Melanin-concentrating hormone as a mediator of intestinal inflammation

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Communicated by Susan E. Leeman, Boston University School of Medicine, Boston, MA, May 12, 2008 (received for review September 13, 2007)

Melanin-concentrating hormone (MCH) is expressed primarily in the hypothalamus and has a positive impact on feeding behavior and energy balance. Although MCH is expressed in the gastrointestinal tract, its role in this system remains elusive. We demonstrate that, compared to wild type, mice genetically deficient in MCH had substantially reduced local inflammatory responses in a mouse model of experimental colitis induced by intracolonic administration of 2,4,6 trinitrobenzene sulfonic acid (TNBS). Likewise, mice receiving treatments with an anti-MCH antibody, either prophylactically or after the establishment of colitis, developed attenuated TNBS-associated colonic inflammation and survived longer. Consistent with a potential role of MCH in intestinal pathology, we detected increased colonic expression of MCH and its receptor in patients with inflammatory bowel disease. Moreover, we found that human colonic epithelial cells express functional MCH receptors, the activation of which induces IL-8 expression. Taken together, these results clearly implicate MCH in inflammatory processes in the intestine and perhaps elsewhere.

experimental colitis | IL-8 | inflammatory bowel disease | neuropeptides | MCH deficient mice

Melanin-concentrating hormone (MCH) is a 17- to 19-aa cyclic neuropeptide conserved from fish to human (1) and predominantly localized in the brain (2). Several pharmacological and genetic studies revealed a role for this peptide in the regulation of feeding behavior and energy expenditure toward a positive energy balance (3–5). More recent studies extended the physiological functions of MCH as a broad regulator of cognitive and autonomic aspects related to rewarding behaviors (6, 7). Outside the brain, MCH is localized in the pancreas (8), skin (9), and gastrointestinal tract (10). It has been also found in tissular and circulating immune cells (11–14). However, the physiological role of MCH in these peripheral tissues has yet to be established.

In humans, two G-protein-coupled receptors for MCH have been identified, MCHR1 (also known as SLC1 or GPR24) (15–18) and MCHR2 (19–21), whereas rodents express only MCHR1. In the rodent brain, MCHR1 is expressed in areas important for feeding, learning and motivated behavior, integration of sensory and gustatory inputs, autonomic control, and arousal (22, 23). MCHR1 mRNA is also expressed in the thyroid, kidney, adipose tissue, lung, testes, and tongue (23), whereas functional MCHR1 is also present in lymphocytes (12, 14), insulin-producing cell lines (24), and mouse and human pancreatic islets (8).

Several neuropeptides that are part of the neuroendocrine system exhibit important immunomodulatory effects and mediate inflammation in various organs, including the intestine (25, 26). There is little evidence to indicate expression of MCHR of either type in the intestine of animals or humans, and the role of MCH in inflammatory responses in the gut or elsewhere has not been evaluated. Based on these considerations and because MCH is also expressed in immune cells (12, 14), we hypothesized that MCH might be involved in the pathophysiology of intestinal inflammation.

To address this hypothesis we used the 2,4,6 trinitrobenzene sulfonic acid (TNBS) mouse model of experimental colitis that is characterized by the infiltration of the intestine by immune cells and production of T helper 1 cytokines resembling Crohn's disease (CD) (27). The same model has been previously used to demonstrate the role of neuropeptides such as substance P (SP), vasoactive intestinal peptide, and endogenous opioids in inflammatory bowel disease (IBD) (26). We treated mice with an anti-MCH antibody to explore whether MCH plays any role in intestinal inflammatory responses. To further support an involvement of MCH in colitis, we studied TNBS-induced colitis in MCH-deficient mice (28). We also examined whether patients with IBD had altered intestinal MCH expression.

PHYSIOLOGY

Results

An Antibody to MCH Reduces Colonic Mucosal Inflammation in Response to Intracolonic TNBS Administration. We treated mice with 1 mg/kg anti-MCH or control antibody 24 h before TNBS exposure and daily thereafter for a total of 3 days. Compared with injection of a control antibody, anti-MCH antibody administration resulted in 40% reduced intestinal inflammation as indicated by histological analysis (Fig. 1). In a separate cohort, mice were injected with an anti-MCH or control antibody starting 24 h before TNBS exposure and continuing daily thereafter for 7 more days. During that period, 50% (6/12) of mice treated with an anti-MCH antibody survived, whereas only 9% (1/11) of mice that received control antibody survived ($P < 0.05$ by χ^2), supporting a detrimental role for MCH in experimental colitis.

To gain some understanding about the time course of the MCH-dependent response during TNBS-induced colitis, mice were treated daily with anti-MCH antibody after the establishment of colitis, starting at day 3 post-TNBS. Our results indicate that at the end of the study, mice receiving anti-MCH treatment had less intestinal inflammation as assessed by the degree of colon shortening (Fig. 2*A*) and the macroscopic (Fig. 2*B*) and histological colonic damage scores (Fig. 2*C*). To examine whether the effects of anti-MCH antibody treatment were coupled to a reduction in appetite and weight loss, as is the case with several MCH antagonists developed to treat obesity (29), mice received daily injections of anti-MCH antibody or control antibody for 1 week, and their food intake and body weight changes were monitored over time. We found weight gain [supporting information (SI) Fig. S1*A*] and food intake

Author contributions: E.K., A.C.M., S.L., and C.P. designed research; E.K., A.C.M., D.T., I.K., and S.L. performed research; A.C. and E.M.-F. contributed new reagents/analytic tools; E.K. and M.O. analyzed data; and E.K. and C.P. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0804536105/DCSupplemental.

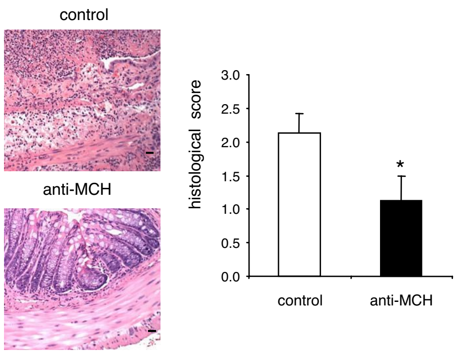

Fig. 1. Mice receiving prophylactic anti-MCH treatment develop attenuated TNBS colitis. Mice were treated daily with anti-MCH or control antibody (1 mg/kg) starting the day before TNBS exposure for a total of three doses. Histological scoring of colonic inflammation was performed in H&E-stained sections under a light microscope. *, $p < 0.05$ compared to control. (Calibration bar = 100 μm.)

(Fig. S1*B*) patterns to be indistinguishable between the two mice groups and, at the end of the study, both mouse groups exhibited similar adiposity (Fig. S1*C*).

MCH-Deficient Mice Develop Reduced Colonic Inflammatory Responses in Response to Intracolonic TNBS Administration. To further assess the potential importance of MCH in colitis, we administered TNBS to MCH-deficient mice (28), and after 48 h we evaluated histological parameters of colitis and mRNA expression of several cytokines in the colon. Histological analysis of the mouse colon revealed normal mucosal architecture of $MCH^{-/-}$ mice compared with $MCH^{+/+}$ (WT) mice at baseline (data not shown). TNBS administration resulted in severe mucosal damage and immune cell infiltration in WT mice. In contrast, $MCH^{-/-}$ mice exhibited 50% less inflammation (Fig. 3*A*). Consistent with the histological evidence, colonic mRNA levels of the proinflammatory cytokines TNFα, IL-1β, and IL-6 were 60–85% lower in TNBS-treated $MCH^{-/-}$ mice compared with their WT littermates (Fig. 3*B*). Baseline cytokine levels in WT and $MCH^{-/-}$ mice, such as IL-1β [100 ± 44 vs. 66 ± 21 arbitrary units (AU), respectively] and TNF-α (100 ± 24 vs. 148 ± 26 AU, respectively) were not different. A significant 4-fold increase in colonic MCH mRNA expression was evident 48 h after induction of colitis in WT mice (Fig. 3*C*). These results further support a role for MCH in the pathophysiology of acute, TNBS-induced experimental colitis.

MCH and MCHR1 Are Expressed in the Enteric Nervous System. Previous reports have described *de novo* synthesis of MCH within intrinsic cells throughout the rat digestive system, but failed to further characterize these cells (10). In the present study, we used double labeling to clearly demonstrate MCH immunoreactivity in a subpopulation of neurons in the rat myenteric (Fig. 4 *A1–A3*) and submucosal plexus (Fig. 4 *B1–B3*). Expression of MCH was also found in the guinea-pig myenteric (Fig. 4*C*) and submucosal (Fig. 4*D*) plexus. The majority of the rat myenteric (Fig. 4 *F1–F3*) and submucosal (Fig. 4 *G1–G3*) neurons were positive for MCHR1 expression. The above results, together with the reported presence of MCH and MCHR1 in immune cells (12, 14), further support a role for MCH in mediating neuroimmune interactions in the intestine.

MCH and MCHR1 mRNA Expression Are Increased in Human Colitis. In light of the evidence for a proinflammatory effect of MCH in experimental colitis, we then investigated whether expression of MCH and its receptors is altered in the colonic mucosa of IBD patients. Paired biopsies from both normal and inflamed mucosa of 10 patients with ulcerative colitis (UC) and 6 with CD were obtained during colonoscopy and MCH, MCHR1, and MCHR2 mRNA expression was determined by real-time PCR. Compared with samples from uninvolved intestine of the same patients, we found several fold up-regulation of MCH and its receptors in the inflamed mucosa of IBD patients (Fig. 5).

MCH Functional Receptors Are Expressed in Colonocytes. Our previous studies on the role of additional neuropeptides such as substance P, neurotensin, and members of the corticotropin-releasing hormone (CRH) family of peptides in intestinal inflammation demonstrated that receptors for these molecules are expressed in intestinal epithelial cells and their expression is increased during the course of inflammation (30–34). Hence, to examine epithelial cell expression of MCH and its receptors, we used a laser capture microdissection (LCM) approach to isolate colonic epithelial cells from cryopreserved surgical specimens obtained from patients with IBD and controls (35). Real-time RT-PCR analysis indicated that human colonic epithelial cells express MCHR1 (Fig. 6*A*), but not MCHR2 or MCH mRNA (data not shown). Notably, a >5-fold increase in MCHR1 mRNA expression was observed in patients with CD ($P < 0.01$). In patients with UC, a similar trend was found but it did not reach statistical significance (Fig. 6*A*). Consistent with these findings, in HT-29 human colorectal adenocarcinoma cells we detected mRNA expression of MCHR1 (Fig. 6*B*), but not MCHR2 or MCH, either at baseline or upon stimulation (data not shown). Moreover, a >7-fold induction of MCHR1 expression was observed in these cells in response to IL-1β exposure (Fig. 6*B*). Most importantly, stimulation of HT-29 colonocytes with MCH resulted in a 50% up-regulation of IL-8 mRNA expression (152 ± 23 AU vs. 100 ± 14 AU in control treatment; $P < 0.05$; Fig. 6*C*), confirming a functional role of MCHR1 in these cells. Taken together, these results suggest that MCH acting on

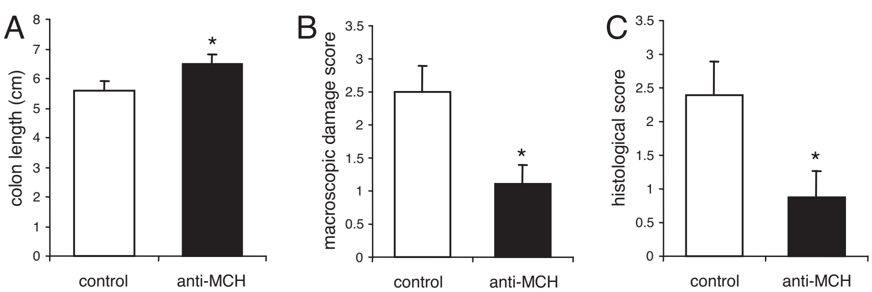

Fig. 2. Treatment with anti-MCH antibody attenuates established TNBS colitis. Starting at day 3 post-TNBS exposure, after colitis had been established, mice received daily injections of anti-MCH or control antibody for a total of four doses. The severity of colitis was assessed by measuring colon length (*A*) and scoring colonic macroscopic inflammation (*B*) and microscopic inflammation (*C*). *, $p < 0.05$ compared to control.

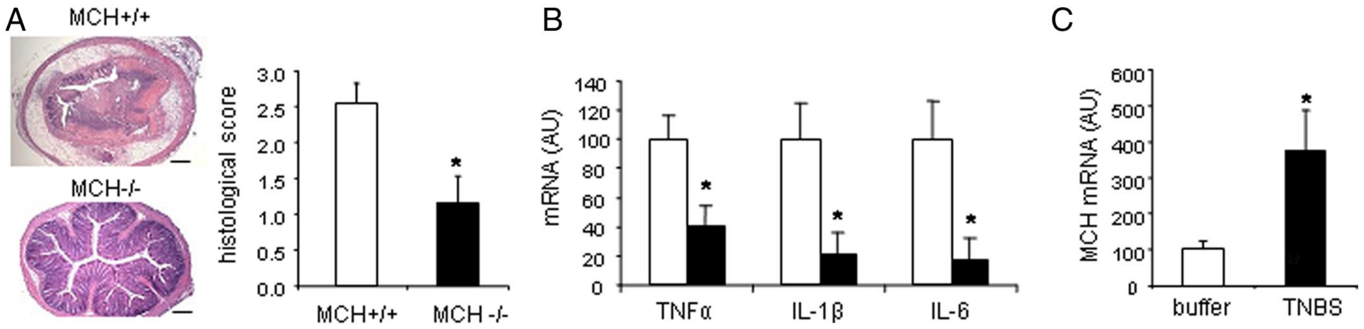

Fig. 3. MCH-deficient mice develop attenuated TNBS-induced acute colitis. (*A*) Histological scoring of inflammation in H&E-stained colonic cross-sections from TNBS-treated MCH $^{-/-}$ mice and their WT littermates. (Calibration bar = 400 μm.) (*B*) Colonic expression of various proinflammatory cytokines at 48 h post-TNBS exposure was measured in MCH $^{-/-}$ and MCH $^{+/+}$ mice by real-time PCR. (*C*) Colonic MCH expression at 48 h post-TNBS in WT mice was measured by real-time PCR. *, $p < 0.05$ compared to control.

colonocytes might play an important role in the pathophysiology of IBD, most likely by contributing to immune cell recruitment via chemokine release.

Discussion

The present study reveals a link between MCH-related pathways and intestinal inflammation. In mice, MCH deficiency conferred a significant degree of protection from TNBS-induced colitis, suggesting that MCH mediates proinflammatory effects in the intestine and perhaps in other systems. The clinical significance of this finding is supported by the increased expression of MCH and its receptor in the affected colonic mucosa of patients with IBD and is further underscored by our results demonstrating that treatment of mice with anti-MCH antibody attenuates the development of experimental colitis.

One potential mechanism by which MCH amplifies inflammatory processes seems to be via direct action on colonocytes. We found that MCHR1 expression on human colonic epithelial cells is up-regulated under inflammatory conditions. MCH stimulates colonocyte IL-8 production, which in turn further promotes local recruitment of immune cells. The pathways leading to up-regulation of MCHR1 expression in colitis can only be speculated at this point. An interesting possibility is via cytokine, driven NF-κB-dependent mechanisms as shown in Fig. 6*B*, and previously described for substance P (36) and CRH (34) receptors. An important question relates to the mechanisms by which MCH-MCHR1 interactions mediate proinflammatory responses in the intestinal mucosa, including colonic epithelial cells. Very little is known about the intracellular targets for MCHR1 besides its coupling via multiple G protein subunits (Gi, Go, and Gq), which results in inhibition of forskolin- or β-agonist-stimulated cAMP production, increase of intercellular calcium and phosphoinositides, and phosphorylation of PKC and erk (37, 38). The PKC and Erk pathways might be quite relevant to the MCH signaling in colonocytes. Previous studies from our group have demonstrated that in the same cells, substance P, neurotensin, and ghrelin stimulate IL-8 gene expression through protein kinase C-mediated NF-κB activation and parallel erk1/2 activation (39–41).

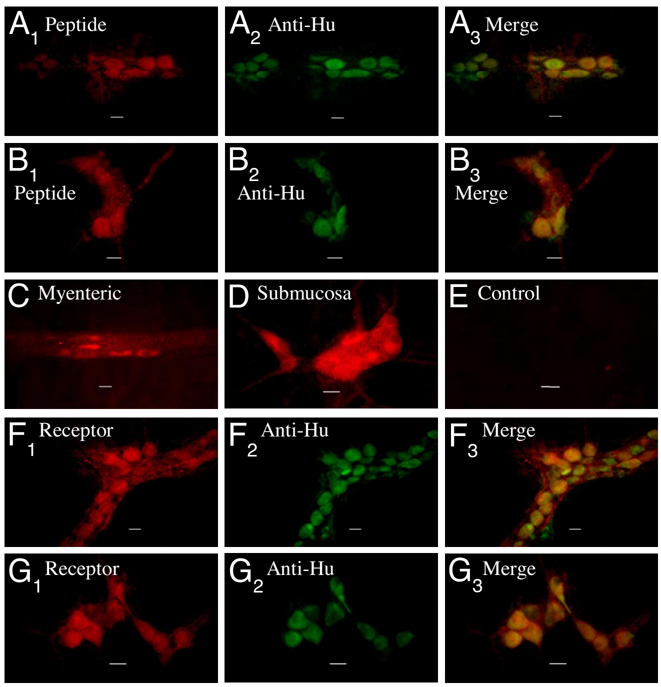

Fig. 4. MCH and MCHR1 immunoreactivity in the rat enteric nervous system. (*A 1–3*) Double staining for MCH and the enteric neuron marker anti-Hu was performed in whole-mount preparations of the rat myenteric plexus. (*B 1–3*) Double staining for MCH and the enteric neuron marker anti-Hu was performed in whole-mount preparations of the rat submucosal plexus. (*C*) MCH staining of the guinea-pig myenteric plexus. (*D*) MCH staining of the guinea-pig submucosal plexus. (*E*) Negative control staining with the primary antibody for MCH omitted. (*F 1–3*) Double staining for MCHR1 and anti-Hu in the rat myenteric plexus. (*G 1–3*) Double staining for MCHR1 and anti-Hu in the rat submucosal plexus. (Calibration bar = 40 μm.)

The effectiveness of anti-MCH antibody treatment in mouse experimental colitis suggests that the MCH-mediated proinflammatory effects are largely peripheral, because the anti-MCH antibody is not likely to cross the blood-brain barrier due to its molecular mass. In further support of this claim, our experiments show that anti-MCH antibody treatment did not affect mouse food intake and weight gain, which are regulated mainly via central mechanisms. Moreover, circulating levels of MCH were not found to be increased in mice with TNBS colitis (data not shown). Thus, the reported increase in local MCH expression in mice with colitis and in patients with IBD further corroborates the involvement of a peripheral MCH-dependent pathway in the pathogenesis of intestinal inflammation. In the gut, likely sources of MCH are the enteric nervous system, enterochromaffin cells, and resident or infiltrating immune cells, based on evidence described here (Fig. 4) and in previous reports (10, 12–14, 42).

There have been few reports in the literature suggesting an immunomodulatory role for MCH based on its source and targets among certain peripheral blood mononuclear cell (PBMC) subpopulations. For example, it has been shown that MCH had a mild inhibitory effect on spontaneous and stimu-

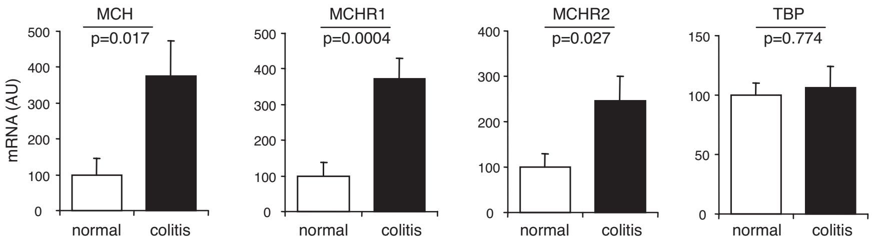

Fig. 5. MCH and MCHR1 mRNA expression are increased in human colitis. MCH, MCHR1, MCHR2, and TBP (control for RNA quality) levels in biopsies of normal colonic mucosa (empty bars) and inflamed mucosa (filled bars) from patients with active IBD (n = 16) were determined by real-time PCR.

lated PBMC proliferation and IL-2, but not IL-4, IFN-γ, or TNF-α production (12, 13). Another study has identified activated human T helper 2 cells as a potential source of MCH, which might be of importance for allergic reactions such as asthma (14). However, implication of MCH in the pathogenesis of any human inflammatory condition, as shown in the present study, has not been previously described to our knowledge, with the exception of MCHR1 serving as an autoantigen in vitiligo, a common depigmenting skin disorder (43).

Our results clearly point to a proinflammatory role of MCH in the development of colitis. They add MCH into an expanding list of neuropeptides and hormones that regulate energy homeostasis and intestinal inflammatory responses (40, 44–46). Among them, leptin seems to have effects similar to MCH. Leptin-deficient mice develop attenuated dextran sulfate sodium- and TNBS-induced experimental colitis (44), and treatment with leptin accelerates colonic inflammation (45). Interestingly, we have previously reported that MCH positively regulates leptin expression and secretion in adipocytes (47). Thus, it is quite tempting to speculate that MCH might have similar effects on leptin expression in colonic epithelial cells (45) during inflammation. In further support of the proinflammatory effects of MCH in the intestine, α-MSH, a functional antagonist of MCH (48), reduces the clinical and histological severity of experimental colitis in rats (49), and a mutation in one of the α-MSH receptors aggravates colitis in mice (50).

In summary, our findings directly support a key role for intestinal MCH and MCHR1 in the pathogenesis of acute experimental colitis and possibly human IBD. We showed that MCH immunoneutralization is an effective treatment for TNBS-induced colitis. Therefore, MCH antagonism as a potential therapeutic approach for IBD, and in particular CD, is a direction worth pursuing. In fact, several pharmaceutical companies in the United States and elsewhere, have developed >96 compounds that antagonize the action of MCH as antiobesity drugs (51). Moreover, by virtue of their antiinflammatory properties, agents blocking peripheral MCH actions might succeed to treat additional inflammatory conditions of nonintestinal origin, where up-regulation of MCH and/or its receptor is a common feature.

Methods

Human Samples. Colonic mucosal biopsies from involved and uninvolved regions, confirmed as such by a pathologist, of the same individual were collected by punch biopsies (three per site). Donors were patients undergoing colonoscopy at the Gastrointestinal Endoscopy Unit of Beth Israel Deaconess Medical Center upon approval by the Institutional Review Broad. Biopsies were immediately frozen in liquid nitrogen. Informed consent was obtained from all study participants. LCM of colonic epithelial cells was performed in cryopreserved 8-μm-thick sections of discarded colonic tissue from five patients with CD, four patients with UC, and seven controls obtained from the Ardais/ Beth Israel Deaconess Medical Center BIGR Tissue Library, using a PixCell II LCM system (Arcturus). An estimated 600–1,000 cells per tissue sample were captured onto CapSure IS ICM caps, and RNA was prepared using a Picopore RNA isolation kit (Arcturus). RNA was treated with DNase I (Qiagen), and cDNA was made using a GeneAmp kit (Applied Biosystems).

Mice. Eight-week-old CD1 male mice were purchased from Charles River Laboratories. The generation of MCH$^{-/-}$ mice has been described (28). For the current experiments, we used 8- to 10-week-old MCH$^{-/-}$ mice and their MCH$^{+/+}$ littermates from our colony, generated by heterozygous breeding and fully backcrossed to the C57BL/6J genetic background (52). Mice were maintained in a controlled environment under an alternating 12-h light/dark cycle, with free access to food and water. All studies were approved by the Beth Israel Deaconess Institutional Animal Care and Use Committee.

TNBS Colitis. A solution (2 mg per mouse) of TNBS (Fluka) in 35% ethanol or saline in 35% ethanol (vehicle) was infused into the colonic lumen (3.5 cm from the anal verge) of anesthetized MCH$^{+/+}$ and MCH $^{-/-}$ mice (n = 8 per group) via a 1-ml syringe fitted with a polyethylene cannula (Intramedic PE-20 tubing; Becton Dickinson). To prevent leakage of the TNBS solution, mice were maintained in a supine Trendelenberg position until recovery from anesthesia. Forty-eight hours after TNBS administration, mice were killed, and the distal colon was harvested for further analysis. In another series of experiments, TNBS (2 mg)-exposed CD1 mice received daily treatments with anti-MCH or control antibody (1 mg/kg) (n = 8 per group) initiated at the day before TNBS treatment for a total of three doses. Mice were killed at 48 h post-TNBS treatment. A separate cohort of mice (n = 11–12 per group) was treated with 4 mg of TNBS per mouse along with daily anti-MCH or control antibody treatments starting the day before TNBS treatment and daily thereafter. Mouse survival was monitored for 7 days post-TNBS treatment. TNBS (2

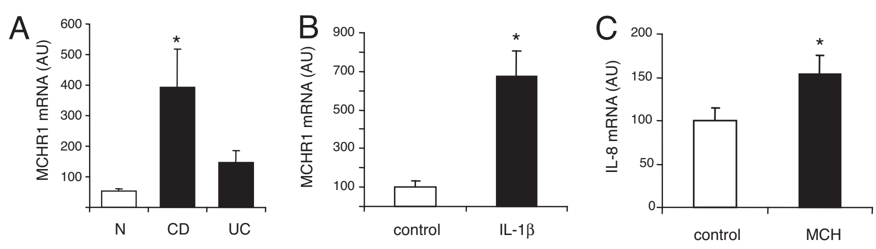

Fig. 6. MCHR1 mRNA is expressed in human colonic epithelial cells and increased during inflammation. (*A*) Colonic epithelial cells from controls (N; n = 7) and patients with CD (n = 5) or UC (n = 4) were isolated from biopsies by LCM, and MCHR1 expression was determined by real-time PCR. (*B*) HT-29 human colorectal adenocarcinoma cells were treated with IL-1β (10 ng/ml) for 2 h, and MCHR1 expression was evaluated by real-time PCR. (*C*) HT-29 cells were treated with MCH (10^{-6} M) for 3 h, and IL-8 expression was evaluated by real-time PCR.

mg per mouse) colitis was induced in CD1 mice ($n = 8$ per group), followed by daily anti-MCH or control antibody treatments (1 mg/kg) at days 3–6 included, post-TNBS exposure. A parallel cohort of mice ($n = 9$–10 per group, single housed) without colitis was treated with anti-MCH or control antibody as above, and mouse body weight and food intake were monitored daily. At the end of the experiment, epididymal fat pads were excised and weighed. The anti-MCH antibody has been raised in rabbits and previously characterized (2, 5). The IgG fraction of the MCH-specific and control antibody (preimmune serum) has been used in the present study.

Inflammation Scoring. Macroscopic damage of the colon was scored for hyperemia, thickness of the colonic wall, and extent of ulceration on a scale from 0 (normal) to 5 (most severe), and the mean value is presented. Histological analysis was performed in H&E-stained transverse sections of paraffin-embedded full-thickness segments of colonic tissue, sampled at 2, 4, and 6 cm from the anus, and three sections per animal and three views per section were scored under a light microscope in a blinded fashion by a pathologist (M.O.). The following scoring system (53) was used to assess the severity of mucosal ulceration: 0, no ulcer; 1, erosions or single ulceration not exceeding lamina muscularis mucosae; 2, multifocal ulcerations not exceeding the submucosa; and 3, ulcerations exceeding the submucosa. Scoring for inflammatory cell infiltration was: 0, no inflammatory cell infiltration; 1, mild inflammatory cell infiltration with few scattered cells; 2, moderate inflammatory cell infiltration; and 3, dense inflammatory infiltration. The histological score presented is the mean of the above two individual scores.

Cell Stimulations. HT-29 human colorectal adenocarcinoma cells (ATCC) were cultured in McCoy's 5A medium supplemented with 10% FBS and 1% antibiotic–antimycotic. Serum-starved HT-29 cells were treated with IL-1β (10 ng/ml) (R&D Systems) for 2 h or with MCH (10^{-6} M) for 3 h, followed by RNA extraction and real-time RT-PCR analysis for MCHR1 and IL-8, respectively. Each experimental condition was run in six replicas.

Real-Time PCR. Total RNA was isolated with the RNeasy mini-kit (Qiagen) followed by DNase treatment. One microgram of RNA was subjected to cDNA synthesis by using the Advantage RT for PCR reagents and oligo(dT) as primer (Clontech) unless indicated otherwise. cDNA was diluted 1:10 and used in a real-time PCR reaction (SybrGreen PCR master mix; Applied Biosystems). The following primers were used for the amplification of human MCH (hMCH) and its receptors: hMCH sense, 5′-cattcaggttggggaaagg-3′; hMCH antisense, 5′-gaaattatgtttggagcctgtgtt-3′; hMCHR1 sense, 5′- ggctggatggacctggaa-3′; hMCHR1 antisense, 5′- gcgaagatgaccgtggag-3′; hMCHR2 sense, 5′-acccttgattttg-gtgtgct-3′, hMCH2 antisense, 5′-gtgtgggctgttccatctgt-3′. The following primers for the amplification of mouse ppMCH gene by *Taq*Man were used as described (23): mMCH, sense 5′-ATTCAAAGAACACAGGCTCCAAAC-3′; mMCH antisense, 5′-CGGATCCTTTCAG AGCAAGGTA-3′; mMCH probe, 6FAM-AATCTT-GTAACTCACGGGCTGCCACTGAGT-TAMRA. For the amplification of mouse cytokine genes, 50 ng of RNA was subjected to real-time RT-PCR by using the *Taq*-Man One Step RT-PCR reagents and respective primers and labeled probes supplied as predeveloped assays (Applied Biosystems). All reactions were run in duplicate in a 5700 Sequence Detector System (Applied Biosystems) and results were normalized by either rodent GAPDH expression, human TATA-binding protein (TBP) (Applied Biosystems), or mouse TBP by using the following primers: mTBP sense, 5′- acccttcaccaatgactcctatg-3′; mTBP antisense, 5′- tgactgcagcaaatcgcttgg-3′. Results were expressed as normalized arbitrary mRNA units (AU), unless otherwise indicated.

Immunostaining. Preparation of whole mounts of myenteric and submucosal plexus has been described (54). For double immunofluoresence staining, tissue fixed in Zomboni's solution (4% formaldehyde, 0.2% picric acid, 0.1 M sodium phosphate buffer, pH 7.0) was incubated with 10% normal donkey serum. This was followed by incubation with a mixture of anti-Hu dilution 1:50 (Invitrogen) and MCHR1 or MCH (2, 5) (at a dilution 1:5,000) overnight at room temperature; and by incubation with a mixture of secondary antibodies conjugated with FITC or Cy3, respectively, dilution 1:100 (Jackson Immunoresearch) for 1 h at room temperature. The anti-MCHR1 antibody was developed in rabbits (BioSource International) against a conserved peptide (ASQRSIRL-RTKRVTR). In some sections, the primary antibody was omitted or replaced with control antibody (preimmune serum). Slides were visualized under a Nikon Eclipse E-600 fluorescence microscope.

Statistical Analysis. Results are reported as group means ± SE. Statistical significance was assessed by two-tail unpaired Student's *t* test, paired *t* test, ANOVA factorial with Bonferroni/Dunn correction for multiple comparisons, and χ^2 as appropriate by using STATVIEW software (Abacus Concepts).

ACKNOWLEDGMENTS. We thank Nasima Mustafa for excellent technical assistance with the animal studies and Rachel Diamond and Charles Vanderburg at the Advanced Tissue Resource Center, Harvard Center for Neurodegeneration and Repair, for performing the LCM studies. This work was supported by Broad Medical Research Program Grant IBD-0165, The Eli and Edythe L. Broad Foundation (E.K.), the AGA Institute (E.K. and A.M.), Pilot and Feasibility Program Grant P30-DK040561 from the Harvard Clinical Nutrition Research Center (E.K.), and National Institutes of Health Grants DK080058 (E.K.) and PO-1 DK 33506 (C.P.).

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