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Melanin-concentrating hormone (MCH) modulates *C difficile* toxin A-mediated enteritis in mice

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Abstract

Objective—Melanin-concentrating hormone (MCH) is a hypothalamic orexigenic neuropeptide that regulates energy balance. However, the distribution of MCH and its receptor MCHR1 in tissues other than brain suggested additional, as yet unappreciated, roles for this neuropeptide. Based on previous paradigms and the presence of MCH in the intestine as well as in immune cells, its potential role in gut innate immune responses was examined.

Methods—In human intestinal xenografts grown in mice, changes in the expression of MCH and its receptors following treatment with *Clostridium difficile* toxin A, the causative agent of antibiotic-associated diarrhoea in hospitalised patients, were examined. In colonocytes, the effect of *C difficile* toxin A treatment on MCHR1 expression, and of MCH on interleukin 8 (IL8) expression was examined. MCH-deficient mice and immunoneutralisation approaches were used to examine the role of MCH in the pathogenesis of *C difficile* toxin A-mediated acute enteritis.

Results—Upregulation of MCH and MCHR1 expression was found in the human intestinal xenograft model, and of MCHR1 in colonocytes following exposure to toxin A. Treatment of colonocytes with MCH resulted in IL8 transcriptional upregulation, implying a link between MCH and inflammatory pathways. In further support of this view, MCH-deficient mice developed attenuated toxin A-mediated intestinal inflammation and secretion, as did wild-type mice treated with an antibody against MCH or MCHR1.

Conclusion—These findings signify MCH as a mediator of *C difficile*-associated enteritis and possibly of additional gut pathogens. MCH may mediate its proinflammatory effects at least in part by acting on epithelial cells in the intestine.

Toxins A and B are the virulent factors of *Clostridium difficile*, the leading cause of antibiotic-associated diarrhoea and pseudomembranous colitis in hospitalised patients.¹ It is estimated that 3% of the general population and 30% of patients admitted to hospitals in the USA and Northern Europe have been colonised by this anaerobe, and about one-third of them become symptomatic.^{2,3} Since 2001, several outbreaks of *C difficile* infections have

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been reported, associated with more severe disease manifestations that might lead to colectomy, admission to the intensive care unit or even death.

Toxin A is a 308 kDa protein with enterotoxic and proinflammatory effects. At the cellular level, toxin A causes disaggregation of actin microfilaments resulting in changes in cell morphology and disruption of tight junctions.^{4,5} In parallel, it stimulates the release of various cytokines and chemokines, prostaglandins and additional inflammatory mediators from enterocytes and monocytes, and activates apoptotic pathways in immune and epithelial cells, resulting in mucosal damage.^{6–8}

Studies from our group and others have revealed a critical role for locally released neuropeptides and hormones in the amplification of inflammatory responses associated with *C difficile* toxin A.^{9–11} The most frequently used experimental model in such studies has been intraluminal administration of purified toxin A in closed intestinal loops of rats and mice. This treatment causes inflammatory diarrhoea, mucosal damage and necrosis, and infiltration by neutrophils, closely mimicking the situation in humans.^{4,12} Following toxin A exposure, neuropeptides such as substance P (SP), calcitonin gene-related peptide (CGRP), neurotensin and corticotrophin-releasing hormone (CRH) are secreted from sensory afferent and enteric neurons. In parallel, there is an upregulation of their respective receptors in target cells, primarily monocytes, mast cells and enterocytes. Upon binding to these receptors, neuropeptides activate proinflammatory signalling cascades which regulate cytokine expression. The significance of the above neuropeptides in the pathogenesis of intestinal inflammation in general, and in that mediated by *C difficile* toxin A in particular, was further underscored by experiments showing reduced disease susceptibility in mice genetically deficient for SP receptor NK-1R,¹³ CRH¹⁴ and CRH receptor 2,¹⁵ and in experiments using SP, neurotensin and CRH pharmacological antagonists.^{15–17}

Melanin-concentrating hormone (MCH) is a 19 amino acid evolutionarily conserved neuropeptide expressed primarily in the hypothalamus. MCH has been implicated in the regulation of feeding behaviour and energy balance.¹⁸ MCHR1 is the predominant MCH receptor in humans and the only functional one in rodents.¹⁹ It belongs to the seven-transmembrane family of G-protein-coupled receptors and can interact with G_{ai} or G_{aq} subunits. In addition to brain, MCH and its receptor MCHR1 are expressed in peripheral tissues, though at lower levels compared with brain, including the gastrointestinal tract and immune cells,^{20–25} with physiological roles that have just started to be appreciated. In the present study, we examined the potential link between MCH and innate immune responses in the gut using a mouse model of acute ileitis induced by *C difficile* toxin A. We analysed MCH-deficient mice, as well as wild-type mice treated with an anti-MCH or anti-MCHR1 antibody, and demonstrate that MCH can amplify intestinal inflammatory responses. The same experimental model has been used previously to reveal a role for leptin, another body weight-regulating hormone, in the pathogenesis of acute intestinal inflammation.²⁶ Furthermore, we describe the in vitro direct proinflammatory effects of MCH signalling in intestinal epithelial cells.

MATERIALS AND METHODS

Cell treatments

NCM460 non-transformed human colonic epithelial cells were grown in M3D medium (INCELL Corporation, San Antonio, Texas, USA) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic (Invitrogen, Carlsbad, California, USA). At 80% confluence, NCM460 cells in 24-well plates were serum starved for 16 h and then treated with MCH (10^{-6} M) (Bachem Bioscience, King of Prussia, Pennsylvania, USA) or *C*

difficile toxin A (3 µg/ml) for the indicated times, as previously described.^{27,28} Each condition was run six times.

Mice

CD1 mice were purchased from Charles River Laboratory (Wilmington, Massachusetts, USA). MCH^{-/-} mice and their MCH^{+/+} littermates from our colony were generated by heterozygous breeding and the line has been fully backcrossed to the C57BL/6 genetic background.²⁹ Mice were maintained in a controlled environment under an alternating 12 h light/dark cycle, with free access to food and water.

C. difficile toxin A-induced mouse ileitis

A middle line laparotomy was performed in 12-week-old male MCH^{-/-} and MCH^{+/+} mice (n = 6–8/group), and a 2.5–3 cm long loop was formed at the terminal ileum as previously described.¹⁴ Loops were injected with either 0.15 ml of phosphate-buffered saline (pH 7.4) containing 10 µg of purified toxin A or buffer alone (control). The abdomen was then closed and 4 h later tissues were harvested and preserved for histology or RNA extraction. Twelve-week-old male CD1 mice (n = 6/group) were treated with toxin A as above and ileal loops were harvested after 2 h for RNA analysis of MCH and MCHR1 expression. In another series of experiments, 12-week-old male CD1 mice (n = 8/group) were treated intraperitoneally at 16 h and 2 h prior to toxin A exposure with 1 mg/kg of anti-MCH, anti-MCHR1 or control (preimmune) antibody, and 4 h after toxin A treatment tissues were harvested. The purified immunoglobulin G (IgG) fraction of each antibody was used in the present study. The characterisation of the anti-MCH antibody has been described elsewhere.^{30,31} The anti-MCHR1 antibody was developed in rabbits (BioSource International, Camarillo, California, USA) against a conserved peptide (ASQRSIRLRTKRVTR) corresponding to amino acids 242–256 of the mouse (accession number: NP_660114.1) and rat sequence (NP_113946.1) and amino acids 311–325 of the human MCHR1 sequence (NP_005288.3). Histological severity of enteritis (epithelial cell damage, congestion and oedema, and neutrophil infiltration) was graded blindly in H&E-stained transverse ileal sections by a gastrointestinal pathologist (MOB) as previously described.¹⁴

Human intestinal xenografts

Human fetal (mean (SEM) 14.2 (1.6) weeks) intestine was obtained after therapeutic abortions from Brigham and Women's Hospital (Boston, Massachusetts, USA). Colonic tissue was dissected and xenotransplanted subcutaneously into C.B-17 *scid* mice (Charles River Laboratory) as previously reported.³² Twelve weeks after xenografting, the graft lumen was injected with 400 µl of purified *C. difficile* toxin A (40 nmol/l) or buffer alone (n = 4/group). Six hours later, the grafted tissues were collected for RNA analysis. All animal experiments were approved by the Institutional animal care and use committee and the local ethics committee.

Real-time PCR

Total RNA was isolated using the RNeasy mini kit and treated with DNase I (Qiagen, Valencia, California, USA). A 50 ng aliquot of RNA was subjected to reverse transcription-PCR (RT-PCR) using the Taq-Man One Step RT-PCR reagents (Applied Biosystems, Foster City, California, USA). A 1 µg aliquot of RNA was subjected to cDNA synthesis using the Advantage RT for PCR reagents and oligo(dT) as primer (Clontech, Mountain View, California, USA). cDNA was diluted 1:10 and used in a real-time PCR analysis (SYBR Green PCR master mix, Applied Biosystems). The following primers were used for the amplification of human MCH and its receptors: hMCH sense, 5'-cattcaggttgggaaagg-3'; hMCH antisense, 5'-gaaattatgtttggagcctgtgtt-3'; hMCHR1 sense: 5'-ggctggatggacctgaa-3';

hMCHR1 antisense: 5'-gcgaagatgaccgtggag-3'. The rest of the human and mouse genes described in this study were amplified using predeveloped assays (Applied Biosystems). PCRs were run in duplicate in a 5700 Sequence Detector System (Applied Biosystems). Results were normalised by either glyceraldehyde phosphate dehydrogenase (GAPDH) or TATA box-binding protein (TBP) expression (Applied Biosystems) and presented as arbitrary mRNA units (AU). For mouse TBP, the following primers were used: mTBP sense, 5'-acccttcaccaatgactcctatg-3', mTBP antisense: 5'-tgactgcagcaaatcgttg-3'.

cAMP assay

Full-length cDNA for hMCHR1 was cloned by PCR into a pcDNA3 vector and confirmed by sequencing. HEK293 (hamster embryo kidney) cells were transfected with this vector and stable transfectants were selected and maintained based on their resistance to geneticin, as previously described.³³ Serum-starved HEK293/hMCHR1 cells were preincubated with anti-MCH, anti-MCHR1 or control antibody (10 µg/ml) for 30 min, and then stimulated with MCH (10^{-6} M), forskolin (10^{-5} M) or both, as previously described.³⁴ Non-acetylated cAMP levels were measured 20 min after stimulation using a cAMP enzyme immunoassay kit (Cayman Chemical, Ann Arbor, Michigan, USA) according to the manufacturer's instructions. Likewise, cAMP levels were measured in NCM460 cells treated with MCH, forskolin or both (nine replicas per condition), as above.

Statistical analysis

Values are reported as group mean (SEM). Statistical significance was evaluated by analysis of variance (ANOVA) or non-parametric tests (Mann–Whitney and Kruskal–Wallis), as appropriate, using STATVIEW (Abacus Concepts, Berkeley, California, USA).

RESULTS

Functional MCHR1s are upregulated in human colonic epithelial cells by a bacterial toxin

Several neuropeptide receptors, including those for SP, neurotensin, CRH, and ghrelin, are present in colonocytes and mediate proinflammatory signalling, as has been shown in *in vitro* studies using NCM460 and HT-29 cells.^{15,27,35,36} In NCM460 minimally transformed human colonic epithelial cells we could detect by real-time RT-PCR low level transcripts for MCHR1, but not MCHR2, the other low affinity MCH receptor in humans.¹⁹ Most importantly, MCHR1 expression in these cells was several fold upregulated in response to *C. difficile* toxin A (386 (57) AU in toxin A vs 127 (32) AU in buffer-treated cells for 2 h, fig 1A, $p<0.001$). The same cells were not found to be positive for MCH expression, indicating that other cells of immune^{23,25} or neuronal origin^{20,21,37} might be responsible for MCH release in the inflamed intestine. Moreover, MCH-mediated activation of MCHR1 on NCM460 colonocytes resulted in complete inhibition of forskolin-induced increases in intracellular cAMP (fig 1B). Forskolin is an activator of the enzyme adenylate cyclase which catalyses the transformation of ATP into cAMP. This result confirms the presence of functional MCHR1 in colonocytes, as has been previously described for adipocytes³⁸, pancreatic β -cells³⁹ and immune cells.²³ The significance of MCHR1 signalling on colonocytes was demonstrated by treating naïve NCM460 cells with MCH. Upon binding to its receptor, MCH stimulated a robust and time-dependent induction of IL8 gene expression (371 (25) AU vs 100 (17) AU, MCH vs control, respectively, 4 h post-MCH treatment, $p<0.001$) (fig 1C).

Increased expression of MCH and MCHR1 mRNA in response to *C difficile* toxin A in a human intestinal xenograft and in a mouse ileal loop model

Toxin A from *C difficile* activates the release of a cascade of mediators from various intestinal cell types resulting in acute diarrhoea, accompanied by severe, neutrophil-driven, acute inflammation.⁴ We have recently described the use of subcutaneously transplanted human intestinal xenografts in immunocompromised mice to generate a chimeric animal model for *C difficile* toxin A-induced-pathology in the human intestine.³² In this model, epithelial cells in the grafted intestine are exclusively of human origin, and injection of toxin A induces an acute inflammatory response, similar to that seen in animal models and patients with *C difficile*-mediated colitis.³² We injected human colonic xenografts with toxin A or buffer and assessed expression of MCH and its receptor by real-time RT-PCR. Both MCH (324 (26) AU vs 100 (29) AU, MCH vs control, respectively, $p < 0.005$) and MCHR1 (272 (44) AU vs 100 (44) AU, MCHR1 vs control, respectively, $p < 0.05$) were significantly upregulated at 6 h after toxin A administration (fig 2A). Likewise, in mouse ileal loops injected with toxin A, a significant upregulation of MCH (232 (35) AU vs 100 (13) AU, MCH vs control, respectively, $p < 0.005$) and of MCHR1 (292 (34) AU vs 100 (17) AU, MCHR1 vs control, respectively, $p < 0.001$) was apparent at 2 h post-treatment (fig 2B).

MCH^{-/-} mice have diminished *C difficile* toxin A-mediated intestinal inflammation

To assess directly the role of MCH in intestinal inflammation, we injected ileal loops of anaesthetised wild-type and MCH^{-/-} mice with purified toxin A. In wild-type mice, toxin A treatment increased ileal fluid secretion, stimulated granulocyte transmigration and caused histological damage characterised by depletion of enterocytes, oedema and neutrophil accumulation (fig 3A). In contrast, MCH^{-/-} mice had significantly reduced ileal microscopic mucosal damage and immune infiltrates (fig 3A). The histological damage score in toxin A-treated wild-type mice was 5.6 (1.6) and in MCH^{-/-} mice was 2.4 (0.5) ($p < 0.05$; fig 3B). Since neutrophil infiltration and neutrophil-derived mediators represent important proinflammatory components of this model,^{40,41} these results might suggest that MCH plays an important role in neutrophil recruitment at sites of inflammation. In line with the histological picture, and compared with wild-type mice, toxin A-exposed MCH^{-/-} mice had 2–3 times lower intestinal mRNA expression of tumour necrosis factor α (TNF α ; 100 (23) AU vs 48 (8) AU, $p < 0.05$), IL1 β (100 (14) AU vs 29 (6) AU, $p < 0.005$) and interferon γ (IFN γ ; 100 (40) AU vs 31 (8) AU, $p = 0.071$), while baseline cytokine mRNA levels were no different between the two genotypes (fig 3C, right panel).

Treatment with anti-MCH or anti-MCHR1 antibody reduces *C difficile* toxin A-induced ileitis and fluid secretion in wild-type mice

Having shown that MCH^{-/-} mice had reduced toxin A-induced enteritis, we next examined whether neutralisation of MCH or its receptor MCHR1 might also confer protection in wild-type mice. Mice were injected intraperitoneally with anti-MCH, anti-MCHR1 or control antibody, at 16 and 2 h prior to ileal loop formation. Ileal loops were then ligated, injected with either toxin A or buffer, and after 4 h tissues were evaluated for established toxin A-associated enterotoxic responses. Compared with toxin A-treated mice that received control antibody, mice receiving antibody against MCH or its receptor exhibited significantly reduced inflammatory and secretory responses (fig 4). At the microscopic level, the mucosal architecture of antibody-treated mice was well preserved, compared with severe tissue damage and inflammation observed in mice treated with control antibody (fig 4A). Overall, anti-MCH-treated mice had 50% less inflammation (3 (0.8) vs 6.4 (0.7) in controls, $p < 0.005$), while anti-MCHR1 treatment followed a similar trend (4.4 (0.8)) (fig 4B). Moreover, specific antibody-treated mice exhibited 50–90% reduction in intestinal levels of proinflammatory cytokines such as TNF α (100 (21) AU in control vs 39 (7) AU in anti-MCH, $p < 0.01$; and 51 (12) AU in anti-MCHR1, $p < 0.05$) and IFN γ (100 (35) AU in control

vs 20.7 (4.1) AU in anti-MCH, $p < 0.05$; and 8.3 (1.5) (AU) in anti-MCHR1, $p < 0.01$) (fig 4C). Consistent with the overall attenuated inflammatory responses in the toxin A-exposed and specific antibody-treated mice, these mice also had diminished intestinal fluid secretion (120 (19) mg/cm in control IgG vs 50 (5.2) mg/cm in anti-MCH, $p < 0.001$; and 56 (10) mg/cm in anti-MCHR1-treated mice, $p < 0.001$) (fig 4D).

MCH-activated intracellular signalling pathways have been previously identified using HEK293 cells stably overexpressing human MCHR1.³³ Treatment of these cells with MCH abrogated forskolin-stimulated cAMP accumulation.³⁴ Thus we used the same system to characterise further the neutralising antibodies used in our studies in mice. As shown in fig 5, incubation with the anti-MCH or anti-MCHR1 antibody resulted in a 40–50% increase in intracellular cAMP levels, only in the group of cells treated with forskolin plus MCH. This result indicates that the presence of anti-MCH or MCHR1 antibodies could specifically reverse the MCH-mediated inhibition of forskolin-stimulated cAMP accumulation.

DISCUSSION

Data presented in this study place MCH among the neuropeptides that play a critical role in toxin A-induced intestinal inflammatory responses, primarily by virtue of attenuated toxin A-mediated intestinal secretion, immune cell infiltration and epithelial cell damage in the MCH-deficient mice. Our results are also consistent with our recent report demonstrating that MCH also plays a proinflammatory role in the TNBS (2,4,6-trinitrobenzene sulfonic acid) model of acute colitis that resembles Crohn's disease.³⁷ Most probably, MCH contributes to intestinal inflammation primarily via peripheral mechanisms, since treatment of mice with anti-MCH or MCHR1 antibodies, which do not cross an intact brain barrier due to their high molecular mass, was as effective as MCH genetic ablation in attenuating toxin A-mediated ileitis. However, based on the extensive projections of MCH neurons throughout the brain and spinal cord,³⁰ one cannot entirely exclude the possibility of centrally mediated proinflammatory effects of MCH. For example, it is known that α -MSH (α -melanocyte-stimulating hormone), a functional antagonist of MCH,⁴² modulates peripheral inflammation, at least in part, via central neurogenic mechanisms.⁴³

Further mechanistic insights on the importance of MCH and its receptor in toxin A-mediated intestinal pathogenesis were provided in the present study by demonstrating direct proinflammatory effects of MCH on colonocytes. Intestinal epithelial cell responses to toxin A are the hallmarks of *C. difficile* infection, and previous studies from our group have shown a significant neuropeptide receptor upregulation in these cells.⁴ In turn, the activation of these receptors by their cognate ligands further amplifies intestinal inflammation by stimulating secretion of proinflammatory mediators from epithelial cells.^{15,27,44} MCH seems to follow exactly the above paradigm. In vitro experiments using NCM460 human colonocytes provide evidence that MCHR1 is dramatically upregulated in response to toxin A exposure. Most importantly, our experiments indicate that treatment of colonocytes with MCH stimulates transcription of IL8, an important mediator of neutrophil recruitment (this study and Kokkotou et al³⁷).

Very little is known about the signalling pathways engaged by MCHR1, a seven-transmembrane G-protein-coupled receptor; most of these studies were performed in receptor-negative HEK293 or Chinese hamster ovary (CHO) cells which were engineered to overexpress this receptor.¹⁹ MCH treatment of adipocytes, which express native MCHR1, leads to erk1/2 and p70S6 activation, and stimulation of leptin synthesis and secretion.^{38,45} In a more recent study, MCH was found to increase intracellular calcium and phosphorylation of erk and Akt in isolated islets from C57BL/6 mice.³⁹ Another report describes that MCH-mediated erk1/2 activation could be substantially decreased by

inhibiting protein kinase C (PKC).⁴⁶ The erk1/2 and PKC pathways might be quite relevant for the MCH signalling in colonocytes, as previous studies from our group have demonstrated that SP, neurotensin and ghrelin stimulate IL8 gene expression through erk1/2 and PKC-mediated nuclear factor (NF)- κ B activation.^{36,44,47}

Accumulating evidence supports an interaction between MCH and the immune system that might be of importance for gut innate immune responses. MCHR1 transcripts have been detected in thymus, spleen and tonsils, and functional receptors for MCH have been described in granulocytes, monocytes (CD14⁺), T cells (CD3⁺) and B cells (CD19⁺).^{20,23,25,48} Of interest, putative binding sites for transcription factors that are considered master regulators of immune cell functions, such as Ikaros and IFN γ , have been found in the MCHR1 and MCH genes, respectively.²³

The exact source of increased levels of MCH in the intestine in response to toxin A, as described in the present study, remains elusive. In contrast to MCHR1, intestinal epithelial cells were found to be negative for MCH expression, even during inflammation. However, we have recently reported that a significant percentage of neurons of the rat myenteric and submucosal plexus exhibit immunoreactivity for MCH and MCHR1,³⁷ in addition to the previously described MCH-positive enterochromaffin cells.²¹ Upregulation of the expression of various neuropeptides (NPY, CRH, CGRP and neurotensin) in the enteric nervous system has been recognised as a critical component of toxin A-mediated intestinal pathogenesis.¹² A potential mechanism which might account for MCH accumulation in the intestine during toxin A-mediated inflammation, in addition to increased expression of MCH, could be decreased activity of the MCH-degrading enzyme neutral endopeptidase (NEP).⁴⁹ It has been shown that NEP is downregulated during intestinal inflammation⁵⁰ and that genetic ablation or pharmacological inhibition of NEP exacerbates toxin A-induced intestinal pathology in mice, most probably due to diminished degradation of proinflammatory neuropeptides which are substrates for NEP, including SP, neurotensin and MCH.^{51,52}

Though more work is needed to delineate the exact MCH-mediated neuroendocrine-immune interactions which take place during *C. difficile* infection, results presented in this and previous studies suggest the following sequence of events: *C. difficile* toxin A disrupts the integrity of the epithelial barrier and stimulates the release of cytokines, chemokines, prostaglandins and reactive oxygen species by epithelial and resident immune cells.^{4,12,28} These proinflammatory mediators diffuse into the lamina propria and activate enteric and sensory neurons to release neuropeptides, including MCH. MCH in turn amplifies the inflammatory response by interacting with its receptors which have been upregulated by toxin A on epithelial cells, as shown in the present study, and perhaps on immune cells. Yet, in the above scheme, it remains unclear whether MCH per se can induce intestinal inflammation. In experiments where mice received daily intraperitoneal injections of MCH (20 μ g per mouse per day) for 7 days, we found that this manipulation was not sufficient to induce intestinal inflammation (data not shown). Nevertheless, based on paradigms of additional appetite-regulating hormones, for example leptin⁵³ and adiponectin,⁵⁴ one cannot exclude the possibility that treatment with MCH might worsen intestinal inflammation in cases where the epithelial barrier has been compromised, such as following TNBS or dextran sodium sulfite (DSS) exposure.

In aggregate, the present work provides the first evidence that in mammals MCH regulates not only energy balance, but also innate immune responses, both essential mechanisms for the maintenance of homeostasis and the survival of any organism. Indeed, MCH is highly conserved through evolution and in teleost fish, in addition to its effects on hormonal control

of skin pigmentation in response to sensing a predator, MCH has been reported to enhance leucocyte phagocytic activity.^{55,56}

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Figure 2.

Intestinal melanin-concentrating hormone (MCH) and MCH receptor 1 (MCHR1) are upregulated in response to toxin A treatment. MCH and MCHR1 expression was determined by real-time reverse transcription-PCR. (A) Human fetal colonic xenografts growing in *scid* mice were injected with toxin A or buffer (n = 4/group) and xenografts were harvested after 6 h. (B) Closed ileal loops were prepared in CD1 mice and injected with toxin A or buffer (n = 6/group). Tissues were analysed at 2 h post-toxin A exposure. *p<0.05; **p<0.01.

Figure 3.

Melanin-concentrating hormone-lacking (MCH^{-/-}) mice develop attenuated *C. difficile* toxin A-induced enteritis. In anaesthetised MCH^{+/+} and MCH^{-/-} mice, closed ileal loops were injected with toxin A or buffer. At 4 h mice were sacrificed and tissues harvested for histology and gene expression analysis. (A) Representative H&E-stained transverse sections of ileal loops from MCH^{+/+} (left panel) and MCH^{-/-} mice treated with buffer or toxin A (magnification $\times 40$ objective). (B) Histological scoring of toxin A-mediated inflammation in the ileal mucosa of MCH^{+/+} and MCH^{-/-} mice. (C) Cytokine mRNA expression in the ileum of toxin A- or buffer-exposed MCH^{+/+} and MCH^{-/-} mice, as measured by real-time reverse transcription-PCR. *p < 0.05; **p < 0.01 among genotypes. IFN γ , interferon γ ; IL1 β , interleukin 1 β ; TNF α , tumour necrosis factor α .

Figure 4.

Effect of melanin-concentrating hormone (MCH) and MCH receptor 1 (MCHR1) neutralisation in *C difficile* toxin A-induced enteritis in mice. Wild-type (CD1) mice were treated twice (–16 h and –2 h) with anti-MCH, anti-MCHR1 or control antibody (1 mg/kg) followed by ileal loop exposure to toxin A or buffer for 4 h. (A) Representative H&E-stained ileal sections of mice treated with control, anti-MCH or anti-MCHR1 antibody. Upper panel, magnification $\times 4$ objective; lower panel, magnification $\times 40$ objective. (B) Histological scoring of the H&E-stained ileal sections for epithelial cell damage, congestion and oedema, and neutrophil infiltration. (C) RNA from full-thickness ileum was prepared and levels of the proinflammatory cytokines tumour necrosis factor α (TNF α), interleukin 1 β (IL1 β) and interferon γ (IFN γ) were measured by real-time reverse transcription-PCR. (D) Toxin A-exposed closed ileal loops were excised and the accumulated fluid secretion was calculated as the loop weight to length ratio. * $p < 0.05$; ** $p < 0.01$.

Figure 5.

HEK293/hMCHR1 (HEK293 cells stably overexpressing human melanin-concentrating hormone receptor 1) cells were treated with MCH, forskolin, both, or buffer in the presence of control immunoglobulin G (IgG), anti-MCH or anti-MCHR antibody. Intracellular cAMP levels were evaluated by enzyme immunoassay. Results are expressed as a percentage of control IgG values. ** $p < 0.01$; *** $p < 0.001$ compared with control IgG.