Supporting Information

Bhargava et al. 10.1073/pnas.1219733110

SI Materials and Methods

Immunofluorescence Staining and Confocal Microscopy. A cross-section of loop tissue from rats treated with toxin A (TxA) or vehicle was fixed in 4% paraformaldehyde. Tissue was washed in PBS, immersed in 30% sucrose in PBS overnight, embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek), and stored at −80 °C. Frozen sections (5–8 μm) were thaw-mounted onto Superfrost Plus slides (Fisher Scientific). Sections were pretreated with PBS containing 5% normal goat serum and reacted with rabbit antibody raised against a peptide corresponding to the terminal 18 residues of rat calcitonin receptor-like receptor (CLR) (RK11) (1) at 1:500 and mouse antibody raised against rat calcitonin gene-related peptide (CGRP) (4901) (2) at 1:400 at 4 °C overnight. After being washed, they were reacted with FITC-labeled donkey anti-rabbit IgG and rhodamine red-X (RRX)-labeled donkey anti-mouse IgG at 1:200 (Jackson ImmunoResearch). For colocalization of CLR (RK11) with PGP9.5 (Abcam; used at 1:200), sections were similarly incubated with both primary antibodies and then reacted with FITC-labeled goat anti-mouse IgG and RRX-labeled goat anti-rabbit IgG at 1:200 (Jackson ImmunoResearch). Sections were mounted with ProLong Gold Antifade Reagent (Invitrogen) before imaging. Sections were viewed on a Zeiss LSM510 Meta microscope equipped with a Plan Apochromat 20× (n.a. 0.8), an ECPlan Neofluor 40× (n.a. 1.3), and a Plan Apochromat 63× (n.a. 1.4) objective. FITC was detected by using a 488 λ argon laser with a 505–530 λ BP filter, and RRX was detected by using a 543 λ HeNe laser and a 560–615 λ BP filter. A scan speed of 6–7 was used, with a pinhole of 0.59–1 airy unit. Unless stated otherwise, the sum of five optical sections is shown, to maximize visualization of nerve fibers.

Image Processing. Bright-field images were acquired using a Zeiss Axioplan microscope equipped with a Fluor 20× (n.a. 0.75) objective or a NeoFluor 40× (n.a. 1.3) objective, and a SPOT digital camera. Images of immunoreactivity using fluorescence detection were acquired with a Zeiss LSM510 confocal microscope. Images of tissue sections from the ileal loops of rats pretreated with non-CLR dsRNA and subsequently treated with TxA were acquired first at settings to prevent saturation of signal. Immediately thereafter, identical configurations were used to acquire all other images. Because of less intense and diffuse cytosolic staining of NF-κB in buffer-treated controls, a bigger pinhole and a larger gain needed to be used to acquire this particular image. All images were minimally processed in the same manner with Adobe Photoshop CS. Images shown were obtained after quantitative scoring and are from rats that had the average response of the experimental group to TxA based on intestinal secretion and myeloperoxidase (MPO), to safeguard against investigator bias.

RNA Analysis. A portion of the ileal loop was homogenized in TRIzol (Invitrogen) buffer, and RNA was isolated according to the manufacturer's specifications. Primers used for TNF-α have been previously described: TNF-α, forward 5′-cgtcgtagcaaaccaccaagca-3′ and reverse 5′-accagggcttgagctcagctc-3′, 70 °C (3). In addition, 4 μL of the RT product was used to amplify cyclophilin (forward 5′-tgcagacgccgctgtctc-3′ and reverse 5′-tgctctcctgagctacag-3′, annealed at 66 °C), a housekeeping gene whose expression is unaltered by TxA treatment. The PCR products were analyzed by agarose gel electrophoresis and were sequenced to confirm identity. The band intensity was quantified by use of National Institutes of Health Image and after background subtraction was normalized against cyclophilin for each lane to safeguard against loading error.

Western Blot Analysis. A portion of the ileal loop was homogenized in lysis buffer containing 30 mM Tris·HCl (pH 7.4), 1 mM EGTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 0.1 mM Na_3VO_4, 1 minitablet protease inhibitor mixture (Roche), 1:100 phosphatase inhibitor mixture (Sigma), 0.04% Triton X-100. Lysates (40 μg of total protein) were separated by SDS/PAGE (12% acrylamide gels). Proteins were transferred to PVDF membranes (Immobilon-FL; Millipore) and blocked for 1 h at room temperature (Odyssey Blocking Buffer; LI-COR). Membranes were incubated with antibodies to pERK1/2 (E-4, a mouse antibody recognizing pERK2 p42 and pERK1 p44; 1:1,000), ERK2 (C-14 from Santa Cruz, a rabbit affinity-purified antibody recognizing ERK2 p42 and ERK1 p44; 1:5,000), mouse NF-κB (Cell Signaling; 1:1,000), and mouse β-actin (Sigma; 1:10,000) overnight at 4 °C (Odyssey Blocking Buffer). Membranes were washed for 30 min (1× PBS, 0.1% Tween 20) and incubated with the secondary antibodies goat anti-mouse conjugated to Alexa Fluor 680 (Invitrogen) and goat anti-rabbit conjugated to IRDyeTM800 (Rockland Immunochemicals) (both 1:20,000, 1 h, room temperature) and analyzed with the Odyssey Infrared Imaging System (LI-COR).

1. Cottrell GS, et al. (2005) Localization of calcitonin receptor-like receptor and receptor activity modifying protein 1 in enteric neurons, dorsal root ganglia, and the spinal cord of the rat. *J Comp Neurol* 490(3):239–255.
2. Wong HC, et al. (1993) Monoclonal antibody to rat alpha-CGRP: Production, characterization, and in vivo immunoneutralization activity. *Hybridoma* 12(1):93–106.
3. Clifton MS, et al. (2007) Role of calcitonin receptor-like receptor in colonic motility and inflammation. *Am J Physiol Gastrointest Liver Physiol* 293(1): G36–G44.

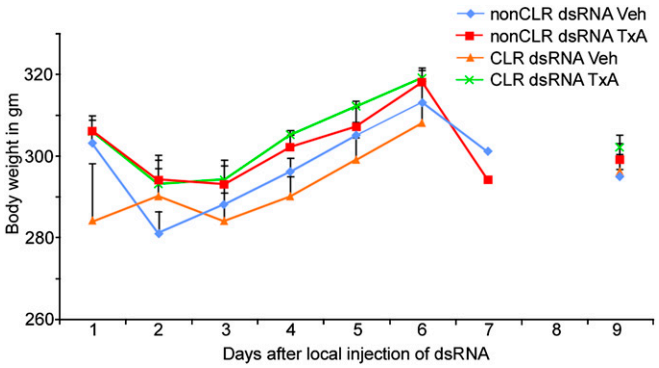

Fig. S1. Weight gain is unchanged by treatment with dsRNA. Rats were weighed before dsRNA injection and daily thereafter for 9 d. Surgery induced a slight decrease in weight. An initial drop in weight 1 d after surgery was consistent between rats pretreated with non-CLR dsRNA and rats treated with CLR dsRNA, and the recovery pattern was similar among all groups. Thus, dsRNA treatment did not alter body weights. Values are mean ± SEM, n = 8–12 per group.

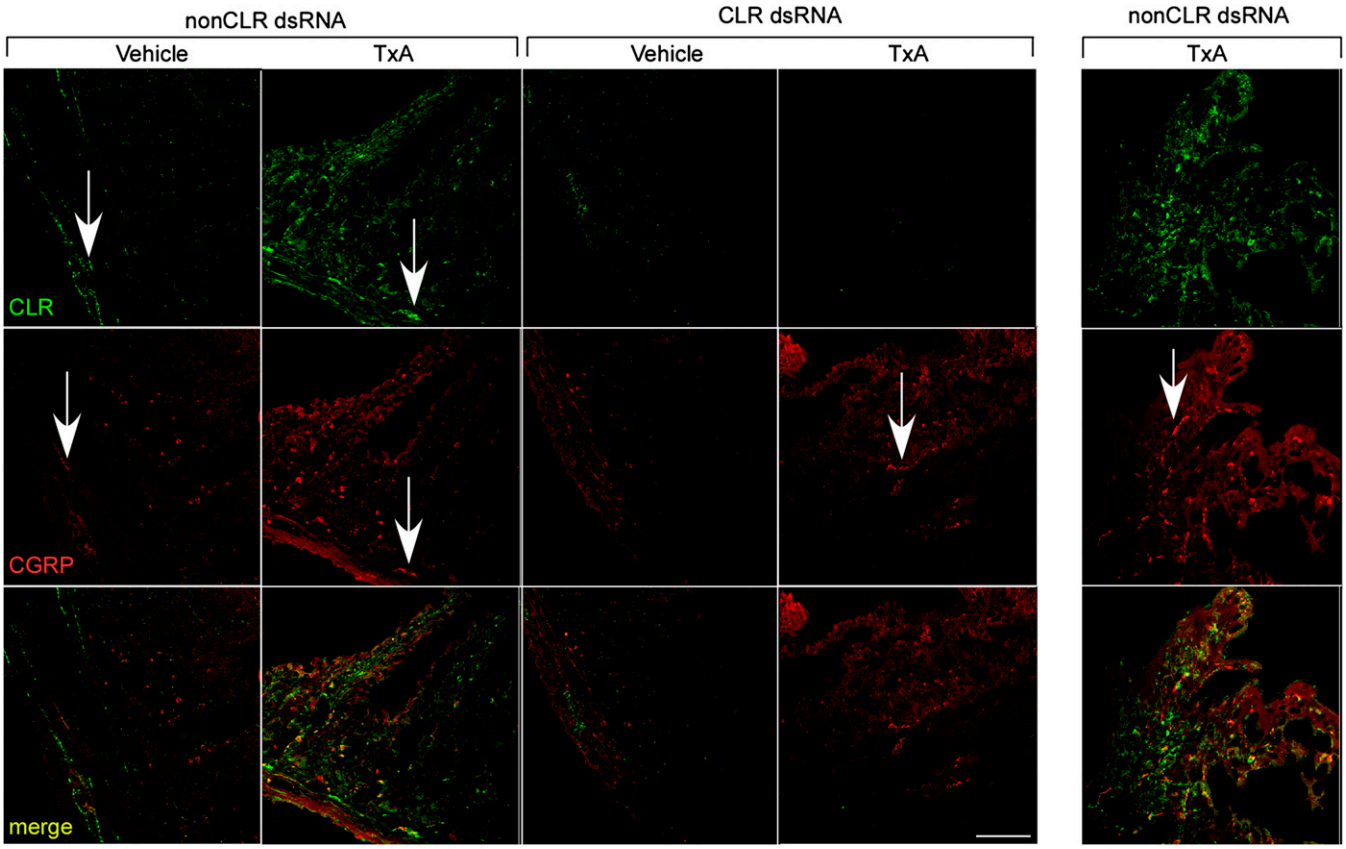

Fig. S2. CGRP immunoreactivity (IR) increases with TxA. Seven days after pretreatment with vehicle, non-CLR dsRNA, or CLR dsRNA, the rats received an injection of vehicle or purified TxA into ileal loops. After 4 h, the rats were killed. CGRP and CLR were detected in loop tissue by immunofluorescence and confocal microscopy. Optical sections were acquired under identical parameters; a single optical section is shown. Loop tissue taken from rats pretreated with non-CLR dsRNA and vehicle showed CGRP-IR and CLR-IR that did not differ from basal levels. Thus, pretreatment with non-CLR dsRNA had no effect on basal CGRP-IR. TxA treatment increased CGRP-IR in the ileum of rats treated with non-CLR dsRNA or vehicle at sites adjacent to locations of increased CLR-IR. Loop tissue from rats injected with CLR dsRNA showed basal CGRP-IR. TxA did not increase CLR-IR in the ileum of rats pretreated with CLR dsRNA, but did increase CGRP-IR. Arrows indicate CLR-IR or CGRP-IR. (Scale bar, 67 μm.)

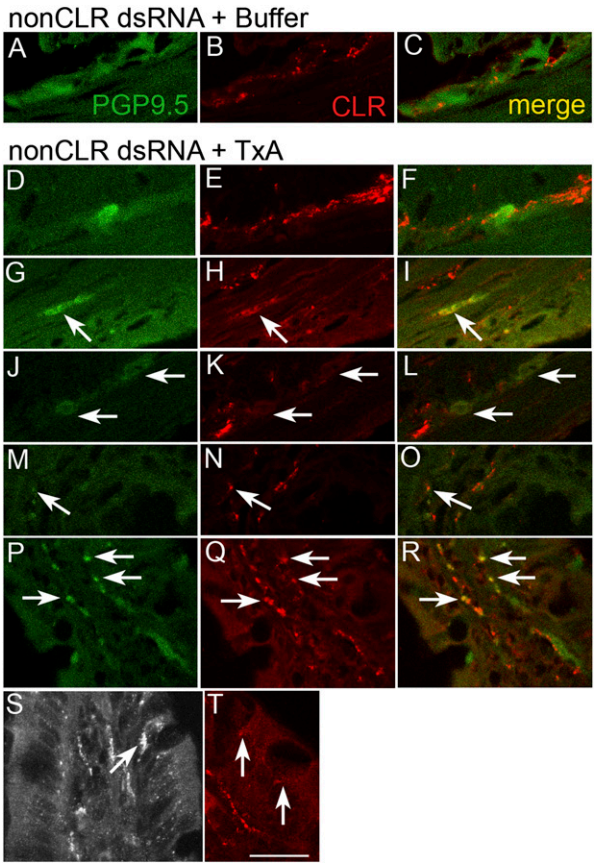

Fig. S3. CLR immunoreactivity increased by TxA colocalized with PGP9.5. Seven days after pretreatment with vehicle, non-CLR dsRNA, or CLR dsRNA, the rats received an injection of vehicle or purified TxA into ileal loops. After 4 h, the rats were killed. The neuronal marker PGP9.5 and CLR were detected in loop tissue by immunofluorescence, using a FITC- and RRX-labeled secondary antibody, respectively, and confocal microscopy. Optical sections were acquired under identical parameters, but all PGP9.5 images were minimally adjusted for brightness. The sum of three optical sections is shown unless otherwise stated. (*A*–*C*) Images from loops with non-CLR dsRNA and buffer treatment. CLR-IR was prominent in nerve fibers of the myenteric plexus (*B*). Most neurons were positive for PGP9.5-IR (*A*) but showed less prominent CLR-IR, a pattern reported before by us for CLR staining in the rat ileum (1). (*D*–*T*) Images from loops with non-CLR dsRNA and TxA treatment. (*D*–*O*) After TxA treatment, CLR-IR was increased in nerve fibers in the myenteric plexus that frequently did not colocalize with PGP9.5-IR (*D*–*F*). (*G*–*I*) In some cases, prominent CLR-IR was found in soma of myenteric neurons and colocalized with PGP9.5-IR. Single optical sections are shown in *J*–*O*, and confirm CLR-IR colocalized with PGP9.5-IR in myenteric neurons (*J*–*L*) and in nerve fibers of the myenteric plexus (*M*–*O*). (*P*–*R*) TxA treatment markedly increased CLR-IR in the lamina propria, where CLR-IR was colocalized with PGP9.5-IR. (*S*) CLR-IR was also detected in epithelial cells of the ileum. (*T*) A single optical section confirms CLR-IR in epithelial cells. (Scale bar, 34 μm.)