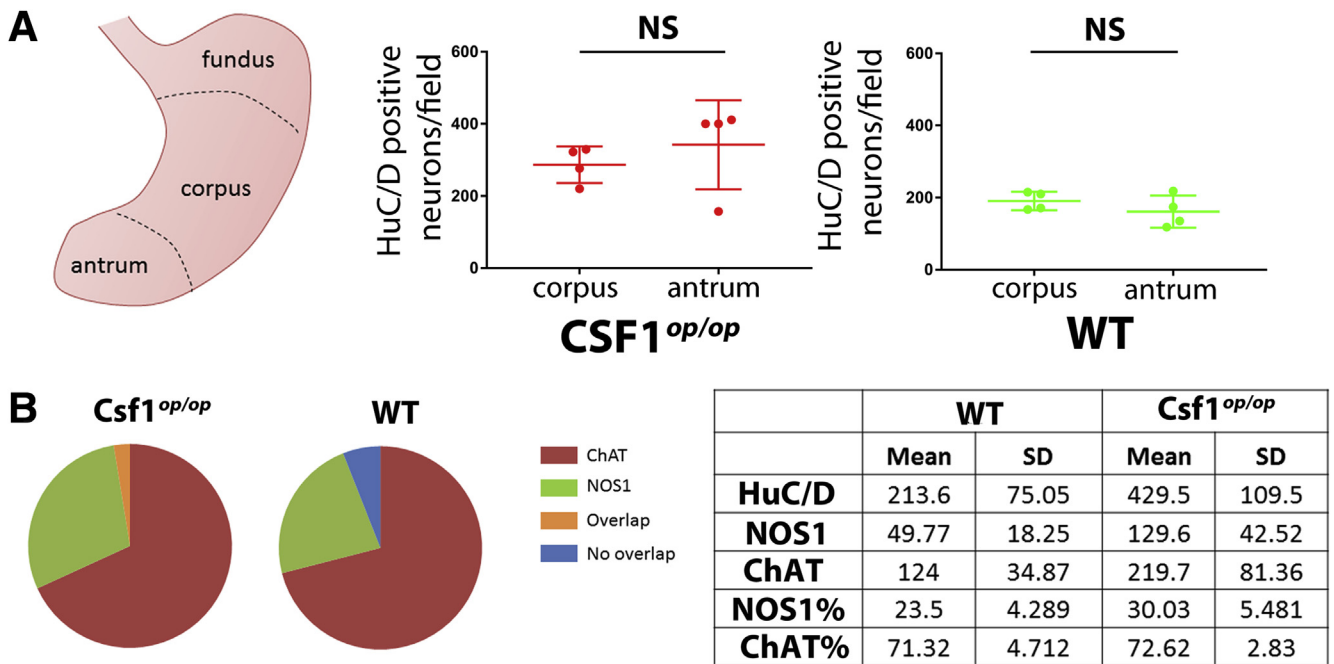
Supplementary Figure 2. (*A*) **Quantification of the HuC/D+ myenteric neurons in the gastric corpus and antrum of WT and Csf1**$^{op/op}$ **mice.** (*B*) Percentage of myenteric neurons identified in Csf1$^{op/op}$ and WT mice. Table shows numbers per field and proportions of different types of myenteric neurons in Csf1$^{op/op}$ and WT mice.

Supplementary Table 1. Sources of Commercial Antibodies Used in Immunohistochemistry Experiments

	Supplier	Final titer	Host	Clonality	Catalog number	Research resource initiative identifier
Primary antibody						
Embryonic lethal, abnormal vision, Drosophila-like protein 3/4	Gift from Dr V. Lennon (Mayo Clinic)	1:500	Human			AB_2314657
NOS1	Millipore	0.33 µg/mL	Rabbit	Polyclonal	AB5380	AB_91824
ChAT	Millipore	1:100	Goat	Polyclonal	AB144P	AB_2079751
F4/80 direct conjugate	Thermo Fisher	0.4 µg/mL	Rat	Polyclonal	MF 48020	AB_10376287
Major Histocompatibility Complex II	eBioscience	1.0 µg/mL	Rat	Monoclonal	14-5321-81	AB_467560
Protein Gene Product 9.5	Thermo Fisher	1:400	Rabbit	Polyclonal	38-1000	AB_2533355
Secondary antibody						
Cy3 anti-goat	Jackson ImmunoResearch	1.75 µg/mL	Donkey	Polyclonal	705-165-147	AB_2307351
Alexa Fluor–488 anti-rat	Jackson ImmunoResearch	2.33 µg/mL	Donkey	Polyclonal	712-545-150	AB_2340683
Cy3 anti-rabbit	Jackson ImmunoResearch	1.75 µg/mL	Donkey	Polyclonal	711-165-152	AB_2307443
Cy5 anti-human	Jackson ImmunoResearch	1.75 µg/mL	Donkey	Polyclonal	709-175-149	AB_2340539

Supplementary Table 2. List of Antibodies Used for Sorting Experiments and List of Primers Used for Quantitative Reverse-Transcription Polymerase Chain Reaction

Antibody	Fluorophore	Catalog number	Company
F4/80 monoclonal antibody (BM8)	Phycoerythrin–cyanine 5	15-4801-82	eBioscence
Anti-mouse CD11b	Alexa Fluor 488	53-0112-82	eBioscence
Anti-mouse CD45	Alexa Fluor 450	48-0451-82	eBioscence
Rat IgG2b K isotype control	APC	17-4031-81	eBioscence
Rat IgG2a K isotype control	PE-cyanine 7	25-4321-81	eBioscence

Supplementary Table 3. List of Primers used for RT-PCR			
Gene symbol	Unigene title	Forward	Reverse
BMP2	Bone morphogenetic protein 2	GGTGATGGCTTCCTTGTACC	AGTGAGGCCCATACCAGAAG
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Qiagen	Qiagen