HHS Public Access

Author manuscript

Mucosal Immunol. Author manuscript; available in PMC 2014 September 01.

Published in final edited form as:

Mucosal Immunol. 2014 March ; 7(2): 335–347. doi:10.1038/mi.2013.52.

Central cholinergic activation of a vagus nerve - to spleen circuit alleviates experimental colitis

Hong Ji[1,*], **Mohammad F Rabbi**[1,*], **Benoit Labis**[1], **Valentin A Pavlov**[2], **Kevin J Tracey**[2], and **Jean-Eric Ghia**[1,3,#]

[1]University of Manitoba, Department of Immunology and Internal Medicine section of Gastroenterology, Winnipeg, Manitoba, Canada

[2]Center for Biomedical Science, The Feinstein Institute for Medical Research, Manhasset, New York, United States of America

[3]McMaster University, Hamilton, Ontario, Canada

Abstract

The cholinergic anti-inflammatory pathway is an efferent vagus nerve-based mechanism that regulates immune responses and cytokine production through α7nicotinic-acetylcholinereceptor (α7nAChR) signaling. Decreased efferent vagus nerve activity is observed in inflammatory bowel disease (IBD). We determined whether central activation of this pathway alters inflammation in mice with colitis and the mediating role of a vagus nerve-to spleen circuit and α7nAChR signaling. Two experimental models of colitis were used in C57BL/6 mice. Central cholinergic activation induced by the acetylcholinesterase inhibitor galantamine or a muscarinic acetylcholine receptor agonist treatments resulted in reduced mucosal inflammation associated with decreased MHC II level and pro-inflammatory cytokine secretion by splenic $CD11c^+$ cells mediated by α7nAChR signaling. The cholinergic anti-inflammatory efficacy was abolished in mice with vagotomy, splenic neurectomy or splenectomy. In conclusion, central cholinergic activation of a vagus nerve–to spleen circuit controls intestinal inflammation and this regulation can be explored to develop novel therapeutic strategies.

Keywords

Galantamine; vagus nerve; cholinergic anti-inflammatory pathway; experimental colitis; dendritic cells

[#]Address for correspondence: Dr. Jean-Eric Ghia, University of Manitoba, 750 McDermot Avenue, Winnipeg, Manitoba, Canada R3N 0T5, jeghia@yahoo.fr.

[*]Equal contribution

Disclosure:

The authors have nothing to disclose.

Author contribution:

JE.G. designed the study. H.J, M.F.R, JE.G, carried out the majority of the experiments, B.L carried out the remaining experiments. K.J.T., V.A.P. and JE.G. analyzed the data. JE.G. wrote the first draft of the manuscript. K.J.T. and V.A.P. reviewed the draft and provided comments. All authors approved the final submission and declare that no potential competing interests exist.

Introduction

Inflammatory bowel diseases (IBD), consisting of Crohn's disease (CD) and ulcerative colitis (UC), are characterized by a chronic relapsing and remitting course as a result of intestinal inflammation[1]. The release of inflammatory mediators, including pro-inflammatory cytokines from immune cells mediate tissue injury and exacerbation of IBD[2]. Accordingly, several therapeutic approaches targeting inflammatory cytokines in IBDs have been investigated[3]. Cholinergic signaling along the vagus nerve has been shown to control interleukin (IL)-6, -1β, tumor necrosis factor (TNF)-α and other pro-inflammatory cytokine production in different inflammatory conditions including IBD[4–6]. α7 nicotinic acetylcholine receptors (α7nAChR) on macrophages, monocytes and mast cells have been shown to mediate cholinergic anti-inflammatory output[7, 8]. This regulation is a part of the current working model of the "inflammatory reflex" controlling immune responses and cytokine levels[9]. In this model afferent vagus neurons sensing peripheral inflammatory molecules convey the signal to the brain[9]. Consequent activation of efferent vagus neurons results in increased cholinergic α7nAChR-dependent anti-inflammatory output and suppressed pro-inflammatory cytokine release. Recent findings have suggested a role for the splenic nerve and the spleen in this cholinergic anti-inflammatory pathway[8].

IBD is associated with an autonomic imbalance and up to 35% of patients with UC exhibit autonomic dysfunction with impaired efferent vagus nerve activity[10]. Although current animal models do not sufficiently recapitulate IBD, we have previously reported that the vagus nerve has a tonic inhibitory role on acute inflammation in murine models of colitis mimicking UC (dextran sulfate sodium (DSS) model) and CD (2, 4 Dinitrobenzenesulfonic acid (DNBS) model)[4]. In this context, the absence of the vagus nerve worsened acute DSS and DNBS-colitis through a macrophage-mediated mechanism, associated with the release of higher levels of IL-6, IL-1β and TNF-α, without affecting the level of the anti-inflammatory cytokine IL-10. Electrical vagus nerve stimulation suppresses myeloperoxidase activity (a marker of neutrophil infiltration) and TNF-α inflammatory cytokine levels in experimental colitis and endotoxemia respectively[11]. Moreover, in line with the role of α7nAChR in mediating anti-inflammatory cholinergic signals, it has been demonstrated that smoking, ameliorates inflammation in UC patients[12]. Conversely, smoking exacerbates inflammation in Crohn's disease[13, 12].

Dendritic cells (DCs) are key cells of the innate immune system that bridge innate with adaptive immune responses. Strategically positioned in the lamina propria in proximity to a number of luminal bacteria and antigenic stimuli, these cells perform a key role in activation of the immune response and generation of gut inflammation via their passage into the spleen and interaction with T cell[4]. Human studies have revealed that there is a significant increase in the numbers of antigen presenting cell (APC), including DCs within the inflamed tissue and the peripheral blood of patients with CD or UC[15, 16]. Furthermore, DCs depletion in dextran sulfate sodium (DSS)-treated CD11c-DT receptor transgenic mice almost completely inhibited experimental colitis[17]. Nicotinic receptors, including α7nAChR are also expressed by human monocytes[18] and mouse DCs[19].

The cholinergic anti-inflammatory pathway can be activated in the central nervous system (CNS) by muscarinic acetylcholine receptor (mAChR) ligands or acetylcholinesterase (AChE) inhibitors [20, 21]. Galantamine (GAL) is a reversible, competitive AChE inhibitor, which crosses the blood-brain barrier, increases brain cholinergic network activity[22] and is widely used in the treatment of Alzheimer's disease. GAL activates efferent vagus nerve activity[23] and its anti-inflammatory activity has been associated with brain mAChR-mediated activation of the cholinergic anti-inflammatory pathway[21]. In addition, it was very recently demonstrated that a treatment with another centrally-acting cholinesterase inhibitor–rivastigmine suppresses IL-6 levels and decreases the severity of murine DSS- and TNBS-induced colitis[24]. A role for mAChRs in the CNS and macrophages in mediating the effects of rivastigmine was also indicated.

Recent findings have highlighted a key role of the spleen in mediating vagus nerve anti-inflammatory signaling during endotoxemia[25]. In the context of endotoxemia, the absence of the intact vagus nerve or the spleen results in abrogation of the beneficial effect of the vagus nerve activation. However, the implication of a vagus nerve-to spleen anti-inflammatory axis in the regulation of intestinal inflammation remains to be determined. To provide insight, here we studied whether central activation of the cholinergic anti-inflammatory pathway by the AChE inhibitor GAL or mAChR ligands alters the severity of DSS-colitis and the specific role of the spleen and DCs. We report that treatments with GAL or McN-A-343 (a M1mAChR agonist) significantly ameliorate disease severity and inhibit inflammation in the context of experimental colitis. These effects were entirely dependent on vagus nerve and splenic nerve integrity and associated with inhibition of splenic $CD11c^+$ cell pro-inflammatory cytokine production. In line with the importance of α7nAChR-mediated signaling in the cholinergic regulation of inflammation, we found that direct stimulation of DCs with a α7nAChR agonist decreases the release of pro-inflammatory cytokines. Similar cholinergic anti-inflammatory mechanisms were demonstrated using the DNBS-model, indicating the broader scope of our findings in the regulation of intestinal inflammation.

Methods

Animals

Male C57BL/6 (7–9 weeks old) were purchased from Charles Rivers (Canada) and maintained in the animal care facility at the University of Manitoba under specific pathogen-free conditions. No differences in food intake or body weight were observed between the groups. All experiments were approved by the University of Manitoba animal ethics committee (10-073) and conducted under the Canadian guidelines for animal research.

Induction of DSS and DNBS colitis

DSS (molecular weight [MW], 40 kilodaltons: ICN Biomedicals Inc) was added to the drinking water in a final concentration of 5% (wt/vol) for 5 days[26, 27]. Controls were all time-matched and consisted of mice that received normal drinking water only. Mean DSS consumption was noted per cage each day. For the DNBS study, mice were anaesthetized with Isoflurane (Abbott, Abbott Park, IL). A 10cm long PE-90 tubing (ClayAdam,

Parsippany, NJ), attached to a tuberculin syringe, was inserted 3.5cm into the colon. Colitis was induced by administration of 100Rl of 4mg of DNBS solution (ICN) in 50% ethanol and left for 3 days[28]. Control mice (without colitis) received saline administration. Mice with colitis were supplied with 6% sucrose in drinking water to prevent dehydration.

Surgical procedures and drug treatments

Mice were anaesthetized using ketamine (150 mg/kg, i.p) and xylazine (10 mg/kg, i.p). I.c.v. implantation of the cannula, splenectomy (SPX), splenic neurectomy (NRX) or subdiaphragmatic bilateral vagotomy (VXP) was performed on the same day[4]. In sham-operated group: mice implanted with the cannula received vehicle; mice were anaesthetized and laparotomy performed but the spleen was not removed; splenic nerve was exposed but not cut; vagal trunks were exposed but not cut, however, a pyloroplasty was performed. The completeness of vagotomy was verified during post-mortem inspection of vagal nerve endings using microscopic inspection associated with a Bielschowsky silver staining[29]. The completeness of neurectomy was verified postmortem by noradrenaline enzyme-linked immunosorbent assay in sham-operated and NRX animals. Mice were allowed to recover for 10 days. One day before initiation of colitis pharmacological treatments started: (GAL, 1–4 mg/kg/day, intraperitoneal (i.p.)); Huperzine-A: (H-A, 0.4 mg/kg/day, i.p., Sigma, Oakville, ON); Atropine Methyl Nitrate (AMN, injected 20 min prior to GAL, 4 mg/kg/day, i.p., Sigma); Atropine sulfate (AS, injected 20 min prior GAL, 4 mg/kg/day, i.p. Sigma). Micro-osmotic pumps (Alzet, Cupertino, California, USA) were filled with vehicle (saline 0.2%), M1 mAChR agonist McN-A-343 (Sigma) or M2 mAChR antagonist methoctramine (MTT, 5 ng/day, Sigma) solution and placed as previously described[28].

Characterization of inflammation

Disease activity index (DAI) and macroscopic scores and colonic damage were determined using a previously described scoring system for DSS colitis[4, 30] and for DNBS[4, 31] over 5 and 3 days respectively. Samples were collected 5 or 3 days post activation associated with DSS or DNBS respectively and blood was collected by intracardiac puncture under isoflurane anesthesia. Formalin-fixed colon segments coming from the splenic flexure were stained with hematoxylin-eosin[4]. Colonic myeloperoxidase (MPO) activity was determined following an established protocol[32]. Serum C-reactive protein (CRP) and colonic cytokine levels were determined using ELISA commercial kit (R&D Systems, Minneapolis, Minnesota, USA).

Acetylcholine detection

5 or 3 days post activation associated with DSS or DNBS respectively, the amount of acetylcholine was measured using the acetylcholine assay kit (Amplex Red; Molecular Probes). This kit measures the amount of hydrogen peroxide (which in the presence of horseradish peroxidase leads to the oxidation of Amplex Red) produced through the oxidation of choline. The concentration of choline and acetylcholine was determined using the software provided by the manufacturer (KC4; Bio-Tek).

Isolation of splenic CD11c$^+$ cells and culture

5 or 3 days post activation associated with DSS or DNBS respectively, the spleens were digested in 2mg/ml^{-1} collagenase D (Roche Diagnostics, Meylan, France) in RPMI 1640 for 30min at 37°C. EDTA at 5mM was added during the last 5min to disrupt DC–T cell complexes, and the cell suspension was filtered. Total splenocytes after RBC lysis with ACK lysis buffer (150mM NH4Cl, 10mM KHCO3, 0.1mM EDTA) were incubated with CD11c$^+$ microbeads (Miltenyi Biotec, Auburn, CA) for 15min at 48°C. The cells were then washed, resuspended in cell separation buffer (Dulbecco's Phosphate-Buffered Saline [D-PBS] without Ca21 and Mg21 containing 2% FBS and 2mM EDTA, Life Technology) and passed through magnetic columns for positive selection. After passing consecutively through two columns, the collected splenic CD11c$^+$ cell preparations showed greater than 95% purity. splenic CD11c$^+$ cell isolated from different groups of mice were cultured in complete RPMI 1640 medium containing 10% heat-inactivated FBS, 25mg/ml^{-1} gentamicin, 2mM L-glutamine in 12-well plates at 1.10^{+6} cells/well for 24hrs, and the supernatants were measured for IL-12p40, IL-6 and TNF-α by ELISA (R&D Systems).

In some experiments lipopolysaccharide (LPS, Sigma) was added to the cultures at a final concentration of 100 ng/ml^{-1}. In a separated set GAL or the a7nAChR agonist GST-21 were added to medium at a final concentration of 10^{-6} M.

Flow cytometry

Surface staining of MACS isolated splenic CD11c$^+$ cell (MHC II-Alexa 647, CD40-FITC, CD86-PE, CD80-V450) (BD Biosciences) of different *in vivo* treatments were subjected to standard multi-color flow cytometry procedures[33]. In brief, fluorescent-labeled antibodies were added to the splenic CD11c$^+$ cell (10^6) and incubated at 4 °C for 30 min in all surface staining procedures. After excessive washing in flow buffer to remove unbound antibodies, the cells were acquired in a BD FACS Calibur Flow Cytometer. Cell viability was assessed using DAPI. Data analysis was performed using the Flowjo software.

Statistical analysis

Results are presented as means ±SEM. Statistical analysis was performed using one or two way ANOVA followed by the Tukey-Kramer multiple comparisons *post hoc* analysis and a p value of <0.05 considered significant with n=8 to 12 depending on the groups tested (Prism 4, GraphPad).

Results

Centrally-acting acetylcholinesterase inhibitor treatment ameliorates the severity of colitis

Previous findings have characterized GAL as a central activator of the cholinergic anti-inflammatory pathway[23, 21, 34]. Daily administration of GAL (1–4 mg/kg/day, i.p.), starting one day before disease induction, dose-dependently reduced the severity of colitis (Figure 1A–D). GAL-treated mice with colitis showed a significantly lower DAI for the last 2 days as compared to saline-treated controls with the disease (Figure 1A). This drug effect was dose-dependent and the highest reduction was achieved with a dose of 4mg/kg/day, which has been previously shown to inhibit mouse brain AChE activity by 43%[34]. The decreased

severity of colitis in GAL (4 mg/kg/day)-treated mice as compared to saline–treated controls was also further demonstrated by the 3.2-fold decrease in the macroscopic damage score, in the 3.4-fold decrease in MPO activity and in the 1.25-fold decrease in serum CRP levels (Figure 1B,C,D). GAL (4 mg/kg/day) treatment of mice with DSS-induced colitis also significantly lowered the histological manifestation of the disease (Figure 2B, C, G). Colonic IL-1β levels in GAL-treated mice were 4-fold lower as compared to the DSS-saline treated group (Figure 3D). Accordingly, colonic IL-6 and TNF-α levels were 2- and 2.3-fold lower (Figure 3E, F). No significant changes were detected for colonic IL-10 (Supplementary Figure 1A).

To characterize the specificity of brain AChE inhibition in preventing the development of colitis, we utilized Huperzine-A (Hup A), a structurally distinct, highly selective, centrally-acting AChE inhibitor. Hup A administration (0.4 mg/kg/day, i.p.) significantly reduced the disease severity and inflammation in mice with DSS-colitis (Supplementary Table 1, Figure 2D, G). In addition to their efficacy in DSS colitis, GAL and Hup A treatments decreased the severity of DNBS colitis (Supplementary Figure 2). Taken together, these findings indicate that centrally-acting AChE inhibitor treatments result in disease-alleviating and counter-inflammatory effects in two experimental models of colitis.

Central muscarinic receptors are essential for alleviation of colitis and inhibition of colonic inflammation by an acetylcholinesterase inhibitor

We next studied whether central mAChRs, which critically mediate brain cholinergic pathways and vagus nerve activation[21], are required for the anti-inflammatory effect of GAL. Pretreatment with atropine methyl nitrate (AMN), a mAChR antagonist that does not cross the BBB, failed to alter the beneficial effects of GAL on colitis (Figure 3). In contrast, pretreatment with atropine sulfate (AS), a mAChR antagonist that penetrates the BBB, abrogated the suppressive GAL effect on colitis development (Figure 3). No significant differences were detected in colonic IL-10 levels (Supplementary Figure 1A). Neither AMN nor AS alone significantly modified the expression of colitis. These results indicate that the anti-inflammatory effects of GAL in the context of colitis are mediated through a brain mAChR-dependent mechanism. Although the presence of mAChRs has been reported on immune cells[19], our data indicate that direct modulation of peripheral mAChRs has no significant effect on the magnitude of colonic inflammation. In the context of DNBS colitis, results were similar for MPO activity (Supplementary Figure 2A). However AS and AMN treatments resulted in a slight, but statistically significant decrease in colonic IL-1β levels (Supplementary Figure 2B).

Central administration of mAChR ligands attenuates the severity of colitis and inhibits colonic inflammation

As central mAChRs are important in mediating the effects of GAL, we further assessed the regulatory involvement of these receptors in the context of colitis. Previously, a role for M1mAChR activation and M2mAChR inhibition in the regulation of inflammation during endotoxemia has been described[20]. Accordingly, we performed experiments with mice subjected to i.c.v. infusions of specific mAChR ligands. Treatment with the M1mAChR agonist (McN-A-343; 5 ng/kg/day) starting one day before induction of DSS-colitis

decreased the macroscopic score, the MPO activity, serum CRP levels, the colonic tissue pro-inflammatory cytokine levels (Figure 4) and the histological score (Figure 2E, G). No significant differences were detected in colonic IL-10 levels (Supplementary Figure 1B).

Then, we examined the anti-inflammatory efficacy of an alternative approach of central cholinergic activation. Central ACh release is negatively regulated by the presynaptic M2mAChR autoreceptor[35, 36]. Therefore, we studied the efficacy of i.c.v. infusion of the M2mAChR antagonist methoctramine (MTT,5 ng/kg/day). MTT administration, initiated one day before induction of colitis resulted in comparable significant decrease of colitis severity (Figure 4, 2F,G). We also demonstrated the beneficial effect of both treatments in the context of DNBS colitis (Supplementary Figure 3). These findings highlight the specific regulatory role of central mAChRs in suppressing colitis pathogenesis and are in line with the previously reported anti-inflammatory effects of these two drugs in the context of endotoxemia[20].

Central cholinergic disease-alleviating and anti-inflammatory efficacy is mediated through vagus and splenic nerve signaling and the release of ACh in the spleen

To study whether the beneficial effects of central cholinergic activation in the context of colitis depend on the vagus nerve-to spleen axis we performed a series of experiments with mice subjected to vagotomy (VXP), splenic neurectomy (NRX) and/or splenectomy (SPX). VXP significantly exacerbated the severity of colitis (Figure 5 & Supplementary Figure 4), thus confirming our previously reported findings[37]. SPX did not alter the development of colitis; however the absence of the spleen abolished the deleterious effect of VXP (Figure 5 & Supplementary Figure 4). The beneficial effects of GAL on disease severity and inflammation were abolished in mice with VXP, SPX, or VXP and SPX (Figure 5 & Supplementary Figure 4). NRX resulted in comparable to VXP exacerbation of disease severity (Figure 5 & Supplementary Figure 4). These effects were abolished in mice simultaneously subjected to SPX (Figure 5 & Supplementary Figure 4). Furthermore, the beneficial effects of GAL were abrogated in mice with NRX, SPX or NRX and SPX (Figure 5, Supplementary Figure 4). No effect of these manipulations was observed on colonic IL-10 levels (Supplementary Figure 1).

In addition, beneficial effects of the M1mAChR agonist McN-A-343 i.c.v. infusion in the context of colitis were abolished in the absence of an intact vagus nerve or splenic nerve, or in mice with SPX (Supplementary Figure 5). Previously, vagus nerve activation functionally associated with signaling along the splenic nerve has been demonstrated to result in an increase of ACh in the spleen[25]. Therefore, we next examined the effects of GAL and McN-A-343 treatments on splenic ACh levels and the mediating role of the vagus nerve and the splenic nerve. GAL and McN-A-343 treatments were associated with a significant increase in ACh levels in the spleen of sham-operated control mice with DSS colitis (Figure 6). ACh levels in the VXP and the NRX group were significantly lower as compared to sham-operated controls in mice with DSS colitis and VXP and NRX significantly abolished the increase in splenic ACh release caused by GAL or McN-A-343 treatments (Figure 6). Together, these results demonstrate that neural signaling through the vagus nerve-to spleen axis is required for central cholinergic activation to alleviate colitis.

Central cholinergic activation decreases splenic $CD11_C^+$ cell cytokine production

To gain further insight into the cellular mechanisms mediating cholinergic anti-inflammatory effects on colitis we studied the role of splenic $CD11_C^+$ cells. IL-12p40, IL-1β and IL-6 levels in the splenic $CD11_C^+$ cells-culture supernatant from colitic GAL-treated (Figure 7) were significantly decreased as compared to colitic non-treated groups. Conversely, VXP or NRX increased IL-12p40, IL-1β and IL-6 levels (Figure 7) and no beneficial effect of GAL (Figure 7) treatment was found in the absence of an intact vagus or splenic nerve. The cell viability in non-colitic condition was 99+2.4%; however in all the groups treated with DSS the cell viability decreased to 82+3.2%. We next performed *ex vivo* experiments to highlight the role of the α7nAChR in mediating cholinergic anti-inflammatory effects in spleen. Splenic $CD11_C^+$ cells were isolated from colitic mice subjected to sham-operation, VXP or NRX and a treatment with GAL (i.p.). Then, these cells were treated *ex vivo* with GTS-21 (a specific α7nAChR agonist). At all conditions the addition of GTS-21 in the culture medium significantly decreased IL-12p40, IL-1β and IL-6 levels production (Figure 7). We also demonstrated the beneficial effects of cholinergic treatments in the context of DNBS colitis (Supplementary Figure 6). Apart from being an AChE inhibitor, GAL has properties of an allosteric positive modulator of nicotinic, including α7nAChR38. Therefore, we evaluated the possibility that stimulation of the α7AChR on splenic $CD11_C^+$ cells by GAL might contribute to anti-inflammatory effects. Splenic $CD11_C^+$ cells isolated from non-colitic mice were activated with LPS and treated with GAL or vehicle *ex vivo*. We did not observe any significant effect of GAL (100 μM) on IL-12p40, IL-1β and IL-6 levels (440±23 *vs* 435±41, 640±78 *vs 712*±65, 910±54 *vs* 876±44 pg/ml respectively) in these settings.

To further assess the effects of *in vivo* treatments and surgeries we next examined the surface expression of MHC II and costimulatory molecules (CD40, CD86, CD80). In the absence of colitis, naïve splenic $CD11_C^+$ cells expressed low levels of CD40, CD86, CD80 and moderate levels of MHC II at cell surface. Upon activation of colonic inflammation, the surface expression of MHC II was upregulated (Figure 8A), and such upregulation was further augmented by VXP and NRX. CD40, CD80 and CD86 expressions were not modified (Figure 8B–D). GAL treatment decreased the level of MHC II in the splenic $CD11_C^+$ cells isolated from mice with colitis, and no beneficial effect of GAL treatment was found in the absence of an intact vagus or splenic nerve (Figure 8A). In the absence of colitis no significant modification of the expression of the different markers was determined in the different groups (data not shown).

Discussion

The results of this study clearly indicate that central cholinergic activation by AChE inhibitors or selective mAChR ligands results in a decreased susceptibility to experimental colitis. These protective effects are dependent on vagus nerve and splenic nerve signaling, which is mediated at a cellular level through cholinergic suppression of splenic DC activation.

In line with previous findings indicating a role for brain mAChRs in the central activation of the vagus nerve-mediated cholinergic anti-inflammatory pathway[20] exogenous GAL failed

to reduce the severity of colitis in mice with pharmacological blockage (by AS) of central mAChRs. The lack of effects of AS or AMN alone on colitis severity suggests that the basal levels of ACh are not associated with protective effects through mAChR-mediated mechanisms. Furthermore, the therapeutic efficacy of McN-A-343 and MTT highlight exploiting central cholinergic activation by M1mAChR agonists or M2mAChR antagonists as novel approaches to alleviate colitis. While it is known that peripheral cholinergic activation may have some effect on colitis[39], in our study we found no evidence for a significant role of peripheral mAChRs in mediating protective cholinergic effects in the context of this experimental plan. Our findings that the effects of GAL and McN-A-343 are mediated through activation of the vagus nerve-based cholinergic anti-inflammatory pathway are consistent with experimental evidence pointing to a protective role of the vagus nerve against acute colitis in animal models[4, 40, 41]. The disease-alleviating and anti-inflammatory effects of both GAL and McN-A-343 treatments were abrogated in mice with VXP, NRX or mice with splenectomy. These results are in line with data demonstrating that vagus nerve stimulation fails to protect against septic shock in rats subjected to common celiac branch VXP, splenectomy or NRX[25, 42]. Both therapeutics (GAL and McN-A-343) increased splenic ACh levels in mice and this effect was not observed in mice with VXP and NRX, indicating that the protective cholinergic effect is dependent on an interaction between the vagus nerve and spleen. These results are consistent with recent observations that splenic ACh is released after vagus nerve activation, which can result in a paracrine effect on antigen presenting cells and anti-inflammatory effect during endotoxemia[25]. Thus, our results identify a vagus nerve –to spleen axis as an important mediator of the central cholinergic regulation of colitis severity. The importance of our findings was additionally substantiated by the similar profile of results in a DNBS-model, which broadens the therapeutic implications of cholinergic modalities in the regulation of intestinal inflammation.

Although previous observations clearly implicate peritoneal macrophages[4, 43] and splenic T cells[44] in the context of the cholinergic anti-inflammatory pathway, it was not previously known whether changes in splenic $CD11_C^+$ cell responses play a role in regulating gut inflammation by cholinergic signaling. Our data demonstrate that activated vagus nerve signaling affects splenic $CD11_C^+$ cells cytokine production and expression of MHC II, which subsequently may play an important role in regulation of gut inflammation through the $CD4^+$T cell population. This observation substantiates previous studies related to the importance of DCs in the context of experimental colitis[45] and demonstrating amelioration of DSS-induced colitis by depletion of DCs after administration of diphtheria toxin in CD11c-DT receptor transgenic mice[17]. This is in agreement with clinical data demonstrating that IBD is characterized by increased expression of IL-12p40, IL-1β and IL-6 pro-inflammatory cytokines in the gut and serum[46].

Our results indicate that splenectomy does not affect colitis. This observation is in line with the hypothesis that appendicular lymphoid tissue, but not the spleen, contributes to the development of colitis[47]. It also suggests that while the gut is the site of disease initiation, the regulation of the cytokine release can be mediated via the spleen. Since the vagus nerve has a tonic inhibitory activity on the release of pro-inflammatory cytokines such as IL-1β, IL-6, from the splenic DCs, its surgical interruption enhances the release of these cytokines

from the spleen. This consequently results in exacerbation of the disease initiated within the colon, and is abolished by VXP, NRX or SPX. These findings are in line with the concept of neural signal modulation of mobile immune cell activation in the spleen[48]. While transiting the spleen, immune cells pass through its vascular matrix in proximity to neuronal endings arising from the splenic nerve. Thus, the inflammatory phenotype of immune cells might undergo neural modulation so that when exiting the spleen, these cells either fail to be recruited to zones of inflammation or fail to mediate damage[49]. This concept has implications for understanding disease pathogenesis and the role of neural regulation in inflammatory conditions initiated or mediated by the trafficking and accumulation of inflammatory cells in peripheral tissues. Although it is theoretically possible that mesenteric lymphoid or lamina propria DCs may also be involved in mediating cholinergic effects in the context of colitis, our findings clearly point to a role of splenic $CD11_C^+$ cells in this regulation. However, the possible contribution of other circulating immune cell (i.e. granulocytes) might also be considered. This regulation could be dysfunctional in the context of IBD with autonomic impairment. Therefore, stimulating vagus nerve activity may contribute to restoring the anti-inflammatory neuro-immune regulation at the level of the spleen.

We have further highlighted the mediating role of the α7AChR on splenic $CD11_C^+$ cells; a specific α7AChR agonist down-regulated *ex vivo* IL-12p40, IL-6 and TNF-a release. This corroborates data demonstrating that *in vitro* immature spleen DCs that mature in a nicotinic environment manifest lower endocytic and phagocytic activities[50]. *In vitro* mature spleen DCs that are exposed to nicotine produce decreased levels of IL-12p40 and displayed reduced ability to induce T-Cell responses[51]. Moreover, our data do not support the possibility that galantamine may act as an allosteric modulator on the α7AChR on splenic $CD11_C^+$ cells to directly affect their activation.

It should be acknowledged that apart from the spleen the vagus nerve targets the proximal colon. Therefore, it is theoretically possible that alterations in the inflammatory state in the vagus nerve-innervated proximal colon affect the enteric nervous system activity in the remainder of the colon[52], which in turn may result in alterations in neurotransmitter release and suppression of inflammation. This may also account for the effect of vagus nerve signaling on colitis observed in this and other studies [4, 40]. However, in our study we did not observe significant effects of GAL or McN-A-343 in the absence of the spleen. It is also conceivable that other factors may contribute to the beneficial effects of these cholinergic treatments on colitis. A dominant sympathetic drive, which can simultaneously occur with impaired vagus nerve activity, has been associated with enhanced colonic inflammation[53]. In addition, vagotomy alters lymphocyte trafficking[54] and the number of mast cells in the gut[55] and influences gut physiology[56]. Therefore, alteration of these parameters may also contribute to changes in colitic severity as a result of cholinergic, vagus nerve-mediated stimulation. Furthermore, the fact that we did not observe significant effects of GAL or McN-A-343 on IL-10 levels suggests that alterations in anti-inflammatory cytokine levels do not play a role in mediating the beneficial effects of cholinergic modalities on colitis severity. This confirms previous data indicating no role of IL-10 in mediating cholinergic regulation of immune responses in models of endotoxemia and colitis[28, 43]. In addition,

cholinergic treatments did not alter the stool consistency in control mice, indirectly pointing to a lack of significant effect on gut physiology.

Our findings prompt close consideration of the relationship between brain cholinergic activation and disease activity in patients with colonic inflammation and have important clinical relevance. Patients with colonic inflammatory conditions might be selected for novel treatment strategies including: 1) Centrally-acting AChE inhibitors, which are already clinically approved for the treatment of Alzheimer's disease; 2) highly selective muscarinic receptor agonists or antagonists[6], which are being actively developed for the treatment of Alzheimer's disease and other neurodegenerative disorders; and 3) electrical vagus nerve stimulation, which is now being used for the treatment of refractory depression and epilepsy[57]. The therapeutic potential of alternative techniques targetting an increase of efferent vagus nerve outflow, such as acupuncture[58, 59], and zen[60, 61] may also be studied in the context of colonic inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant support:

This study was supported by grants from the Manitoba Health Research Council and the Canada Foundation for Innovation to JE.G and by grants from the National Institute of General Medical Sciences, NIH to K.J.T.

The authors thank Drs. Charles N Bernstein and Stephen M. Collins for their discussion and continuous support. The work was supported by the Manitoba Health Research Council, the University of Manitoba, the Canada Foundation for Innovation and the National Institute of General Medical Sciences, NIH.

Abbreviations used in this paper

ACh	acetylcholine
α7nAChR	alpha7 nicotinic acetylcholine receptor
AMN	atropine methyl nitrate
AS	atropine sulfate
CD	Crohn's disease
CRP	C-reactive protein
DAI	disease activity index
DCs	dendritic cells
DNBS	2,4 dinitrobenzene-sulfonic acid
DSS	dextran sodium sulfate
GAL	galantamine
IBDs	inflammatory bowel diseases

i.c.v	intracerebroventricular
IL	interleukin
INF	interferon
LPS	lipopolysaccharide
MLA	methyllycaconitine
MPO	myeloperoxidase
MTT	methoctramine
mAChR	muscarinic acetylcholine receptor
NRX	splenic neurectomy
SPX	splenectomy
TNF	tumor necrosis factor
UC	ulcerative colitis
VXP	vagotomy and pyloroplasty

References

1. Molodecky NA, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, et al. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology. 2012; 142:46–54. e42. quiz e30. [PubMed: 22001864]
2. Strober W, Fuss IJ. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. Gastroenterology. 2011; 140:1756–1767. [PubMed: 21530742]
3. Perrier C, Rutgeerts P. Cytokine blockade in inflammatory bowel diseases. Immunotherapy. 2011; 3:1341–1352. [PubMed: 22053885]
4. Ghia JE, Blennerhassett P, Kumar-Ondiveeran H, Verdu EF, Collins SM. The vagus nerve: a tonic inhibitory influence associated with inflammatory bowel disease in a murine model. Gastroenterology. 2006; 131:1122–1130. [PubMed: 17030182]
5. Goldstein RS, Bruchfeld A, Yang L, Qureshi AR, Gallowitsch-Puerta M, Patel NB, et al. Cholinergic anti-inflammatory pathway activity and High Mobility Group Box-1 (HMGB1) serum levels in patients with rheumatoid arthritis. Mol Med. 2007; 13:210–215. [PubMed: 17597834]
6. The F, Cailotto C, van der Vliet J, de Jonge WJ, Bennink RJ, Buijs RM, et al. Central activation of the cholinergic anti-inflammatory pathway reduces surgical inflammation in experimental post-operative ileus. Br J Pharmacol. 2011; 163:1007–1016. [PubMed: 21371006]
7. Borovikova LV, Ivanova S, Nardi D, Zhang M, Yang H, Ombrellino M, et al. Role of vagus nerve signaling in CNI-1493-mediated suppression of acute inflammation. Auton Neurosci. 2000; 85:141–147. [PubMed: 11189021]
8. Van Der Zanden EP, Boeckxstaens GE, de Jonge WJ. The vagus nerve as a modulator of intestinal inflammation. Neurogastroenterol Motil. 2009; 21:6–17. [PubMed: 19140954]
9. Pavlov VA, Tracey KJ. The vagus nerve and the inflammatory reflex--linking immunity and metabolism. Nature reviews Endocrinology. 2012; 8:743–754.
10. Lindgren S, Stewenius J, Sjolund K, Lilja B, Sundkvist G. Autonomic vagal nerve dysfunction in patients with ulcerative colitis. Scand J Gastroenterol. 1993; 28:638–642. [PubMed: 8362220]
11. Meregnani J, Clarencon D, Vivier M, Peinnequin A, Mouret C, Sinniger V, et al. Anti-inflammatory effect of vagus nerve stimulation in a rat model of inflammatory bowel disease. Auton Neurosci. 2011; 160:82–89. [PubMed: 21071287]

12. Cosnes J. Tobacco and IBD: relevance in the understanding of disease mechanisms and clinical practice. Best Pract Res Clin Gastroenterol. 2004; 18:481–496. [PubMed: 15157822]
13. Frolkis A, Dieleman LA, Barkema H, Panaccione R, Ghosh S, Fedorak RN, et al. Environment and the inflammatory bowel diseases. Can J Gastroenterol. 2013; 27:e18–24. [PubMed: 23516681]
14. Drakes ML, Blanchard TG, Czinn SJ. Colon lamina propria dendritic cells induce a proinflammatory cytokine response in lamina propria T cells in the SCID mouse model of colitis. J Leukoc Biol. 2005; 78:1291–1300. [PubMed: 16204624]
15. Ikeda Y, Ueno A, Naraba H, Oh-ishi S. Involvement of vanilloid receptor VR1 and prostanoids in the acid-induced writhing responses of mice. Life Sci. 2001; 69:2911–2919. [PubMed: 11720094]
16. te Velde AA, van Kooyk Y, Braat H, Hommes DW, Dellemijn TA, Slors JF, et al. Increased expression of DC-SIGN+IL-12+IL-18+ and CD83+IL-12−IL-18− dendritic cell populations in the colonic mucosa of patients with Crohn's disease. Eur J Immunol. 2003; 33:143–151. [PubMed: 12594843]
17. Berndt BE, Zhang M, Chen GH, Huffnagle GB, Kao JY. The role of dendritic cells in the development of acute dextran sulfate sodium colitis. J Immunol. 2007; 179:6255–6262. [PubMed: 17947701]
18. Yoshikawa H, Kurokawa M, Ozaki N, Nara K, Atou K, Takada E, et al. Nicotine inhibits the production of proinflammatory mediators in human monocytes by suppression of I-kappaB phosphorylation and nuclear factor-kappaB transcriptional activity through nicotinic acetylcholine receptor alpha7. Clin Exp Immunol. 2006; 146:116–123. [PubMed: 16968406]
19. Kawashima K, Yoshikawa K, Fujii YX, Moriwaki Y, Misawa H. Expression and function of genes encoding cholinergic components in murine immune cells. Life Sci. 2007; 80:2314–2319. [PubMed: 17383684]
20. Pavlov VA, Ochani M, Gallowitsch-Puerta M, Ochani K, Huston JM, Czura CJ, et al. Central muscarinic cholinergic regulation of the systemic inflammatory response during endotoxemia. Proc Natl Acad Sci U S A. 2006; 103:5219–5223. [PubMed: 16549778]
21. Pavlov VA, Parrish WR, Rosas-Ballina M, Ochani M, Puerta M, Ochani K, et al. Brain acetylcholinesterase activity controls systemic cytokine levels through the cholinergic anti-inflammatory pathway. Brain Behav Immun. 2009; 23:41–45. [PubMed: 18639629]
22. Ellis JM. Cholinesterase inhibitors in the treatment of dementia. J Am Osteopath Assoc. 2005; 105:145–158. [PubMed: 15863734]
23. Waldburger JM, Boyle DL, Edgar M, Sorkin LS, Levine YA, Pavlov VA, et al. Spinal p38 MAP kinase regulates peripheral cholinergic outflow. Arthritis Rheum. 2008; 58:2919–2921. [PubMed: 18759350]
24. Shifrin H, Nadler-Milbauer M, Shoham S, Weinstock M. Rivastigmine alleviates experimentally induced colitis in mice and rats by acting at central and peripheral sites to modulate immune responses. PLoS One. 2013; 8:e57668. [PubMed: 23469045]
25. Rosas-Ballina M, Olofsson PS, Ochani M, Valdes-Ferrer SI, Levine YA, Reardon C, et al. Acetylcholine-Synthesizing T Cells Relay Neural Signals in a Vagus Nerve Circuit. Science. 2011; 334:98–101. [PubMed: 21921156]
26. Ghia JE, Galeazzi F, Ford DC, Hogaboam CM, Vallance BA, Collins SM. Role of M-CSF dependent macrophages in colitis is driven by the nature of the inflammatory stimulus. Am J Physiol Gastrointest Liver Physiol. 2008; 294:770–777.
27. Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology. 1990; 98:694–702. [PubMed: 1688816]
28. Ghia JE, Blennerhassett P, Collins SM. Impaired parasympathetic function increases susceptibility to inflammatory bowel disease in a mouse model of depression. J Clin Invest. 2008; 118:2209–2218. [PubMed: 18451995]
29. Rosenwald A, Reusche E, Ogomori K, Teichert HM. Comparison of silver stainings and immunohistology for the detection of neurofibrillary tangles and extracellular cerebral amyloid in paraffin sections. Acta Neuropathol (Berl). 1993; 86:182–186. [PubMed: 7692696]
30. Cooper HS, Murthy SN, Shah RS, Sedergran DJ. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. Lab Invest. 1993; 69:238–249. [PubMed: 8350599]

31. Appleyard CB, Wallace JL. Reactivation of hapten-induced colitis and its prevention by anti-inflammatory drugs. Am J Physiol. 1995; 269:G119–125. [PubMed: 7631788]
32. Boughton-Smith NK, Wallace JL, Whittle BJ. Relationship between arachidonic acid metabolism, myeloperoxidase activity and leukocyte infiltration in a rat model of inflammatory bowel disease. Agents Actions. 1988; 25:115–123. [PubMed: 2847506]
33. Zhang L, Procuik M, Fang T, Kung SK. Functional analysis of the quantitative expression of a costimulatory molecule on dendritic cells using lentiviral vector-mediated RNA interference. J Immunol Methods. 2009; 344:87–97. [PubMed: 19303417]
34. Bickel U, Thomsen T, Fischer JP, Weber W, Kewitz H. Galanthamine: pharmacokinetics, tissue distribution and cholinesterase inhibition in brain of mice. Neuropharmacology. 1991; 30:447–454. [PubMed: 1865992]
35. Stillman MJ, Shukitt-Hale B, Kong RM, Levy A, Lieberman HR. Elevation of hippocampal extracellular acetylcholine levels by methoctramine. Brain Res Bull. 1993; 32:385–389. [PubMed: 8221128]
36. Lachowicz JE, Duffy RA, Ruperto V, Kozlowski J, Zhou G, Clader J, et al. Facilitation of acetylcholine release and improvement in cognition by a selective M2 muscarinic antagonist, SCH 72788. Life Sci. 2001; 68:2585–2592. [PubMed: 11392630]
37. Ghia JE, Blennerhassett P, Collins SM. Vagus nerve Integrity and Experimental Colitis. Am J Physiol Gastrointest Liver Physiol. 2007; 293:G560–G567. [PubMed: 17585014]
38. Texido L, Ros E, Martin-Satue M, Lopez S, Aleu J, Marsal J, et al. Effect of galantamine on the human alpha7 neuronal nicotinic acetylcholine receptor, the Torpedo nicotinic acetylcholine receptor and spontaneous cholinergic synaptic activity. Br J Pharmacol. 2005; 145:672–678. [PubMed: 15834443]
39. Miceli PC, Jacobson K. Cholinergic pathways modulate experimental dinitrobenzene sulfonic acid colitis in rats. Auton Neurosci. 2003; 105:16–24. [PubMed: 12742187]
40. Mazelin L, Theodorou V, More J, Fioramonti J, Bueno L. Protective role of vagal afferents in experimentally-induced colitis in rats. J Auton Nerv Syst. 1998; 73:38–45. [PubMed: 9808369]
41. van Westerloo DJ, Giebelen IA, Florquin S, Bruno MJ, Larosa GJ, Ulloa L, et al. The vagus nerve and nicotinic receptors modulate experimental pancreatitis severity in mice. Gastroenterology. 2006; 130:1822–1830. [PubMed: 16697744]
42. Xue N, Liang H, Yao H, Song XM, Li JG. The role of spleen in vagus nerve stimulation for treatment against septic shock in rats. Zhongguo Wei Zhong Bing Ji Jiu Yi Xue. 2011; 23:263–266. [PubMed: 21805738]
43. Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, et al. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature. 2000; 405:458–462. [PubMed: 10839541]
44. Karimi K, Bienenstock J, Wang L, Forsythe P. The vagus nerve modulates CD4+ T cell activity. Brain Behav Immun. 2010; 24:316–323. [PubMed: 19887104]
45. Abe K, Nguyen KP, Fine SD, Mo JH, Shen C, Shenouda S, et al. Conventional dendritic cells regulate the outcome of colonic inflammation independently of T cells. Proc Natl Acad Sci U S A. 2007; 104:17022–17027. [PubMed: 17942668]
46. Sanchez-Munoz F, Dominguez-Lopez A, Yamamoto-Furusho JK. Role of cytokines in inflammatory bowel disease. World J Gastroenterol. 2008; 14:4280–4288. [PubMed: 18666314]
47. Krieglstein CF, Cerwinka WH, Laroux FS, Grisham MB, Schurmann G, Bruwer M, et al. Role of appendix and spleen in experimental colitis. J Surg Res. 2001; 101:166–175. [PubMed: 11735272]
48. Tracey KJ. Understanding immunity requires more than immunology. Nat Immunol. 2010; 11:561–564. [PubMed: 20562838]
49. Saeed RW, Varma S, Peng-Nemeroff T, Sherry B, Balakhaneh D, Huston J, et al. Cholinergic stimulation blocks endothelial cell activation and leukocyte recruitment during inflammation. J Exp Med. 2005; 201:1113–1123. [PubMed: 15809354]
50. Guinet E, Yoshida K, Nouri-Shirazi M. Nicotinic environment affects the differentiation and functional maturation of monocytes derived dendritic cells (DCs). Immunol Lett. 2004; 95:45–55. [PubMed: 15325797]

51. Nouri-Shirazi M, Tinajero R, Guinet E. Nicotine alters the biological activities of developing mouse bone marrow-derived dendritic cells (DCs). Immunol Lett. 2007; 109:155–164. [PubMed: 17368810]

52. Jacobson K, McHugh K, Collins SM. Experimental colitis alters myenteric nerve function at inflamed and noninflamed sites in the rat. Gastroenterology. 1995; 109:718–722. [PubMed: 7657099]

53. McCafferty DM, Wallace JL, Sharkey KA. Effects of chemical sympathectomy and sensory nerve ablation on experimental colitis in the rat. Am J Physiol. 1997; 272:G272–280. [PubMed: 9124351]

54. Antonica A, Ayroldi E, Magni F, Paolocci N. Lymphocyte traffic changes induced by monolateral vagal denervation in mouse thymus and peripheral lymphoid organs. J Neuroimmunol. 1996; 64:115–122. [PubMed: 8632053]

55. Gottwald TP, Lhotak S, Stead RH. Effects of subdiaphragmatic vagotomy on mucosal mast cell densities in stomach and jejunum of rats. Adv Exp Med Biol. 1995; 371A:303–306. [PubMed: 8525930]

56. Dapoigny M, Cowles VE, Zhu YR, Condon RE. Vagal influence on colonic motor activity in conscious nonhuman primates. Am J Physiol. 1992; 262:G231–236. [PubMed: 1539658]

57. Diamond A, Kenney C, Jankovic J. Effect of vagal nerve stimulation in a case of Tourette's syndrome and complex partial epilepsy. Mov Disord. 2006; 21:1273–1275. [PubMed: 16703589]

58. Huang ST, Chen GY, Lo HM, Lin JG, Lee YS, Kuo CD. Increase in the vagal modulation by acupuncture at neiguan point in the healthy subjects. Am J Chin Med. 2005; 33:157–164. [PubMed: 15844844]

59. Sakai S, Hori E, Umeno K, Kitabayashi N, Ono T, Nishijo H. Specific acupuncture sensation correlates with EEGs and autonomic changes in human subjects. Auton Neurosci. 2007; 133:158–169. [PubMed: 17321222]

60. Kubota Y, Sato W, Toichi M, Murai T, Okada T, Hayashi A, et al. Frontal midline theta rhythm is correlated with cardiac autonomic activities during the performance of an attention demanding meditation procedure. Brain research Cognitive brain research. 2001; 11:281–287. [PubMed: 11275489]

61. Paul-Labrador M, Polk D, Dwyer JH, Velasquez I, Nidich S, Rainforth M, et al. Effects of a randomized controlled trial of transcendental meditation on components of the metabolic syndrome in subjects with coronary heart disease. Arch Intern Med. 2006; 166:1218–1224. [PubMed: 16772250]

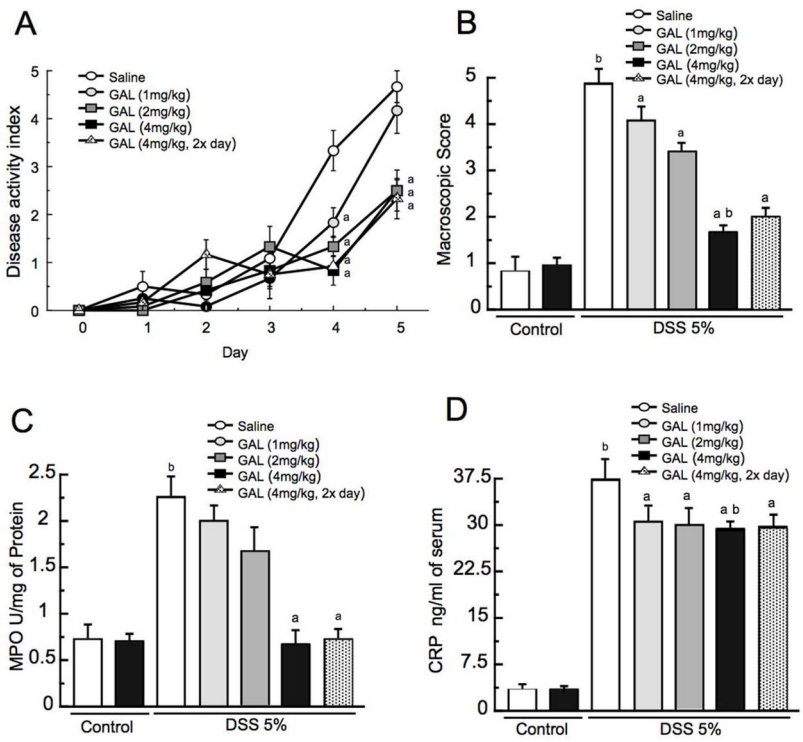

Figure 1. Galantamine (GAL) alleviates the severity of dextran sulphate sodium (DSS)-induced colitis

GAL (6 days, i.p.) treatment was started one day prior to colitis induction. ***A:*** Disease activity index; ***B:*** Macroscopic scores; ***C:*** Colonic myeloperoxidase (MPO) activity; ***D:*** Serum C-reactive protein (CRP). Values are shown as means±SEM. Samples were collected on day 5 post-DSS; mice per group ≥8. $^{a}P<0.05$ as compared to saline DSS-treated group, $^{b}P<0.05$ as compared to control (H2O)-treated group.

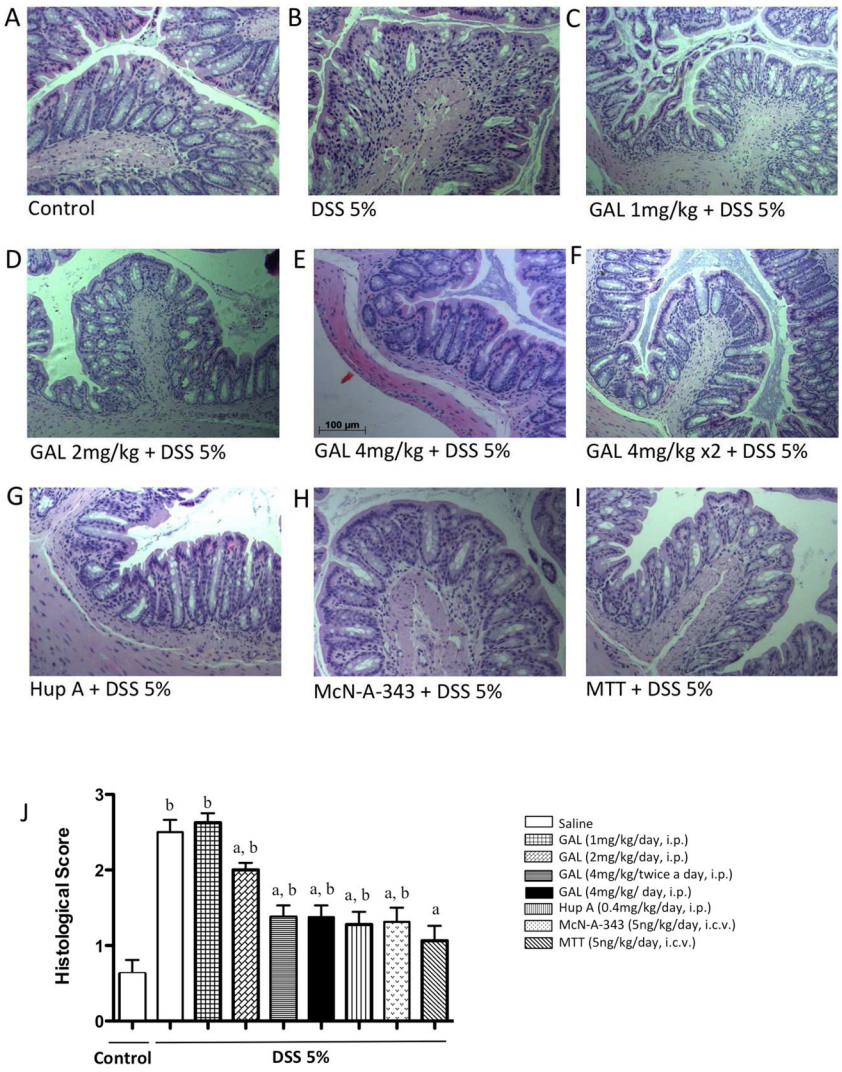

Figure 2. Centrally-acting acetylcholinesterase inhibitors and mAChR ligands alleviate the severity of dextran sulphate sodium (DSS)-induced colitis

Appearance of a colon in mice ***A:*** in absence of colitis (control group); ***B:*** in mice with DSS-induced colitis; ***C:*** in galantamine (GAL) (4mg/kg, i.p. for 6 days)-treated mice with DSS-induced colitis ***D:*** in Huperzine A (Hup A) (0.4mg/kg, i.p. for 6 days)-treated mice with DSS-induced colitis ***E:*** in McN-A-343 (M1mAChR agonist) (5 ng/kg/day, i.c.v., for 6 days)-treated mice with DSS-induced colitis ***F:*** in methoctramine (MTT, M2mAChR antagonist) (5 ng/kg/day, i.c.v., for 6 days)-treated mice with DSS-induced colitis ***G:*** Histological score; values are shown as means±SEM. Samples were collected on day 5 post-DSS; mice per group ≥8. $^{a}P<0.05$ as compared to saline DSS-treated group, $^{b}P<0.05$ as compared to control (H2O)-group. Hematoxylin and eosin staining, 100X magnifications.

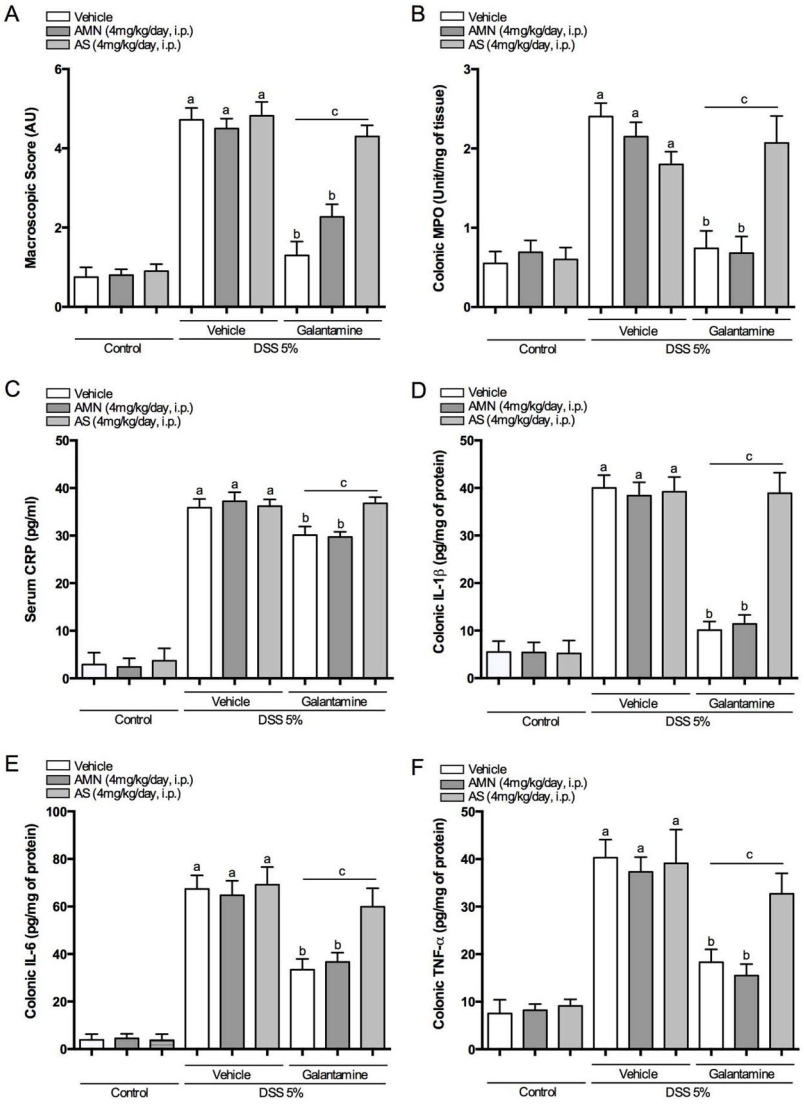

Figure 3. Galantamine (GAL) effects in dextran sulphate sodium (DSS)-induced colitis are mediated through central mAChRs

Atropine methyl nitrate (AMN, a mAChR antagonist that does not cross the blood-brain barrier) (4mg/kg, i.p. for 6 days) or atropine sulfate (AS, a mAChR antagonist that crosses the blood-brain barrier) were administered as a single daily injection 20 min prior to every GAL administration (4mg/kg, i.p., for 6 days). ***A:*** Macroscopic score; ***B:*** Colonic myeloperoxidase (MPO) activity; ***C:*** Serum C-reactive protein (CRP); ***D:*** Colonic Interleukin (IL)-1β amount; ***E:*** Colonic IL-6 amount; ***F:*** Colonic tumor necrosis factor (TNF)-α amount. Values are shown as means±SEM. Samples were collected on day 5 post-DSS; mice per group ≥8. $^{a}P<0.05$, compared to non DSS-treated group (control, H20) respectively, $^{b}P<0.05$ as compared to vehicle DSS-treated group respectively, $^{c}P<0.05$ as compared to GAL-DSS-treated group.

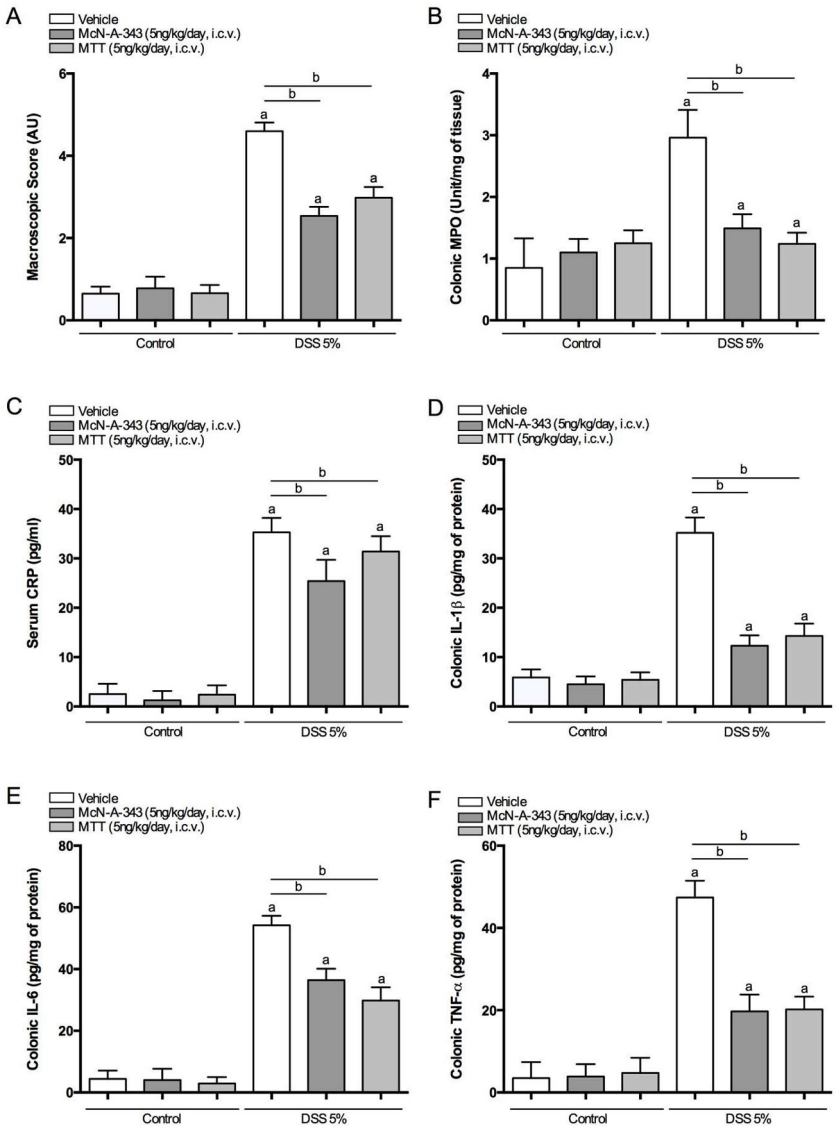

Figure 4. Central administration of a M1mAChR agonist or a M2mAChR antagonist alleviates the severity of dextran sulphate sodium (DSS)-induced colitis

McN-A-343 (M1mAChR agonist) or methoctramine (MTT, M2mAChR antagonist) (5 ng/kg/day, i.c.v., for 6 days) treatments were started one day before induction of colitis. ***A:*** Macroscopic score; ***B:*** Colonic myeloperoxidase (MPO) activity; ***C:*** Serum C-reactive protein (CRP); ***D:*** Colonic Interleukin (IL)-1β amount; ***E:*** Colonic IL-6 amount; ***F:*** Colonic tumor necrosis factor (TNF)-α amount. Values are shown as means±SEM. Samples were collected on day 5 post-DSS; mice per group ≥8. $^{a}P<0.05$, compared to non DSS-treated group (control, H20) respectively, $^{b}P<0.05$ as compared to vehicle DSS-treated group.

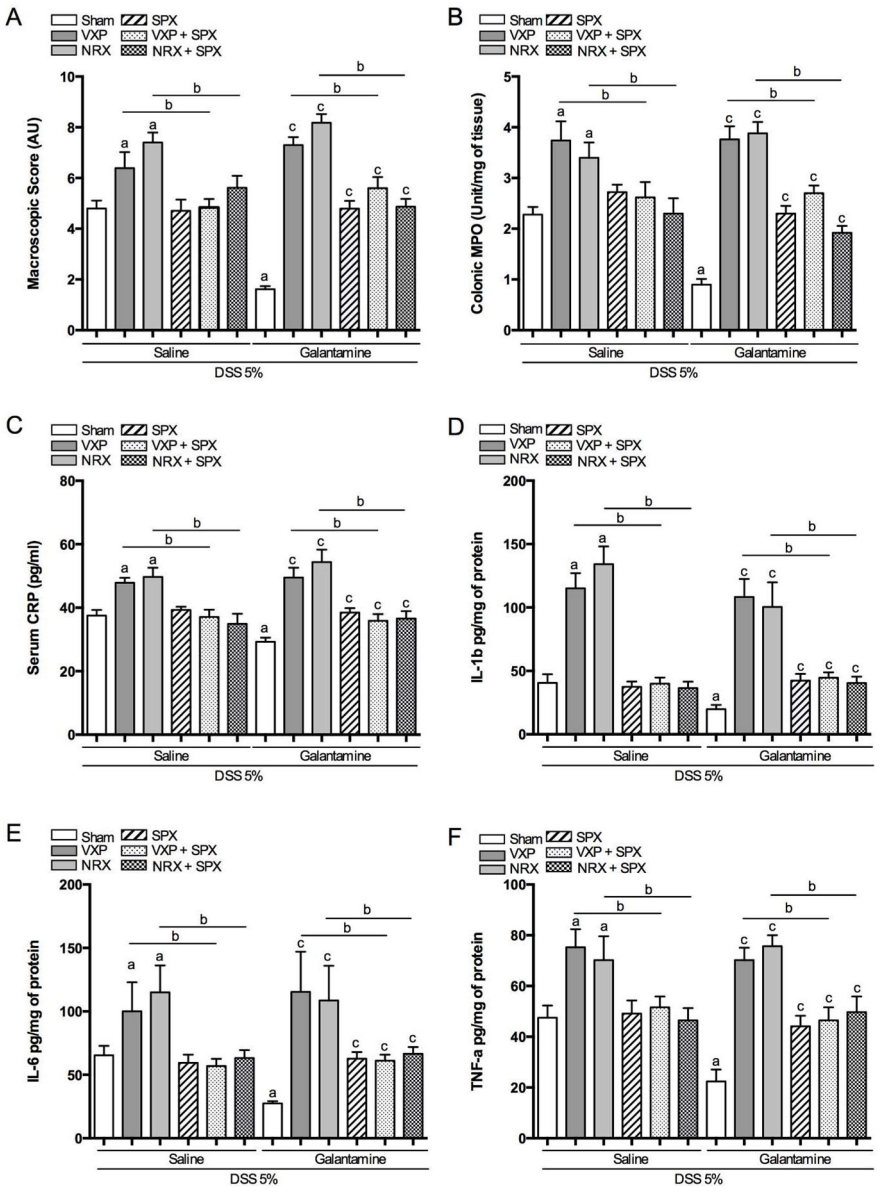

Figure 5. Galantamine's effects in mice with dextran sulphate sodium (DSS)-induced colitis are mediated through vagus nerve and splenic nerve signaling to the spleen

Vagotomy (VXP) and/or splenectomy (SPX), splenic neurectomy (NRX) and/or splenectomy (SPX) were performed 10 days prior to initiating galantamine (4 mg/kg/day, i.p.) treatment and/or colitis induction as described in Material and Methods. *Sham represents data obtained in sham SPX mice, because no significant differences were determined between this group and any other sham group of animals; ***A:*** Macroscopic score; ***B:*** Colonic myeloperoxidase (MPO) activity; ***C:*** Serum C-reactive protein (CRP); ***D:*** Colonic Interleukin (IL)-1β amount; ***E:*** Colonic IL-6 amount; ***F:*** Colonic tumor necrosis factor (TNF)-α amount. Values are shown as means±SEM. Samples were collected on day 5 post-DSS; mice per group ≥8. $^{a}P<0.05$ as compared to sham-saline-DSS-treated

group, [b]$P<0.05$ as compared to VXP-DSS-treated group or NRX-DSS-treated group, [c]$P<0.05$ as compared to sham-GAL-DSS-treated group.

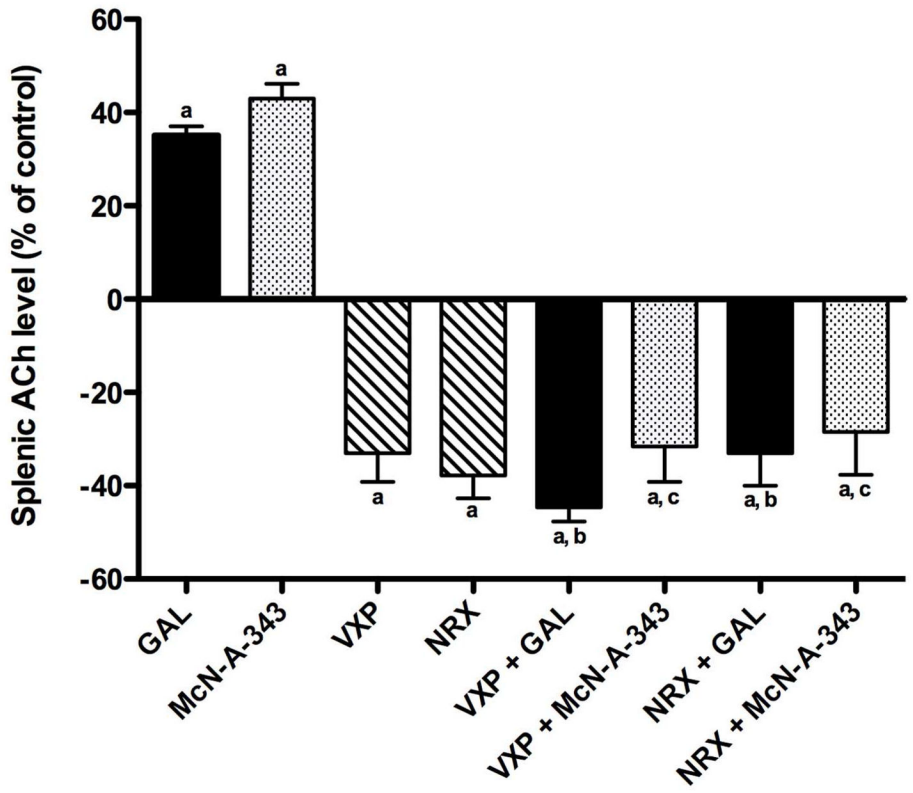

Figure 6. Cholinergic treatments with galantamine (GAL) or McN-A-343 result in increased splenic acetylcholine (ACh) levels, mediated through vagus nerve and splenic nerve signaling

Vagotomy (VXP) and/or splenic neurectomy (NRX) were performed 10 days prior to GAL (4mg/kg/day, i.p. for 6 days) or McN-A-343 (5ng/kg/day, i.c.v., for 6 days) treatments and splenic ACh levels were analyzed as described in Material and Methods. Splenic ACh levels were determined on day 5 post-colitis induction with **dextran sulphate sodium** (DSS). In colitic control condition, the level of ACh was 1845±85μM/spleen. Values are shown as means±SEM, 3 independent experiments with 4 mice per group. $^{a}P<0.05$ as compared to control sham-DSS-treated group, $^{b}P<0.05$ as compared to sham GAL-DSS-treated group, $^{c}P<0.05$ as compared to sham MCN-A-343-DSS-treated group.

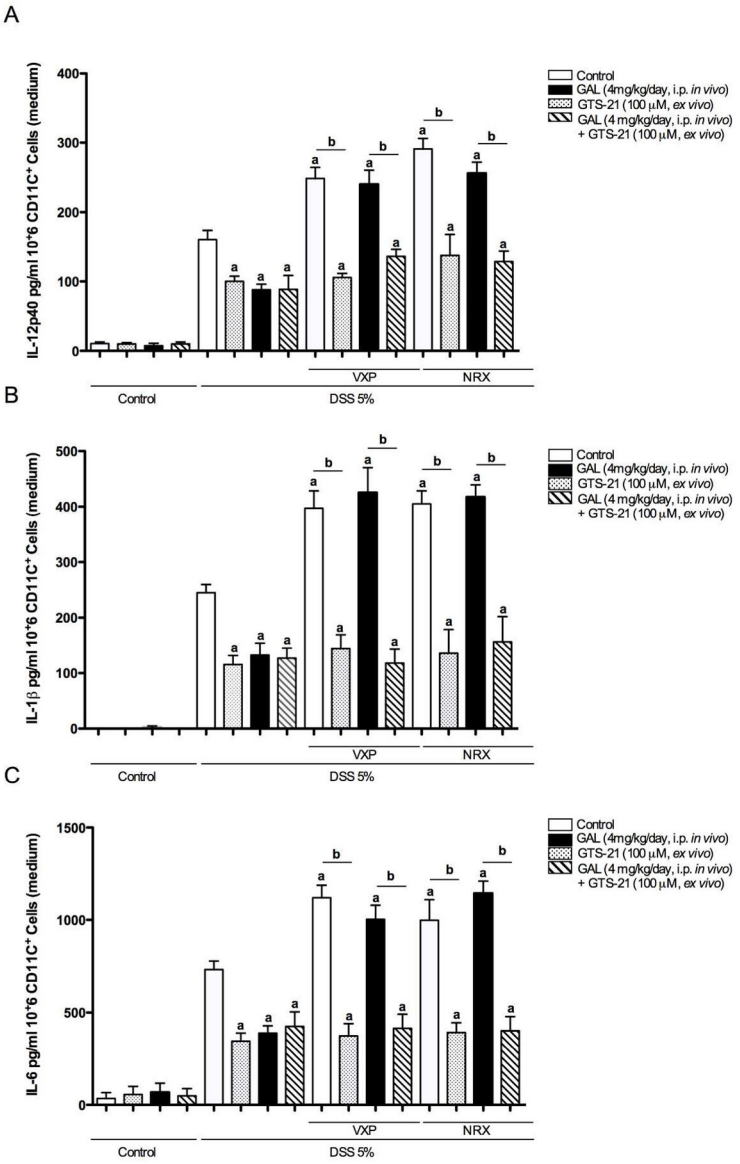

Figure 7. Effects of cholinergic treatment on splenic CD11c+ cells cytokine production in the context of dextran sulphate sodium (DSS)-induced colitis

A: Interleukin (IL)-12p40, IL-1β and IL-6 production from dendritic cells. Splenic CD11c+ cells were isolated from galantamine (GAL, 4 mg/kg/day, i.p. for 6 days)-treated groups of colitic mice subjected to sham-operation, vagotomy (VXP) or splenic neurectomy (NRX) on day 5 post-colitis induction. Splenic CD11c+ cells were also isolated from groups of colitic mice subjected to sham-operation, vagotomy (VXP) or splenic neurectomy (NRX) and incubated *ex vivo* with GTS-21 (a specific α7nAChR agonist, 100 μM). IL-12p40, IL-1β and IL-6 was measured in media at 24h following treatments. Values are shown as means±SEM, 3 independent experiments with 4 mice per group. $^{a}P<0.05$ as compared to DSS control group, $^{b}P<0.05$, n=8.

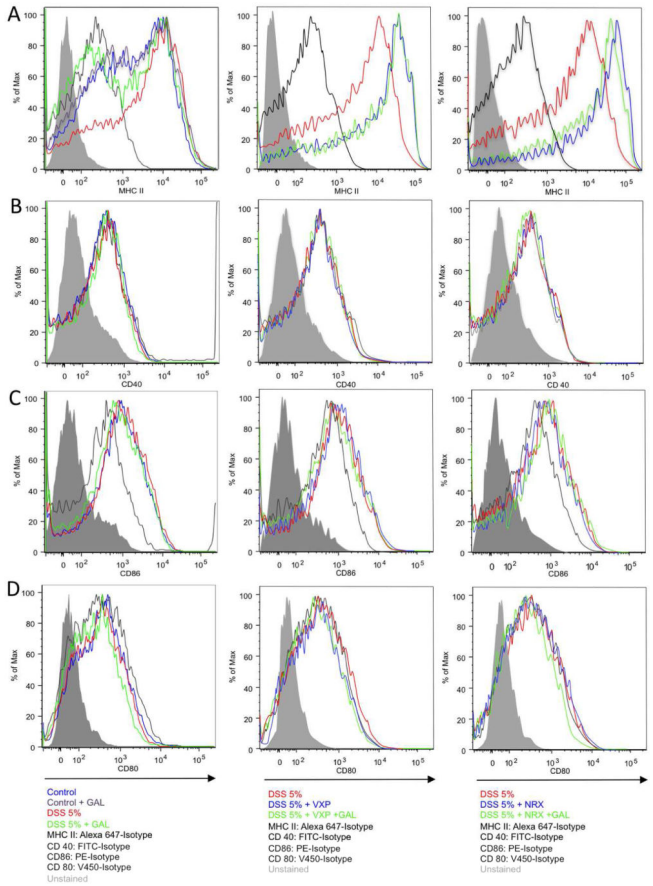

Figure 8. Effect of cholinergic treatments with galantamine (GAL), vagotomy and neurectomy on splenic CD11c^{+} cells phenotype in the context of dextran sulphate sodium (DSS)-induced colitis

Using CD11c^{+} MACS positive selection, splenic CD11c^{+} cells were isolated from galantamine (GAL, 4 mg/kg/day, i.p. for 6 days)-treated or vehicle groups of colitic mice subjected to sham-operation, vagotomy (VXP) or splenic neurectomy (NRX) on day 5 post-colitis induction. Splenic CD11c^{+} cell phenotype as characterized by median fluorescence intensity (% of Max) ***A:*** MHC II (Alexa-647), ***B:*** CD40 (FITC), ***C:*** CD80 (PE) and ***D:*** CD86 (V450) surface expression. Representative results from n=4 per group are shown.