

## Role of calcitonin receptor-like receptor in colonic motility and inflammation

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**Clifton MS, Hoy JJ, Chang J, Idumalla PS, Fakhruddin H, Grady EF, Dada S, Corvera CU, Bhargava A.** Role of calcitonin receptor-like receptor in colonic motility and inflammation. *Am J Physiol Gastrointest Liver Physiol* 293: G36–G44, 2007. First published March 15, 2007; doi:10.1152/ajpgi.00464.2006.—Calcitonin gene-related peptide (CGRP) mediates neurogenic inflammation and modulates intestinal motility. The CGRP receptor is a heterodimer of calcitonin receptor-like receptor (CLR) and receptor-associated modifying protein 1. We used RNA interference to elucidate the specific role of CLR in colonic motility and inflammation. Intramuscular injection of double-stranded RNA (dsRNA) against CLR (dsCLR) into the colonic wall at two sites caused the spatial and temporal downregulation of CLR in the colon within 1 day of dsRNA injection. Knockdown of CLR persisted for 7–9 days, and the effect of knockdown spread to ~2 cm proximal and distal to the injection sites, whereas control dsRNA injection did not affect CLR expression. Measurement of isometric contractions of isolated colonic muscle segments revealed that in control dsRNA-injected rats, CGRP abrogated contractions entirely and decreased resting muscular tone, whereas in dsCLR-injected rats, CGRP decreased muscle tone but slow-wave contractions of varying amplitude persisted. In trinitrobenzene sulfonic acid-induced colitis, rats with knockdown of CLR displayed a significantly greater degree of edema and necrosis than saline- or control dsRNA-injected rats. Levels of the proinflammatory cytokines TNF- $\alpha$  and IL-6 were markedly upregulated by trinitrobenzene sulfonic acid treatment. TNF- $\alpha$  mRNA levels were further increased in CLR knockdown rats, whereas levels of IL-6 were unaltered. Thus this study demonstrates that CLR is a functional receptor for CGRP.

calcitonin gene-related peptide; RNA interference; trinitrobenzene sulfonic acid; cytokines; colon-specific; tumor necrosis factor- $\alpha$

CALCITONIN GENE-RELATED PEPTIDE (CGRP) is widely distributed in both the central and peripheral nervous systems. In the mammalian gastrointestinal (GI) tract, CGRP immunoreactivity is present in the myenteric and submucosal plexuses as well as in the sensory neurons, whose cell bodies are located in the nodose and dorsal root ganglia (13, 30). The functional CGRP receptor has been proposed to be a heterodimer of calcitonin receptor-like receptor (CLR) and receptor-associated modifying protein (RAMP) 1. Association of CLR with RAMP1 or RAMP2 confers specificity for binding either CGRP or adrenomedullin, respectively (23). Previous colocalization studies have demonstrated the presence of CLR and RAMP1 in enteric neurons, including the neurons in the myenteric plexus (5). Additionally, nerve fibers containing CGRP are found in close association with the CLR-positive neurons, suggesting that the effects of CGRP may be mediated via the CLR-RAMP1 heterodimer complex (5).

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CLR knockout mice are embryonic lethal with severe cardiovascular defects (6). Pharmacological antagonists for CLR have been reported, but systemic administration of antagonists does not result in tissue-specific inhibition of receptor function. A small-molecule antagonist that is a specific and potent CGRP receptor antagonist is BIBN4096BS (7), which has been an effective acute treatment for migraine (24). Although BIBN4096BS exhibits a high affinity for the human CGRP receptor, it has a 100-fold lower affinity for the rat CGRP receptor and therefore is of limited use to evaluate the function of the rat receptor. An alternative to using either genetic knockouts or pharmacological agents to study gene function is RNA interference (RNAi). RNAi has recently emerged as a powerful tool to transiently silence gene expression both in vitro and in vivo. Moreover, in vivo RNAi has proved to be a specific, efficient, and nonimmunogenic way to knock down gene expression (19). Thus RNAi provides an attractive way to define whether CGRP mediates its effects via CLR.

CGRP exerts multiple effects, in both the central and the peripheral nervous systems (5, 29, 35). In the GI tract, CGRP exerts anti-inflammatory actions. In rodents, peripheral but not central infusion of CGRP ameliorates trinitrobenzene sulfonic acid (TNBS)-induced colonic inflammation (9, 22). In patients with inflammatory bowel disease (IBD), CGRP immunoreactivity is either decreased (11) or unchanged (27). CGRP also regulates gastric and mesenteric blood flow (15), controls gastric acid secretion (31), and modulates intestinal motility (15, 16). Unlike the predominantly excitatory effect of substance P and other related neurokinins on GI motility (1, 18), the effect of CGRP can be either excitatory or inhibitory, depending on the region of the GI tract (15, 20) and the specific muscle layer (i.e., longitudinal vs. circular) (16). CGRP is involved in the peristaltic reflex elicited by muscle stretch and mucosal stimulation (14). CGRP produces a concentration-dependent relaxation of longitudinal muscle and has a mild inhibitory effect on the peristaltic reflex in guinea pig ileum (16). Studies of experimentally induced colitis in rabbits have shown a time-dependent decrease in CGRP concentration caused by a release from intrinsic and extrinsic enteric nerve fibers (10). Other experiments have shown that this release of CGRP exerts a protective effect by release from sensory neurons that modulate mucosal blood flow (26) and have led to speculation that alterations in CGRP content may affect gut motility in the setting of inflammation (10). In this study, we sought to determine the colon-specific role of CLR in inflammation and motility by creating a transient knockdown of CLR by using RNAi.

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## MATERIALS AND METHODS

**Animals.** Male Sprague-Dawley rats ( $n = 4-8/\text{group}$ ) weighing 260–280 g were individually housed in wire-bottom cages in temperature- and light-controlled rooms. Rats had ad libitum access to chow and water unless stated otherwise. All procedures were in accordance with the Committee on Animal Research at the University of California, San Francisco.

**Experimental design 1: time course and distance of effect.** Rats were anesthetized with isoflurane, and a midline laparotomy was performed. The descending colon was exteriorized, and a marking suture was placed in the colon wall at the splenic flexure (8 cm from the anus). dsRNA (20  $\mu\text{g}$ ) for either  $\beta$ -globin (dsControl) or CLR (dsCLR) (see *Synthesis of dsRNA*) mixed with 1.5  $\mu\text{l}$  of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was injected intramurally into the colon wall at two sites (10  $\mu\text{g}$  dsRNA/125  $\mu\text{l}$  Lipofectamine solution at each site), 1.5 cm proximal and distal to the silk suture, using a 30-gauge needle (Fig. 1A). The abdomen was closed in two layers; ketoprofen (10 mg/kg sc) was given postoperatively, and rats were allowed to recover. Rats were killed at 1, 3, 5, 7, and 9 days after dsRNA injections. Four 1-cm-long colon segments were collected from the regions proximal (*segments A–D*) and distal (*segments W–Z*) to the suture (Fig. 1A). Half of each segment was processed for histological and immunofluorescence analyses, and the other half was snap-frozen for protein and RNA analyses.

**Experimental design 2: inflammation studies using a model of TNBS-induced colitis.** TNBS was used to induce colitis because TNBS mimics Crohn's disease, with areas of "skipped" lesions. Rats were anesthetized briefly with isoflurane, after which a silicone catheter (Mallinckrodt, St. Louis, MO) was inserted to position the tip 8 cm proximal to the anus. An enema consisting of 15 mg TNBS in a 50% ethanol vehicle was then given via a catheter in a 250- $\mu\text{l}$  volume; 50% ethanol enemas (in 250  $\mu\text{l}$ ) served as vehicle controls. Three days after being given the TNBS enema, rats were killed and colon segments were collected for histological examination, myeloperoxidase (MPO) assay, and RNA, protein, and cytokine analyses.

**Experimental design 3: colonic CLR and colitis.** Rats were briefly anesthetized with isoflurane and were divided into the following groups ( $n = 4-8$ ): 1) saline + 50% ethanol; 2) saline + TNBS; 3) dsControl RNA + TNBS; and 4) dsCLR + TNBS. dsRNAs were injected into the colonic wall as in *experimental design 1*. Simultaneously, colitis was induced by a TNBS enema (15 mg/rat). Rats were killed 1 or 3 days later, and colon tissue was collected as described for *experimental design 1*.

**Experimental design 4: motility studies.** CLR and control dsRNAs were administered in the rat colonic wall as described for *experimental design 1* ( $n = 4-6/\text{group}$ ). Naïve rats served as an additional control group. Three days later, four 1-cm segments (2 cm proximal and 2 cm distal to the marking stitch) were collected from each rat and were immediately placed in cold Krebs solution. Silk suture loops (6-0) were tied on either end of the tissue segment and were used to mount the segments on a tension recorder/transducer submerged in Krebs solution bubbled with 95%  $\text{O}_2$ -5%  $\text{CO}_2$ . Approximately 0.5 g tension was applied, and the tissue was allowed to equilibrate for 30–60 min. The effects of nonadrenergic, noncholinergic neurons were isolated by the addition of guanethidine (10  $\mu\text{M}$ ) and atropine (0.7  $\mu\text{M}$ ) to the Krebs bath. Basal tone was increased by the addition of histamine (27  $\mu\text{M}$ ), and the maximum effect at 30 min was recorded. CGRP was added at graded concentrations of 1, 10, and 100 nM, and the response was recorded for 10 min at each interval. All data were acquired by using Acqknowledge software (Acq373) on an MP100 system (Biopac Systems, Goleta, CA). Data are reported as means  $\pm$  SE for each treatment group.

**RT-PCR analyses for CLR and cytokines.** RNA was isolated from the colon tissue by using Trizol reagent (Invitrogen, Carlsbad, CA) as previously described (3). RNA (2  $\mu\text{g}$ ) was reverse-transcribed by using random hexamers and Moloney murine leukemia virus-RT in a

20- $\mu\text{l}$  volume (Applied Biosystems, Branchburg, NJ) as per the manufacturer's specifications. Subsequent PCR reactions were performed by using 4  $\mu\text{l}$  RT product and gene-specific primers. The primer sets and annealing temperatures used in this study were as follows: CLR, forward 5'-TACGGTGTCAACAATCAGCG-3' and reverse 5'-GGTATTCAAGAAACCCATTGCTA-3', annealed at 56°C; TNF- $\alpha$ , forward 5'-CGTCGTAGCAAACCACCAAGCA-3' and reverse 5'-AC-CAGGGCTTGAGCTCAGCTC-3', 70°C; and IL-6, forward 5'-CAGGGAGATCTTGGAAATGAG-3' and reverse 5'-TGATAT-GCTTAGGCATAGCACAC-3', 60°C. In addition, 4  $\mu\text{l}$  of the RT product was used to amplify cyclophilin (forward 5'-TGCAGACGCGCTGTCTC-3' and reverse 5'-TGCTCTCCTGAGCTA-CAG-3', annealed at 66°C), a housekeeping gene whose expression remains unaffected on TNBS treatment. The PCR products were analyzed by agarose gel electrophoresis and were sequenced to confirm identity. The band intensity was quantitated by using NIH Image and was normalized against cyclophilin.

**Synthesis of dsRNA.** CLR PCR product obtained from colon tissue by using the above protocol was cloned in pTOPO vector (Invitrogen) and was sequenced to confirm identity. A BLAST search of CLR sequence against the NR and EST databases revealed that the CLR sequence used for synthesis of long dsRNA did not have any identity or homology with other related or unrelated sequences at the nucleotide level. This cloned cDNA was used as a template to transcribe sense and antisense RNAs in vitro by using the MegaScript RNA kit (Ambion, Austin, TX) according to the manufacturer's specifications and as described previously by us (2). Green fluorescent protein and rat  $\beta$ -globin sequences were used as nonspecific dsRNA controls and also have been described earlier (2).

**Histological evaluation.** Colon tissue was collected in 4% paraformaldehyde (PFA) and then was transferred to 30% sucrose in 4% PFA at 4°C. Tissue was embedded in optimum cutting temperature compound (Tissue-Tek; Sakura Finetek, Torrance, CA), sectioned (5  $\mu\text{m}$ ), and thaw-mounted onto Superfrost Plus slides (Fisher, Pittsburgh, PA). Sections were counterstained with hematoxylin and eosin for histological grading. Inflammation severity was scored in a blinded manner (E. F. Grady). Histological scoring was based on the following gross and microscopic characteristics: 1) intra-abdominal adhesions, 2) mucosal ulceration, 3) submucosal thickening, 4) ulcer size, 5) presence or absence of necrosis, and 6) cellular infiltrate. Submucosal thickening was given a coded score (1, 0–200  $\mu\text{m}$ ; 2, 201–400  $\mu\text{m}$ ; 3, 401–500  $\mu\text{m}$ ; 4, >501  $\mu\text{m}$ ). Depth of lesion was scored as well (0, 0  $\mu\text{m}$ ; 1, 1–350  $\mu\text{m}$ ; 2, 351–600  $\mu\text{m}$ ; 3, >601  $\mu\text{m}$ ). Ulceration scoring was based on number of microscopic fields encompassed by the lesion at  $\times 20$  magnification (0, 0 fields; 1, 0.5–2 fields; 2, 2.5–4.5 fields; 3, 5–7 fields; 4, >7 fields). Necrosis was scored in a binary fashion (0, absent; 1, present). Histological damage was calculated by adding scores from all of these characteristics.

**Immunohistochemistry and Western blot analysis.** Colon tissue was fixed in 4% PFA and was serially sectioned as described above. CLR was detected by using our serum raised against a peptide corresponding to the 18 residues of rat CLR, which we have described previously (5). CGRP was detected by using a mouse antiserum (4910) raised against rat  $\alpha$ -CGRP (33). Tissue sections were incubated at 4°C overnight with the primary antibodies to CLR (1:1,000) and CGRP (1:500). The sections were subsequently washed and incubated with secondary antibodies conjugated to Rhodamine Red-X (Jackson, West Grove, PA) or Alexa 488 (Molecular Probes, Eugene, OR) at a concentration of 1:200–1:1,500 at room temperature for 2 h. The sections were washed and mounted in ProLong Gold anti-fade reagent (Molecular Probes). Sections were analyzed by epifluorescence microscopy, and images were acquired with a Spot digital camera using identical camera settings for control and experimental groups.

**SDS-PAGE and Western Blotting.** Tissue lysates were prepared by sonicating tissue in  $1\times$  PBS containing 0.5% Tween 20 and mini complete protease inhibitor tablet (Roche, Nutley, NJ). Lysates (40  $\mu\text{g}$ ) were separated by SDS-PAGE (16% acrylamide), transferred to



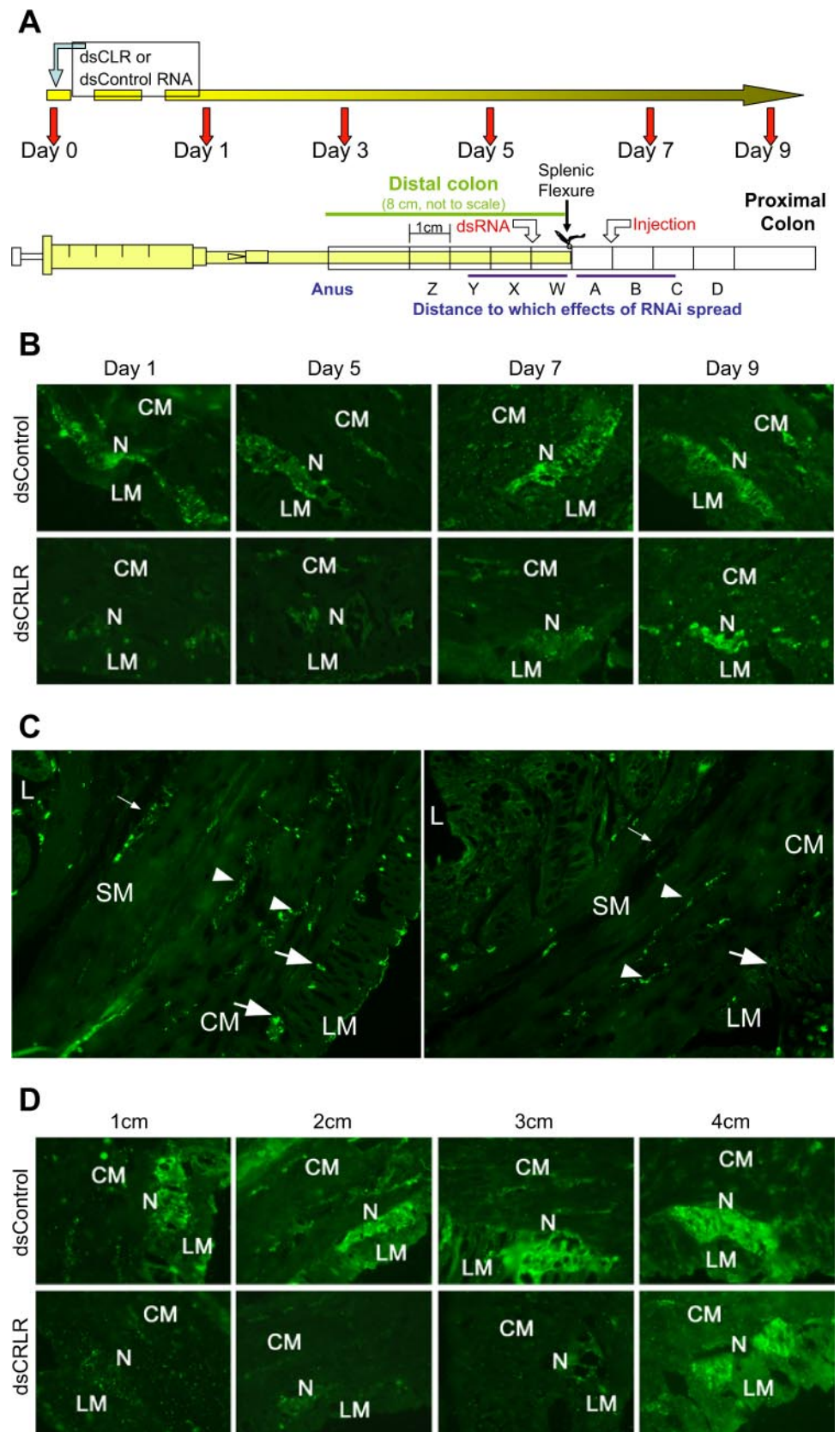


Fig. 1. *A*: schematic of administration of double-stranded RNA (dsRNA) and measurement of spatiotemporal effects of RNA interference (RNAi). dsRNA for control (dsControl) or calcitonin receptor-like receptor (dsCLR) was injected into the colonic musculature at 2 sites proximal and distal to the marking suture (at the splenic flexure) as depicted. Eight 1-cm-long segments (A–D or W–Z) were isolated 1–9 days after injection of dsRNA and were processed for immunofluorescence, RNA analysis, or protein analyses. Knockdown of CLR protein was observed in segments A–C and W–Y. *B*: effects of RNAi last for 7 days after a single bolus of dsRNA injection. Robust CLR immunoreactivity (CLR-IR) is clearly evident in the neurons of the myenteric nerve plexus (N) at all days examined in the dsControl group (*top*). CLR-IR is clearly knocked down for up to 7 days in the dsCLR group (*bottom*), and its expression is back to control levels by *day 9*. CM, circular muscle; LM, longitudinal muscle. *C*: although CLR-IR was detected in the neurons of myenteric (arrows) and submucosal (thin arrow) plexuses in the dsControl group, it was clearly knocked down in neurons of myenteric and submucosal plexuses in the dsCLR group. However, CLR-IR appeared to be unaltered in the nerve fibers that innervate the lamina propria and the CM. L, lumen; SM, submucosa. Magnification,  $\times 40$ . *D*: effects of RNAi encompass 2–3 cm of tissue. Inhibitory effects of RNAi spread to  $\sim 2$  cm proximal and distal to the injection site (*segments C and Y*). CLR-IR was knocked down in *segments A–C* and *W–Y*, whereas *segments D* and *Z* displayed CLR-IR that was qualitatively equivalent to the CLR expression seen in controls (serving as an additional control for specific knockdown). Knockdown at *day 3* in different segments is shown. Magnification,  $\times 40$ .

polyvinylidene difluoride membranes (Immobilon-FL; Millipore, Billerica, MA), and incubated with blocking buffer (LI-COR, Lincoln, NE). Membranes were incubated in blocking buffer with the following primary antibodies: anti-TNF- $\alpha$  (3.5  $\mu$ g/ml; Biosource, Camarillo, CA), anti-IL-6 (2  $\mu$ g/ml; Biosource), and anti- $\beta$ -actin (1:20,000) overnight at 4°C. Membranes were washed and then incubated with secondary antibody coupled to either Alexa Fluor 680 or IRDye™ 800 (1:10,000–1:20,000) for 1 h at room temperature. Immunoreactive proteins were viewed with an Odyssey infrared imaging system (LI-COR).

**MPO assay.** MPO activity was measured in colon tissue lysates in a microtitre plate assay as previously described (17). Briefly, colon tissue was homogenized in hexadecyltrimethylammonium bromide in 50 mM KH<sub>2</sub>PO<sub>4</sub> (pH 6). This suspension was sonicated and centrifuged at 14,000 g. The supernatant was aliquotted and stored at -80°C until it was assayed.

**Statistical analysis.** Data for studies of inflammation were first analyzed by one-way ANOVA. A significant ( $P < 0.05$ ) global effect was followed by a stepwise comparison using Bonferroni's *t*-test. Differences in muscle tone were compared by using the Student's *t*-test. A  $P$  value  $< 0.05$  was considered significant.

## RESULTS

**RNAi knocks down CLR expression circumferentially in the rat colon.** We followed the time course of downregulation of CLR after RNAi induction as depicted in Fig. 1A. Under basal conditions, CLR immunoreactivity (CLR-IR) was abundantly expressed within the neurons of the myenteric plexus and in the fibers of the circular muscle, as previously reported using this antibody (5). Injection of dsControl did not affect expression of CLR-IR in the neurons of the myenteric or submucosal plexuses or in the nerve fibers in the circular muscle (Fig. 1B, top, and data not shown) on all days examined (*days 3, 5, 7, and 9*). In marked contrast, injection of dsCLR resulted in dramatic and specific knockdown of CLR-IR in the myenteric neurons (Fig. 1B, bottom). CLR-IR was low or undetectable in dsCLR rats at *days 3 and 5*. By *day 7*, CLR-IR was weakly discernable in the myenteric neurons and the nerve fibers of the circular muscle. By *day 9*, CLR-IR in rats injected with CLR dsRNA was indistinguishable from that of controls (Fig. 1B). Further analysis of the sections at *days 3 and 5* revealed that CLR-IR was also knocked down in the neurons of the submucosal plexus (Fig. 1C). However, the nerve fibers of the circular muscle in the dsCLR group continued to express CLR-IR, suggesting an extrinsic source of CLR (Fig. 1C).

Next, we determined the distance to which the effects of RNAi spread from the point of dsRNA injection (Fig. 1A). Immunofluorescence revealed that rats given dsControl continued to exhibit CLR-IR in the enteric neurons in all four 1-cm segments (designated as *segments A–D* or *W–Z*); there was no discernable difference in CLR expression within the neurons of the myenteric plexus between dsControl or sham-operated rats (Fig. 1D and data not shown). In dsCLR-administered rats, knockdown of CLR was evident up to 2 cm proximal (*segments A–C*) and distal (*segments W–Y*) to the site of dsRNA injection, whereas at 3 cm from the injection site (*segment D* or *Z*), CLR-IR was indistinguishable between dsControl- and dsCLR-injected colons.

**Knockdown of CLR attenuates CGRP's effect on colonic motility.** We evaluated the functional role of CLR in rat colon pretreated with dsControl or dsCLR RNA and subsequently challenged the tissues with CGRP. Colon segments from rats

treated with dsControl or dsCLR RNA displayed an average baseline tone of  $0.56 \pm 0.03$  g. Pretreatment of colon segments with atropine and guanethidine did not have a measurable effect on spontaneous, slow, and sporadic contractile activity. Histamine increased muscle tone by  $13.73 \pm 2.93\%$  above baseline in both groups. In rats pretreated with dsControl RNA, addition of 1 nM CGRP decreased histamine-augmented tone by  $18 \pm 2.01\%$  over 10 min. In tissues pretreated with dsCLR, 1 nM CGRP was ineffective in decreasing histamine-augmented tone over 10 min (Fig. 2). Higher concentrations of CGRP caused a greater reduction in tone in both control and dsCLR RNA-treated animals (~45% decrease). Thus the inhibitory effect of CGRP seen at 1 nM concentration was lost at high concentrations.

**CLR plays an anti-inflammatory role in rat model of colitis.** Histological changes induced by TNBS-induced colitis in rats included edema, destruction of mucosal crypts, and thickening of the submucosa (Fig. 3A). Tissue histology was unaffected by 50% ethanol treatment (vehicle). Saline and dsControl RNA injection did not alter the development or progression of colitis in rats. Knockdown of CLR worsened the degree of tissue necrosis, whereas destruction of mucosal crypts and thickening of the submucosa were similar in all TNBS treatment groups (Fig. 3A). Histological scoring confirmed that TNBS damaged tissue; when CLR was knocked down before the TNBS enema was given, tissue damage was exacerbated (Fig. 3B). Next, we determined changes in CLR mRNA due to TNBS-induced inflammation. RT-PCR revealed that at *day 3*, CLR mRNA levels in rats treated with TNBS were not significantly different from that of vehicle controls (Fig. 3C). We also ascertained whether treatment with TNBS or dsRNA altered CGRP levels. TNBS treatment by itself did not appear to increase CGRP-IR in the neurons of the myenteric plexus, and CGRP-IR did not differ between different groups of rats treated with both dsRNA and TNBS (Fig. 3D).

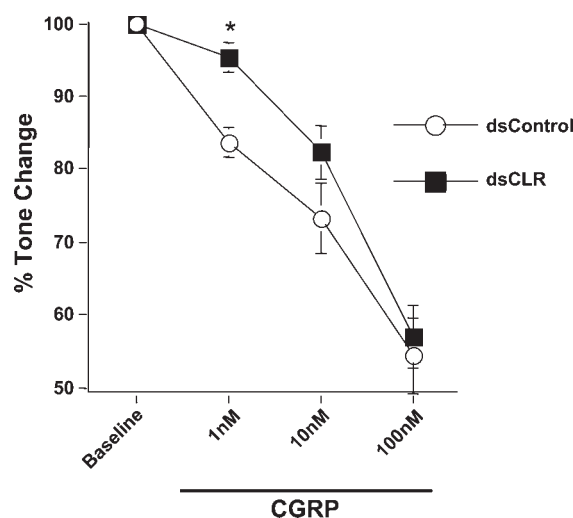


Fig. 2. CLR is a functional receptor for calcitonin gene-related peptide (CGRP). The reduction in smooth muscle tone elicited by the addition of 1 nM CGRP was significantly attenuated in CLR knockdown rats. As the system became saturated with graded increases in the dose of CGRP, the drop in muscle tone measured in the CLR knockdown approached that of controls. Values are means  $\pm$  SE;  $n = 4$ /group (at least 2–3 recordings were made from individual rats, and data were pooled). \* $P < 0.01$  by Student's *t*-test.



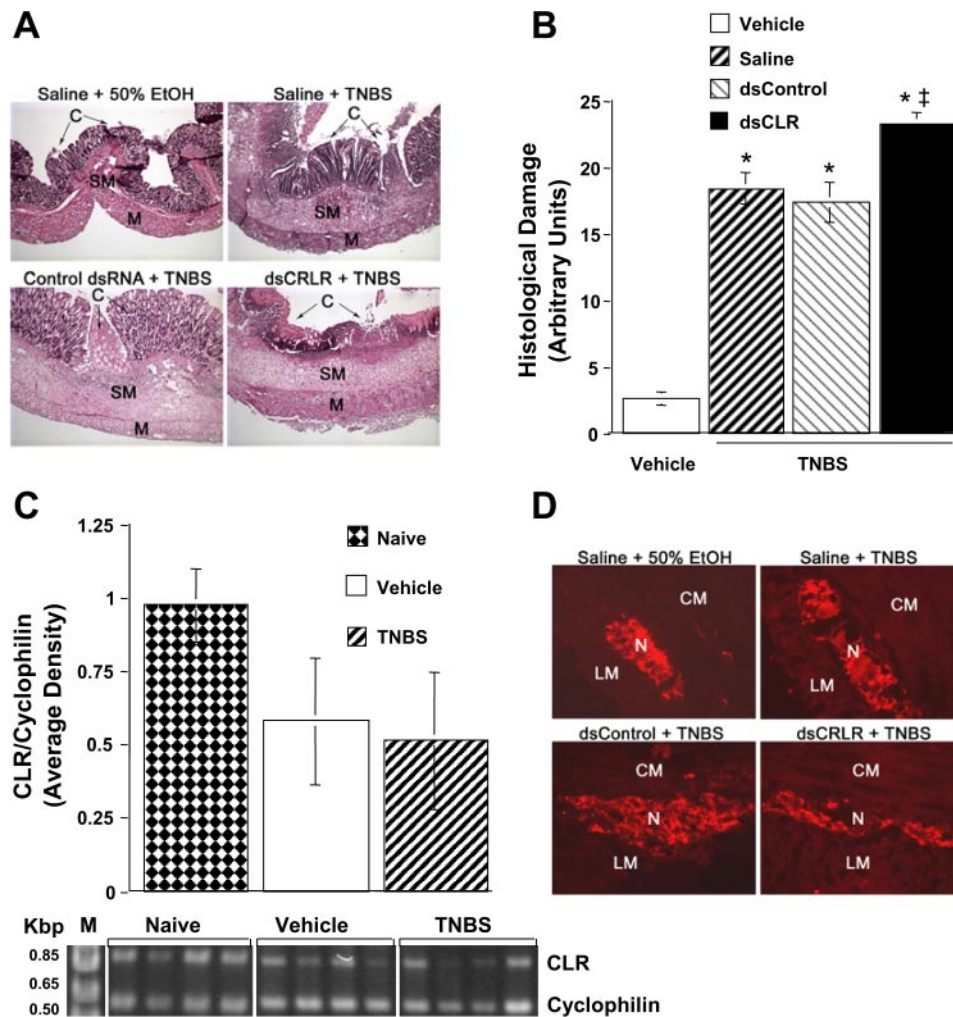


Fig. 3. CLR exerts anti-inflammatory effects in a rat model of colitis. **A**: baseline architecture of the colon in rats receiving vehicle treatment (50% ethanol enema + saline injection) was essentially unperturbed. Rats that received saline + trinitrobenzene sulfonic acid (TNBS) showed changes identical to control dsRNA + TNBS, such as marked thickening of the submucosa with infiltration of inflammatory cells and regions of crypt ulceration. Rats that received dsCLR had an exacerbated inflammatory response with significantly increased tissue necrosis. C, crypts; M, circular and longitudinal muscle layers; SM, submucosal layer. Magnification,  $\times 5$ . **B**: tissue histology scoring of hematoxylin and eosin-stained sections validated our initial findings that knockdown of CLR worsens inflammation. Tissue taken from rats injected with dsCLR had significantly more histological damage than rats injected with saline + TNBS or dsControl + TNBS. Furthermore, all groups treated with TNBS had significantly more tissue damage than that seen in uninfamed, vehicle-treated colon. Values are means  $\pm$  SE;  $n = 4-10$ /group;  $*P < 0.05$  vs. vehicle (50% ethanol enema + saline injection);  $\ddagger P < 0.05$  for dsCLR vs. other TNBS-treated groups as well (ANOVA, Bonferroni). **C** and **D**: colitis does not affect CLR or CGRP levels. **C**: semiquantitative RT-PCR revealed that CLR mRNA levels were not changed on TNBS treatment at *day 3* compared with vehicle control. In fact, ethanol treatment itself apparently inhibited levels of CLR mRNA compared with naïve rats. *Bottom* depicts PCR product from 4 different rats within each treatment group. CLR signal intensity was normalized against that of cyclophilin. **D**: immunostaining of dsRNA + TNBS-treated and vehicle control sections revealed that CGRP levels were not qualitatively different in TNBS-treated colons compared with vehicle controls. Magnification,  $\times 40$ .

TNBS induced MPO activity in the rat colon on *days 1* and *3* of TNBS treatment, whereas MPO activity was very low to undetectable in vehicle controls (Fig. 4), suggesting enhanced neutrophil infiltration into the submucosal layer. Similarly, MPO activity was enhanced in rats treated with dsControl RNA and TNBS. Surprisingly, MPO activity was not further increased in rats with transient knockdown of CLR (Fig. 4).

Next we examined whether levels of proinflammatory cytokines such as TNF- $\alpha$  and IL-6 were changed in rats with knockdown of CLR in response to TNBS-induced inflammation at both *day 1* and *day 3*. RT-PCR revealed that TNF- $\alpha$  and IL-6 mRNA levels were undetectable or expressed at very low levels in vehicle control rats, whereas their levels significantly increased in the colons of rats treated with saline + TNBS or

with dsControl + TNBS. TNF- $\alpha$  levels were further increased in rats that had transient knockdown of CLR (Fig. 5A). In contrast to TNF- $\alpha$  levels, IL-6 mRNA levels were not significantly different between different TNBS treatment groups (Fig. 5B). Western blot analysis further confirmed that both TNF- $\alpha$  and IL-6 protein levels were significantly increased after TNBS treatment. TNF- $\alpha$  antibody detects both the precursor peptide (32–34 kDa) and the mature, cleaved 17-kDa peptide. Although the levels of 17-kDa TNF- $\alpha$  peptide were further increased in rats with transient knockdown of CLR (Fig. 5C), when the two bands were quantitated together, TNF- $\alpha$  levels did not differ significantly between different TNBS treatment groups. In general, protein data were in agreement with the RT-PCR data.

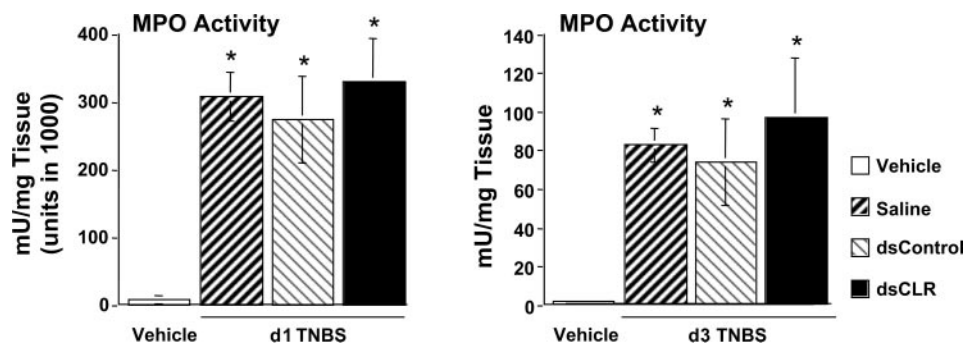


Fig. 4. CLR does not affect myeloperoxidase (MPO) activity in TNBS-induced colitis. MPO assay at *day 1* and *day 3* after rats were given a TNBS enema showed a significant increase in MPO activity in colon tissue homogenates of all groups compared with vehicle controls. There was a nonsignificant, incremental increase in MPO activity in the colons of rats receiving dsCLR + TNBS over other TNBS-treated groups. Values are means  $\pm$  SE;  $n = 5-12$ /group; \* $P < 0.05$ .

*Colitis, not dsRNA, affects body weight gain.* Colitis results in loss of body weight. As expected, TNBS treatment resulted in reduced weight gain in all treatment groups compared with vehicle controls (Fig. 6A). By *day 3* of TNBS treatment, rats in all groups began to gain weight. To ascertain that this reduction in body-weight gain was not due to treatment with dsRNA, food and water intake were measured for the duration of the experiment. As shown in Fig. 6, B and C, dsRNA treatment did not affect food or water intake; rats treated with TNBS across the groups displayed a trend toward reduced food intake (Fig. 6B).

DISCUSSION

In this study, we demonstrate that RNAi resulted in a transient knockdown of CLR in the periphery that was site specific and allowed us to determine that CLR is a functional receptor for CGRP. Although we previously used RNAi to

specifically knock down expression of certain neuropeptides in the rat ileum (19), we did not address the time course or the distance of this effect. In the current study, the effect of dsRNA injection on CLR protein knockdown lasted for  $\sim 7$  days; by *day 9* CLR expression returned to baseline. The distance to which the effect of knockdown spread ranged from 1.5 to 2.0 cm proximal and distal to the dsRNA injection sites. Several factors determine this spatiotemporal knockdown achieved by RNAi. The half-life of mRNA and protein will eventually govern the duration of RNAi's effect. If a protein is transcribed and translated at a different location and is transported to nerve terminals, then knockdown of these transported protein molecules will not be accomplished because RNAi acts locally and at the transcriptional level; this seems to be the case for CLR expressed in the nerve fibers in the lamina propria and circular muscle. Knockout animals are invaluable in studying how a particular gene might function. However, if the lack of gene

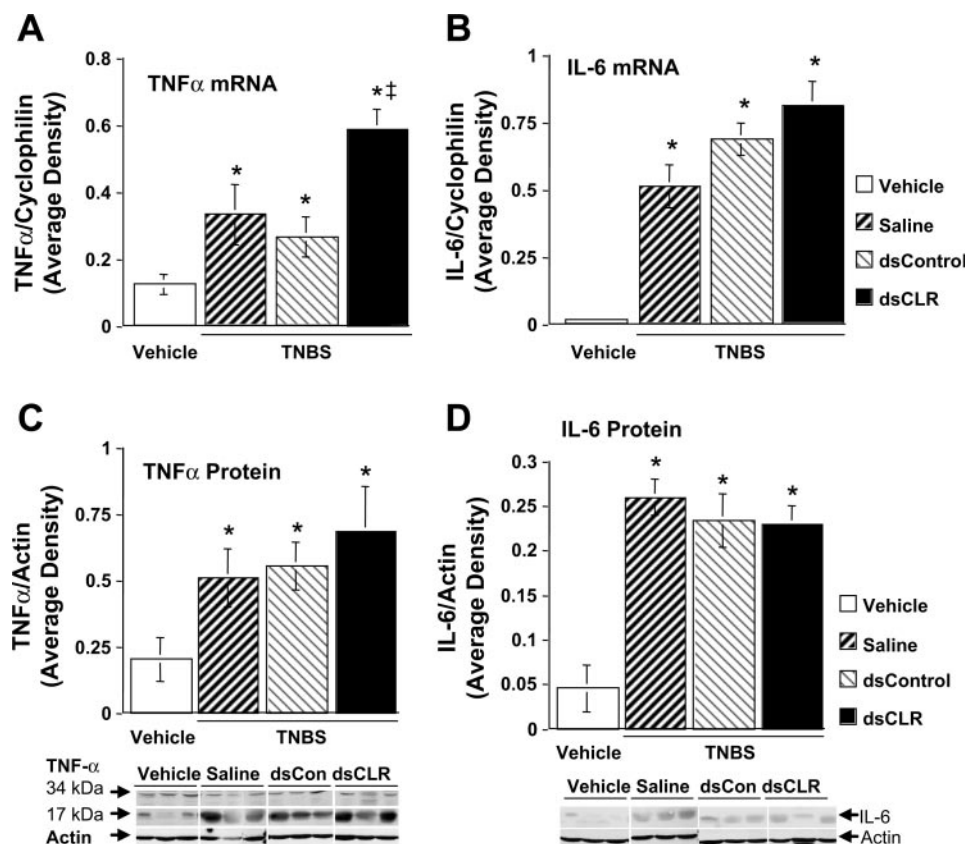


Fig. 5. Knockdown of CLR affects specific Th1 cytokines. A and C: TNBS treatment induced colonic TNF- $\alpha$  mRNA and protein levels compared with vehicle-treated controls (\* $P < 0.05$ ). Prior knockdown of CLR by using RNAi further increased TNF- $\alpha$  mRNA levels in the dsCLR group compared with other TNBS-treated groups ( $\ddagger P < 0.05$ ). B and D: IL-6 mRNA and protein levels were low in the vehicle group and were significantly induced on TNBS treatment (\* $P < 0.05$ ); however, knockdown of CLR did not significantly increase IL-6 levels over other TNBS-treated groups. Vehicle was 50% ethanol enema + saline injection.

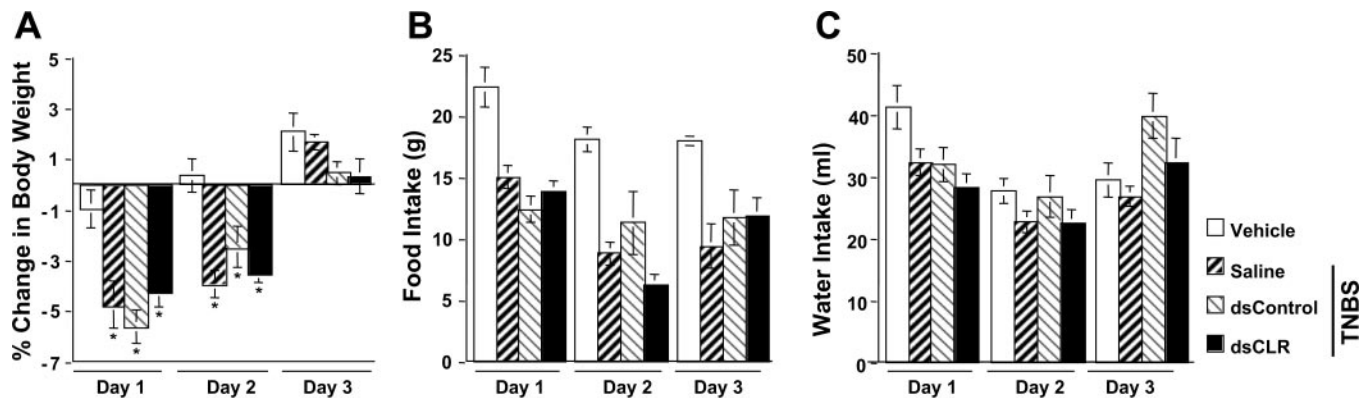


Fig. 6. Colitis, but not dsRNA, affects biometric variables. Groups of rats that received TNBS enemas showed a reduced rate of gain in body weight compared with vehicle-treated controls on the first 2 days examined (A). *Day 1* was the day when either a TNBS enema or a vehicle (50% ethanol) enema was delivered under a brief period of anesthesia ( $*P < 0.05$  for vehicle-treated group vs. TNBS-treated groups on *days 1* and *2*). There were no significant differences between vehicle controls and TNBS-treated groups in food consumption (B) or water intake (C) on all 3 days examined. Values are means  $\pm$  SE;  $n = 5$ – $12$ /group.

product results in embryonic lethality, use of knockouts is limited. Indeed, the recently described CLR knockout mice are embryonic lethal and have severe cardiovascular defects (6). Thus RNAi provides an attractive alternative to study gene function in adult animals.

Neuroactive peptides, such as substance P and CGRP, mediate colonic motor response to pain and inflammation. The effect of CGRP on spontaneous contractions is strikingly different between longitudinal and circular muscle layers. In longitudinal muscles CGRP-mediated stimulation is preceded by a transient inhibition, whereas in circular muscle only inhibition is observed (21). A heterodimer of CLR and RAMP1 is proposed to be the functional receptor for CGRP (23). To demonstrate that CLR indeed mediates CGRP's effect on colonic motility, we generated a transient knockdown of CLR in the rat colon under basal conditions and subsequently measured longitudinal smooth muscle tone in response to graded concentrations of CGRP. At a concentration of 1 nM, CGRP caused a significant decrease ( $\sim 18\%$ ) in histamine-induced tone in control colons, whereas in colons with knockdown of CLR 1 nM CGRP did not decrease muscle tone. At higher concentrations of CGRP, this difference in decrease in tone was lost between the control and dsCLR groups. It is possible that at saturating concentrations of CGRP, the remaining CLR receptors were equally effective at mediating CGRP's effect on tone. It is also possible that the presence of extrinsic CLR receptors on nerve fibers, whose expression remained unchanged, diluted the effect of intrinsic CLR receptors that were knocked down by RNAi. Importantly, at physiologically relevant concentrations, CGRP was ineffective at decreasing muscle tone in rats that lacked CLR. Thus these results suggest that CLR is the functional receptor for CGRP.

Although CGRP plays an integral role in neurogenic inflammation and blood flow, the role of CLR in mediating the effects of CGRP has not been determined. Using the TNBS colitis model, we established that CLR mRNA levels are not altered in inflamed colon compared with levels in colons of 50% ethanol-treated controls but that these levels are inhibited by ethanol treatment by itself when compared with levels in naïve rats. Whether levels of CLR are altered in IBD patients is not known. Paradoxically, in some IBD patients, a pronounced reduction in CGRP had been reported in intestinal muscle

layers compared with normal controls, whereas in IBD patients with moderate-to-severe ulcerative colitis, tissue CGRP levels are not different between patients and controls (11, 27). In this study, CGRP levels did not appear to be increased on TNBS treatment. Interestingly, CGRP levels in rats with colitis and knockdown of CLR also did not differ from that of vehicle controls.

To determine the role of colonic CLR in the development of colitis, we knocked down expression of CLR in the colon by using RNAi before inducing colitis by a TNBS enema. We found that prior inhibition of CLR resulted in exacerbated intra-abdominal adhesions, tissue necrosis, and histological damage to the colon. This was not surprising because in the acute phase of inflammation, histological damage, such as increased transmural neutrophil infiltration, is normally observed. However, unlike histological damage, MPO activity did not differ between the control + TNBS group and the dsCLR + TNBS group on either day of treatment (*day 1* and *day 3*). This finding suggests that CLR does not affect recruitment of neutrophils. Previous studies support our findings in showing that peripheral infusion of  $\alpha$ -CGRP in rats treated with TNBS has a protective effect on colon morphology (22).

Although the etiology of inflammatory diseases is uncertain, activation of the mucosal immune system in response to bacterial antigens with consecutive pathological cytokine production is thought to have a key role (8, 28). In particular, cytokines produced by T lymphocytes appear to initiate and perpetuate chronic intestinal inflammation (4, 12, 25, 32). Interestingly, cytokine production by lamina propria CD4<sup>+</sup> T lymphocytes differs between Crohn's disease and ulcerative colitis. Whereas Crohn's disease is associated with increased production of Th1-type cytokines such as IFN- $\gamma$  and TNF- $\alpha$ , ulcerative colitis is associated with increased production of the Th2-type cytokine IL-5 but IFN- $\gamma$  production is unaffected (12, 25, 32). There appears to be a fragile balance between proinflammatory (TNF- $\alpha$ , IL-6, IL-12) and anti-inflammatory (IL-4, IL-10, IL-11) cytokines secreted under normal conditions in the intestinal mucosa. This balance must be orchestrated by interplay between several signaling molecules.

TNF- $\alpha$  has been the target for therapeutic treatment in IBD patients, and infliximab, a chimeric anti-TNF- $\alpha$  monoclonal antibody, has become a commonly used therapy for patients



with severe or refractory Crohn's disease. Increases in colonic TNF- $\alpha$  levels appear to be the hallmark of IBD; patients with clinical remission display significantly decreased levels of TNF- $\alpha$  and IL-6 levels (34). We determined whether knockdown of CLR affected proinflammatory cytokines such as TNF- $\alpha$  secreted by Th1-type immune cells. Ethanol-treated vehicle control colons expressed very low-to-undetectable levels of TNF- $\alpha$  and IL-6 mRNAs (secreted by monocytes and macrophages, respectively), whereas TNBS treatment significantly induced secretion of TNF- $\alpha$  and IL-6 mRNA and protein levels. Knockdown of CLR before induction of TNBS colitis further increased TNF- $\alpha$  mRNA. However, neither TNF- $\alpha$  protein levels nor levels of IL-6 mRNA or protein were further increased by CLR knockdown, suggesting that the effect on inflammation may be a secondary event. Knockdown of CLR resulted in altered motility (a primary event), which may have resulted in increased exposure to TNBS in this group of rats, thereby exacerbating inflammation.

Administration of long dsRNA to achieve site-specific knockdown in the peripheral gut tissue had no adverse effects on food and water intake in the rats in our study. Although TNBS-induced colitis resulted in reduction of body-weight gain, it did not alter food or water intake. Similarly, rats treated with long dsRNA for control or CLR did not reduce their food or water intake, thereby suggesting that dsRNA does not adversely affect biometric variables. Thus RNAi can be used to downregulate expression of genes in the GI tract. Silencing of CLR exacerbated TNBS-induced colitis, suggesting a protective role for CGRP and its receptor in colonic inflammation.

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