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RESEARCH PAPER

Selective α7 nicotinic acetylcholine receptor agonists worsen disease in experimental colitis

Susanne A Snoek[1], Marleen I Verstege[1], Esmerij P van der Zanden[1], Nigel Deeks[2], David C Bulmer[2], Michael Skynner[2], Kevin Lee[2], Anje A te Velde[1], Guy E Boeckxstaens[1,3] and Wouter J de Jonge[1]

[1]*Tytgat Institute for Liver and Intestinal Research, Academic Medical Center, Amsterdam, the Netherlands,* [2]*Immuno-Inflammation CEDD, GlaxoSmithKline, Stevenage, UK, and* [3]*Catholic University of Leuven, Leuven, Belgium*

Background and purpose: In various models vagus nerve activation has been shown to ameliorate intestinal inflammation, via nicotinic acetylcholine receptors (nAChRs) expressed on immune cells. As the α7 nAChR has been put forward to mediate this effect, we studied the effect of nicotine and two selective α7 nAChR agonists (AR-R17779, (-)-spiro[1-azabicyclo[2.2.2] octane-3,5′-oxazolidin-2′-one and GSK1345038A) on disease severity in two mouse models of experimental colitis.
Experimental approach: Colitis was induced by administration of 1.5% dextran sodium sulphate (DSS) in drinking water or 2 mg 2,4,6-trinitrobenzene sulphonic acid (TNBS) intrarectally. Nicotine (0.25 and 2.50 μmol·kg^{-1}), AR-R17779 (0.6–30 μmol·kg^{-1}) or GSK1345038A (6–120 μmol·kg^{-1}) was administered daily by i.p. injection. After 7 (DSS) or 5 (TNBS) days clinical parameters and colonic inflammation were scored.
Key results: Nicotine and both α7 nAChR agonists reduced the activation of NF-κB and pro-inflammatory cytokines in whole blood and macrophage cultures. In DSS colitis, nicotine treatment reduced colonic cytokine production, but failed to reduce disease parameters. Reciprocally, treatment with AR-R17779 or GSK1345038A worsened disease and led to increased colonic pro-inflammatory cytokine levels in DSS colitis. The highest doses of GSK1345038A (120 μmol·kg^{-1}) and AR-R17779 (30 μmol·kg^{-1}) ameliorated clinical parameters, without affecting colonic inflammation. Neither agonist ameliorated TNBS-induced colitis.
Conclusions and implications: Although nicotine reduced cytokine responses *in vitro*, both selective α7 nAChR agonists worsened the effects of DSS-induced colitis or were ineffective in those of TNBS-induced colitis. Our data indicate the need for caution in evaluating α7 nAChR as a drug target in colitis.
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Keywords: colitis; nicotine; vagus nerve; α7 nicotinic acetylcholine receptor; macrophages

Abbreviations: ACh, acetylcholine; DAI, disease activity index; DSS, dextran sodium sulphate; LPS, lipopolysaccharide; nAChR, nicotinic acetylcholine receptor; TNBS, 2,4,6-trinitrobenzene sulphonic acid; UC, ulcerative colitis

Introduction

Genetic association studies (Barrett *et al.*, 2008) and functional evidence (Dieleman *et al.*, 1994; Watanabe *et al.*, 2003) have increased the recognition that intestinal macrophages play an important role in the initiation and progression of inflammatory bowel disease. In several studies it was demonstrated that resident macrophages in mucosal samples of active ulcerative colitis (UC) and Crohn's disease patients differ phenotypically and functionally from healthy controls (MacDermott, 1996; Rogler *et al.*, 1999; Kamada *et al.*, 2005). Similarly, data obtained from mouse models of colitis have implicated an important role for macrophages: in IL-10-deficient mice that develop colitis spontaneously, intestinal inflammation is prevented by the use of antagonists of chemokine receptors (Tokuyama *et al.*, 2005) that are generally expressed by macrophages, or by elimination of tissue macrophages (Watanabe *et al.*, 2003). Furthermore, colitis can still be induced in the absence of T- and B-cells (Dieleman *et al.*, 1994). Recently, it has been shown that macrophage-

Correspondence: Wouter J de Jonge, Department of Gastroenterology and Hepatology, Academic Medical Center, S2-180, Meibergdreef 9, 1105 AZ Amsterdam, the Netherlands. E-mail: w.j.dejonge@amc.nl
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derived IL-10 is crucial for the induction of regulatory T-cells, thereby controlling intestinal inflammation in colitis (Murai *et al*., 2009).

Recently, the parasympathetic system, in particular the vagus nerve, has been shown to negatively regulate macrophage immune responses via the peripheral release of acetylcholine (ACh) (Borovikova *et al*., 2000; Tracey *et al*., 2001). Activation of the so-called 'cholinergic anti-inflammatory pathway' has been shown to ameliorate disease in various models of inflammation, including sepsis (Borovikova *et al*., 2000), ischaemia reperfusion (Sadis *et al*., 2007), haemorrhage (Luyer *et al*., 2005) and postoperative ileus (de Jonge *et al*., 2005). In mouse models of colitis and postoperative ileus, enhanced parasympathetic output is involved in the negative regulation of intestinal inflammation via efferent activity of the vagus nerve (de Jonge *et al*., 2005; Ghia *et al*., 2006; 2007). Ghia *et al*. (2006; 2007) have recently demonstrated that the vagus nerve controls gut inflammation in two experimental models of colitis. In these studies it was shown that chemical as well as surgical blockade of vagus nerve signalling significantly worsened colitis and enhanced colonic inflammatory mediators. The anti-inflammatory effect of the vagus nerve most likely involves activation of the nicotinic acetylcholine receptors (nAChRs) on immune cells such as macrophages (Borovikova *et al*., 2000; Matsunaga *et al*., 2001; Wang *et al*., 2003; de Jonge *et al*., 2005) or dendritic cells (Kawashima *et al*., 2007; Nouri-Shirazi *et al*., 2007). This notion is supported by clinical observations that smoking, and the administration of nicotine (i.e. via patches) may have a protective effect on colonic inflammation in UC, even though results are generally disappointing due to the significant toxic adverse events (McGrath *et al*., 2004).

The cellular mechanisms that induce nicotinic inhibition of macrophage activation involve the activation of anti-inflammatory Stat3/Socs3 signalling pathways (de Jonge *et al*., 2005) and inhibition of NF-κB signalling (Wang *et al*., 2004). Earlier studies have indicated that the anti-inflammatory effect of ACh is mediated through the α7 nicotinic acetylcholine receptor (α7 nAChR) (Borovikova *et al*., 2000; Wang *et al*., 2003) expressed by human (Wang *et al*., 2003; 2004) and mouse macrophages (Wang *et al*., 2003; 2004; Kawashima *et al*., 2007). Given the purported role of α7 nAChRs in mediating the cholinergic anti-inflammatory pathway (Wang *et al*., 2003; de Jonge *et al*., 2005; Ulloa, 2005), selective α7 nAChR agonists may have more therapeutic potential in ameliorating disease than nicotine. Therefore, we explored the potential of pharmacological activation of the cholinergic anti-inflammatory pathway by treatment with nicotine and two α7 nAChR selective agonists in two mouse models of colitis. In dextran sodium sulphate (DSS)-induced colitis, our results show that nicotine does not affect disease severity. Both of the selective α7 nAChR agonists, AR-R17779 [(-)-spiro[1-azabicyclo[2.2.2] octane-3,5′-oxazolidin-2′-one] and GSK1345038A, affected disease severity in a bell-shaped response curve; low doses aggravated the disease, while high doses ameliorated the disease. In 2,4,6-trinitrobenzene sulphonic acid (TNBS) colitis, treatment with GSK1345038A was ineffective. These data have important repercussions on the therapeutic potential of selective α7 nAChR agonists in colitis.

Methods

Animals

Female C57BL/6 mice (8–10 weeks old and weighing 20–25 g; Charles River, Maastricht, the Netherlands) were housed and maintained under standard conditions at our animal facility. Food and water were available *ad libitum*. All animal experiments were performed according to the guidelines of the Animal Research Ethics Committee of the University of Amsterdam.

Induction of colitis

To induce DSS colitis, 1.5% (w/v) DSS (TdB Consultancy, Uppsala, Sweden) was administered in the drinking water of the mice for 7 days. Body weight was recorded daily, and weight loss on day 7 as compared with day 0 was calculated. Animals were killed on day 7 of DSS administration. Hapten-induced colitis was induced by rectal administration of one dose of 2 mg TNBS (Sigma Chemical Co, St Louis, MO, USA) in 40% ethanol (Merck, Darmstadt, Germany), using a vinyl catheter that was positioned 3 cm from the anus. During the instillation of the catheter, the mice were anaesthetized with isoflurane (1-chloro-2,2,2- trifluoroethyl-isoflurane-difluoromethyl-ether; Abbott Laboratories Ltd., Queenborough, Kent, UK), and after the instillation mice were kept vertically for 30 s. Five days after TNBS instillation, mice were killed. Mice received a daily i.p. injection of nicotine (0.04 or 0.4 $mg \cdot kg^{-1}$, or 0.25 or 2.5 $\mu mol \cdot kg^{-1}$ resp.) (Sigma-Aldrich, Zwijndrecht, the Netherlands), AR-R17779 (0.1, 0.3, 1, 3 or 5 $mg \cdot kg^{-1}$; or 0.6–30 $\mu mol \cdot kg^{-1}$) (kindly provided by Critical Therapeutics, Lexington, MA, USA) or GSK1345038A (3, 10, 30 or 60 $mg \cdot kg^{-1}$or 6–120 $\mu mol \cdot kg^{-1}$) (kindly provided by Glaxo SmithKline, Stevenage, UK) in 1% methylcellulose. The treatment with the agonists was started at the first day of DSS administration.

GSK1345038A pharmacokinetics

GSK1345038A, 60 or 120 $\mu mol \cdot kg^{-1}$, was administered i.p. to C57Bl/6 mice ($n = 4$), and blood samples were taken at time points 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8 and 12 h. Blood samples were analysed for the free base of GSK1345038A using a method based on protein precipitation and HPLC-MS/MS analysis. Acetonitrile: ammonium acetate (10 mM) (8:2, 250 μL) containing an appropriate internal standard was added to the samples of blood (50 μL diluted with 50 μL of water). Samples were mixed thoroughly (mechanical shaking for 20 min), and then centrifuged (2465× *g* for 15 min at room temperature). An aliquot of the resulting supernatant was analysed for GSK1345038A by reverse-phase HPLC-MS/MS using a heat-assisted electrospray interface in positive ion mode (Sciex API 4000) and a ACE-3 C18 column (50 × 4.6 mM ID, 3 uM; Hichrom). The mobile phase was delivered as a linear gradient of 20% to 95% acetonitrile : ammonium acetate (1 mM containing 0.1%v/v formic acid) over 0.8 min. The final composition was held for 0.8 min before return to initial composition. Nominal MRM transition for GSK1345038A was 454–123. Concentration range for the assay was 0.011–22.0 μM with a lower limit of quantification (LLQ) of 0.011 μM.

Assessment of colitis
Faecal blood, diarrhoea and disease activity index (DAI) were scored as described in Table 1. The wet weight of each colon was recorded and used as an index of disease-related intestinal wall thickening. The total length of the colon was measured, and colon shortening as a consequence of DSS-induced colitis was used as a disease parameter. Subsequently, the colons were separated from mesentery and fat and longitudinally divided into two parts for histological examination and measurement of cytokines.

Histological examination
The longitudinally divided colons were fixed in 4% formalin and embedded in paraffin for routine histology. An experienced pathologist microscopically evaluated formalin-fixed haematoxylin tissue sections in a blinded fashion. Rolled colon was evaluated, and graded from 0 to 26 points as an indicator of incidence and severity of inflammatory lesions based on the extent of the area involved, the number of follicle aggregates, oedema, fibrosis, hyperplasia, erosion/ulceration, crypt loss and infiltration of granulocytes and mononuclear cells (Table 2).

Colonic cytokine production
For cytokine measurements, colons were diluted 1:9 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1% Triton X-100, pepstatin A, leupeptin and aprotinin (all 20 ng·mL^{-1}; pH 7.4) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500× *g* at 4°C for 15 min, and supernatants were stored at –20°C until analysed. TNF, IL-6 and IL-17 in supernatants were analysed by mouse ELISA Duoset kits (R&D Systems, Minneapolis, MN, USA). Assays were performed according to the manufacturer's instructions.

Table 1 Scoring of the disease activity index (DAI)

a Weight loss	*b Stool consistency*	*c Bleeding*
0: <1%	0: normal	0: negative
1: 1–5%	2: loose stools	2: positive
2: 5–10%	4: diarrhea	4: gross bleeding
3: 10–15%		
4: >15%		

To determine the DAI, scores for a, b and c were combined and divided by three. Bodyweight loss was calculated as the percentage difference between the body weight on day 0 and the body weight on day 7 of the experiment. The appearance of diarrhoea was defined as mucus/faecal material adherent to anal fur (Cooper *et al.*, 1993).

Whole blood stimulation assays
Whole blood was taken via heart punction following anaesthesia. Aliquots of 50 µL were divided onto round-bottomed 96-well plates and treated with appropriate concentrations of nicotinic agonists diluted in 50 uL of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco-BRL, Breda, the Netherlands), 2 mM L-glutamine, 1000 U·mL^{-1} penicillin, 1000 µg·mL^{-1} streptomycin, 250 ng·mL^{-1} amphotericin B (Gibco) for 15 min at 37°C. Subsequently, heat-killed *Escherichia coli* (1×10^4 per well) or lipopolysaccharide (LPS) (Sigma Chemical Co) at a final concentration of 100 ng·mL^{-1} was added to the wells. After 3 h of stimulation, plates were centrifuged, supernatants were collected, and levels of TNF, IL-6 and IL-17 were analysed by ELISA (R&D Systems).

NF-κB activity assay
Immortalized peritoneal macrophages RAW264.7 were stably transfected with a NF-κB luciferase reporter construct (Clontech, Mountain View, CA, USA) in which a PDNA3.1(+)-derived neomycin resistance TK cassette was inserted (referred to as pNF-κB^{neo}-luc). Transfection was performed using Nucleofactor V (Lonza, Cologne, Germany). Briefly, 0.5 µg per 10^6 cells of constructs pNF-κB^{neo}-luc was suspended in 75 µL of 150 mM sterile NaCl solution. The transfection was allowed to proceed for 16 h, and the medium refreshed. Twenty-four hours after transfection, neomycin resistant clones were selected and subcloned. For assay, cells were pretreated with nicotinic agonists at the concentration indicated for 20 min, washed and subsequently stimulated with LPS (100 ng·mL^{-1}; Sigma) for 6 h. After treatment, the medium was removed; the cells washed three times with ice-cold PBS and cells lysed with Passive Lysis Buffer supplied in the Luciferase™ Reporter Assay Kit (Promega Corporation, Madison, WI, USA) and the lysate was assayed for luciferase activity according to the manufacturer's instructions.

Statistics
The values of the clinical parameters of the groups without DSS and TNBS groups treated with nicotine and α7 nAChR

Table 2 Histopathology scoring

Score	*0*	*1*	*2*	*3*	*4*
Area involved	0%	<10%	10–25%	25–50%	≥50%
Follicles	Normal (0–1)	Little (2–3)	Moderate (4–5)	Extensive (>6)	
Oedema	Absent	Little	Moderate	Extensive	
Erosion/ulceration	0%	<10%	10–25%	25–50%	≥50%
Fibrosis	Absent	Little	Moderate	Extensive	
Hyperplasia	0%	<10%	10–25%	25–50%	≥50%
Crypt loss	0%	<10%	10–25%	25–50%	≥50%
Granulocytes	Normal	Few	Moderate	Extensive	
Mononuclear cells	Normal	Few	Moderate	Extensive	

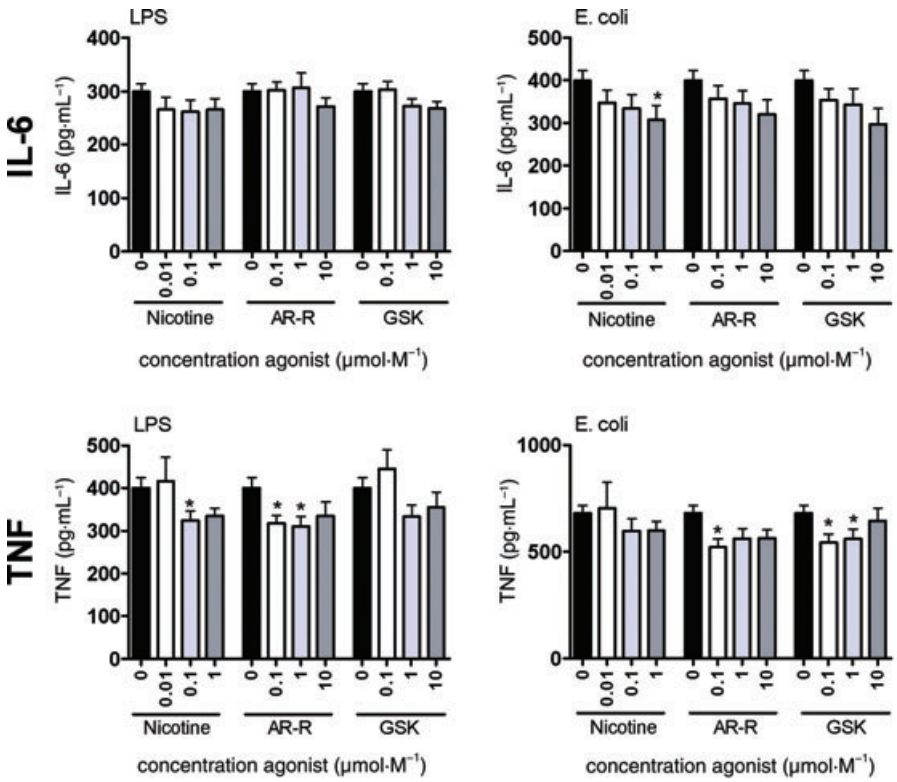

Figure 1 Cytokine production in mouse whole blood induced by lipopolysaccharide (LPS) and *Escherichia coli*. Whole blood from healthy female C57BL/6 mice was stimulated *ex vivo* with nicotine (0–1 μmol·M^{-1}), AR-R17779 [(-)-spiro[1-azabicyclo[2.2.2] octane-3,5′-oxazolidin-2′-one] (0–10 μmol·M^{-1}), GSK1345038A (0–10 μmol·M^{-1}) and subsequently incubated with LPS (100 ng·mL^{-1}) (left panels) or *E. coli*. IL-6 (upper panels) and TNF (lower panels) values are indicated as compared with vehicle. Data represent three independent experiments. Asterisks indicate significant differences (*$P < 0.05$); columns indicate mean ± SEM.

agonists are relative values (%) as compared with the DSS and TNBS groups treated with vehicle. Differences between groups were analysed using the nonparametric Mann–Whitney *U*-test. $P < 0.05$ was considered significant. All analyses were performed using SPSS (SPSS Inc. Chicago, IL, USA)

Nomenclature

All drug/molecular target are termed in accordance with *BJP*'s Guide to Receptors and Channels (Alexander *et al*., 2008).

Results

Macrophage activation was modulated by nicotine and the α7 nAChR agonists GSK1345038A and AR-R17779

Initially, we reproduced experiments showing that nicotine, AR-R17779 (de Jonge *et al*., 2005; The *et al*., 2007) and GSK1345038A reduce TNF and IL-6 release *in vitro* in Biogel-elicited peritoneal macrophages stimulated with heat-killed *E. coli* or LPS in a 0–10 μM concentration range (The *et al*., 2007) (data not shown). In line with these previous observations, AR-R17779 and GSK1345038A, as selective α7 nAChR agonists, were less potent in reducing peritoneal macrophage cytokine release as compared with nicotine (The *et al*., 2007) (data not shown). In whole blood cell preparations (Figure 1), nicotine or the α7 nAChR agonists, AR-R17779 and GSK1345038A, significantly reduced IL-6 and TNF production in response to activation with LPS- or heat-killed *E. coli*, albeit the potency to reduce cytokine production was less pronounced. None of the three agonists significantly reduced IL-6 production after stimulation with LPS (Figure 1). The values for these inflammatory substances in unstimulated cells were below levels of detection (data not shown).

The potential of nicotinic agonists to reduce cytokine production has previously been associated with inhibition of NF-κB activity (Ulloa, 2005; The *et al*., 2007). We explored the ability of GSK1345038A and AR-R17779 to reduce NF-κB transcriptional activity in activated macrophages. To this end, we investigated the effects of nicotine, AR-R17779 and GSK1345038A on NF-κB activation induced by LPS in a reporter assay using the macrophage cell line RAW264.7, which was stably transfected with a NF-κB reporter construct. As shown in Figure 2, LPS induced NF-κB transcriptional activity that was significantly reduced by nicotine, AR-R17779, as well as GSK1345038A.

Treatment with nicotine does not affect clinical parameters in DSS-induced colitis

Given the reported potential of the vagus nerve to reduce disease in various mice models, including colitis (Ghia *et al*., 2006; 2007) and the positive association between smoking and the course of UC (Birrenbach and Bocker, 2004), we next tested whether treatment with nicotine affected the disease

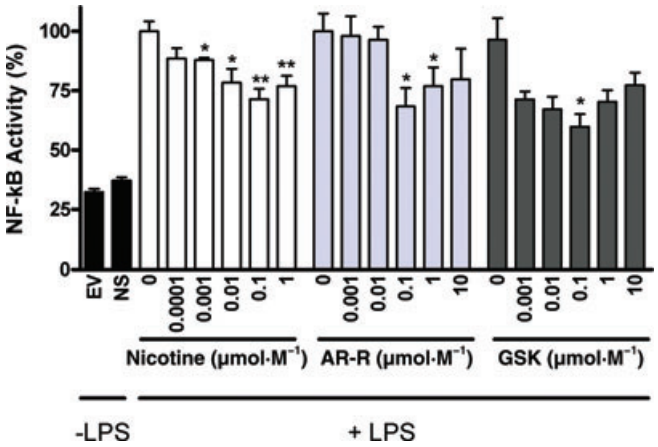

Figure 2 Effect of nicotine, AR-R17779 [(-)-spiro[1-azabicyclo[2.2.2] octane-3,5′-oxazolidin-2′-one] and GSK1345038A on NF-κB activation in RAW264.7 cells. RAW264.7 cells stably transfected with NF-κB luciferase reporter constructs were pretreated with different concentrations of nicotine (0–1 μmol·M^{-1}), AR-R17779 (0–10 μmol·M^{-1}) or GSK1345038A (0–10 μmol·M^{-1}) and stimulated with lipopolysaccharide (LPS) (100 ng·mL^{-1}). Values are relative as compared with vehicle. Data represent three independent experiments. Asterisks indicate significant differences (*$P < 0.05$; **$P < 0.01$) as compared with vehicle. Columns represent mean ± SEM. EV, empty vector; NS, not stimulated.

course of DSS-induced colitis. Daily treatment with nicotine did not alter weight loss (Figure 3A) or DAI (Figure 3B) as compared with the vehicle-treated group. Only colon weight, which represents thickening of the colon by oedema, was significantly reduced by treatment with both 0.25 and 2.5 μmol·kg^{-1} nicotine (Figure 3C) but colon shortening was not affected by nicotine administration (Figure 3D). To test the effect of nicotine on intestinal inflammation we measured the production of TNF, IL-6 and IL-17 in colon homogenates. Although TNF levels were not altered, colonic IL-6 and IL-17 levels were significantly reduced by nicotine treatment (Figure 3). However, this reduced cytokine production was not reflected in a decreased histopathology score (Table 3).

Treatment with α7 nAChR agonists GSK1345038A and AR-R17779 worsened the clinical parameters of colitis

We next determined whether the reason nicotine treatment failed to ameliorate disease in DSS-induced colitis is because nicotine does not selectively target the α7 nAChR. In separate experiments, we therefore tested the efficacy of AR-R17779 and GSK1345038A in DSS-induced colitis. A dose of 6 μmol·kg^{-1} of AR-R17779 results in a C_{max} of 4.6 μM and a half life of approximately 150 min (G. LaRosa, pers. comm.), and should thus reach the effective concentration range to reduce cytokine release in macrophages (The *et al*., 2007) and whole blood (Figures 1 and 2). Hence, we administered AR-R17779 at doses of 1.8–30 μmol·kg^{-1} daily. Daily i.p. injection with 1.8, 6 and 18 μmol·kg^{-1} of AR-R17779 aggravated weight loss (Figure 4A). In contrast, in the group treated with a highest dose of AR-R17779 (30 μmol·kg^{-1}) weight loss was prevented (Figure 4A).

To confirm these data, we next tested another selective α7 nAChR agonist, GSK1345038A in the same model of DSS-induced colitis. Similar to AR-R17779, we first assessed the optimal dosage range for GSK1345038A by measurement of the blood concentration of GSK1345038A. The pharmacokinetics indicated that GSK1345038A has a half live of 2–3 h, and reaches blood concentrations of 5–25 μM in a dosage range of 60–120 μmol·kg^{-1} mouse (Figure 5), that is, the effective dose range to reduce cytokine release in our *in vitro* assays (Figures 1 and 2). Hence, to reach optimal circulation levels *in vivo*, we administered doses of 6, 20, 60 and 120 μmol·kg^{-1} i.p. daily. In line with the results obtained using AR-R17779, weight loss was significantly enhanced by daily injection of 6, 20 or 60 μmol·kg^{-1} GSK1345038A (Figure 4A). In accord with the effect of the highest dose of AR-R17779 on the course of the colitis, weight loss was prevented by daily treatment with the highest dose of GSK1345038A (120 μmol·kg^{-1}) tested (103.1 ± 1.9% relative to vehicle group) (Figure 4A).

The effects of both α7 nAChR agonists on the DAI paralleled those of the effects on weight loss as treatment with AR-R17779 significantly enhanced DAI (Figure 4B). In contrast, DAI was significantly reduced after treatment with the highest dose (30 μmol·kg^{-1}) of AR-R17779 (Figure 4B). Similar results were obtained on treatment with GSK1345038A in that it significantly worsened the disease, as reflected in DAI (Figure 4B), except with the highest dose of 120 μmol·kg^{-1} GSK1345038A, that ameliorated DAI compared with the vehicle control (Figure 4B). In contrast to nicotine treatment, the increase in colon weight was unaffected by either of the α7 nAChR agonists (Figure 4C), while the DSS-induced decrease in colon length was further reduced by AR-R17779 and treatment with GSK1345038A was ineffective (Figure 4D).

The effect of the α7 nAChR agonists GSK1345038A and AR-R17779 on colonic inflammation in DSS-induced colitis

We next measured the effect of the α7 nAChR agonists on colonic cytokine production after 7 days of DSS administration. In line with the augmented disease outcome, nicotine treatment (Figure 3E), but neither of the α7 nAChR agonists reduced colonic TNF and IL-6 levels (Figure 6). In contrast, colonic TNF, IL-6 and IL-17 levels were significantly elevated after treatment with AR-R17779, but not GSK1345038A (Figure 6).

In addition, histopathology scores were assessed for the doses of the α7 nAChR agonists with most pronounced effects on disease severity. As indicated in Table 4, total histopathology scores after treatment with 30 μmol·kg^{-1} AR-R17779 and 120 μmol·kg^{-1} GSK1345038A did not parallel clinical scores as there was no significant difference between groups. Similar effects were observed after administration of lower doses of AR-R17779 (1.8 μmol·kg^{-1}) and GSK1345038A (60 μmol·kg^{-1}); clinical outcome was poorer, but total histopathology scores were not significantly different from vehicle controls, except for crypt loss, which was significantly worsened after treatment with 1.8 μmol·kg^{-1} AR-R17779 or 60 μmol·kg^{-1} GSK1345038A (Table 4).

The effect of the α7 nAChR agonist GSK1345038A in a mouse model of acute TNBS colitis

To investigate whether the effects of α7 nAChR agonists on colitis were specific for the acute DSS colitis model, we

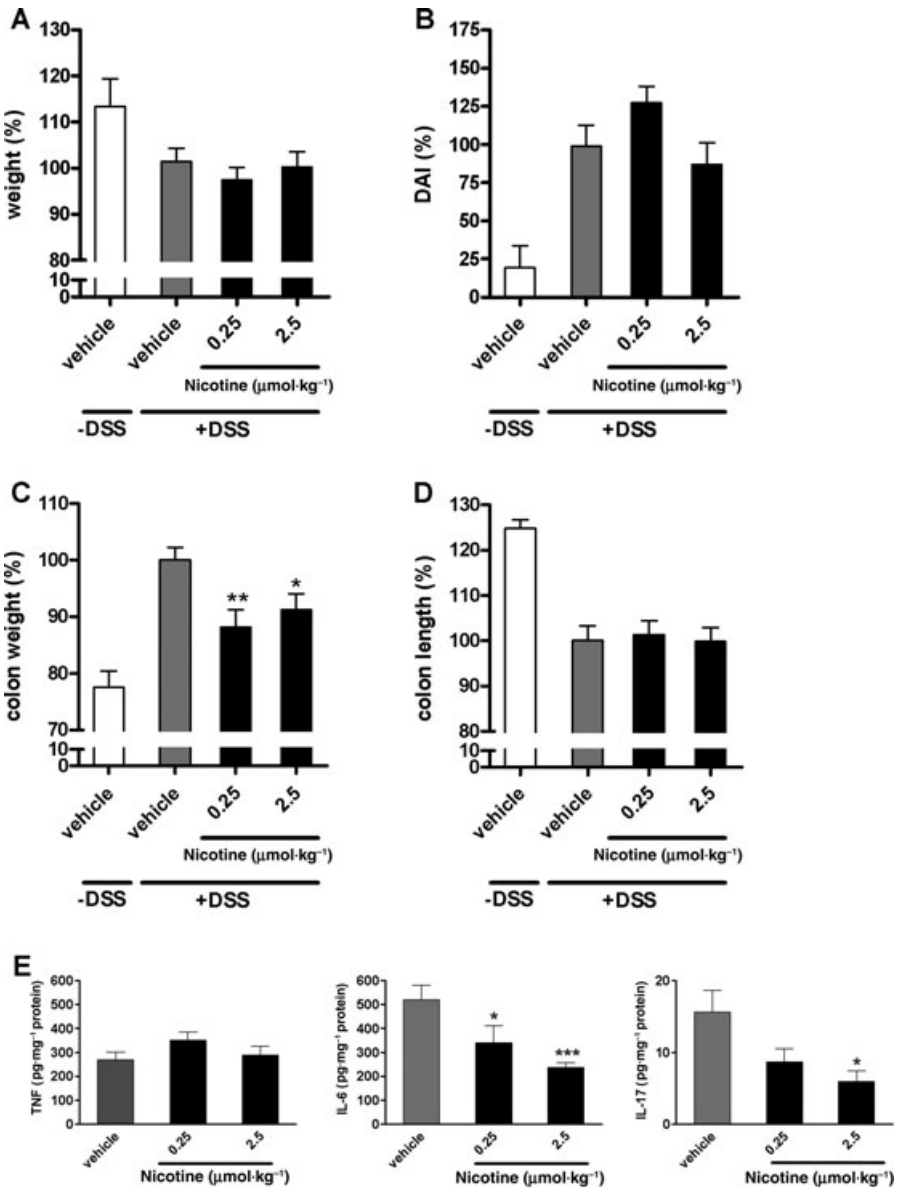

Figure 3 Effects of nicotine on dextran sodium sulphate (DSS)-induced colitis. (A) %Body weight on day 7 as compared with body weight on day 0 of the experiment. (B) Disease activity index (DAI) as described in *Methods*. (C) Colon weight per centimetre colon. (D) Colon length. (E) TNF, IL-6 and IL-17 levels in colon homogenates. Data are expressed as % of those values in mice receiving DSS and treated with vehicle. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) as compared with DSS group treated with vehicle; $n = 10$ per group. Columns indicate mean ± SEM.

tested the effect of GSK1345038A in another acute model of colitis, TNBS-induced colitis. The main indicator for this model is colonic inflammation and not clinical parameters, as the mice are allowed to recover after one dose of TNBS. As indicated in Figure 7A, weight loss 5 days after instillation of TNBS was not significantly different between groups treated with GSK1345038A and vehicle. In addition, histopathology scores were not significantly altered by treatment with 60 or 120 μmol·kg^{-1} GSK1345038A (Table 5). GSK1345038A treatment did not alter colonic production of TNF and IL-17, while IL-6 production was below levels of detection (Figure 7B).

Discussion

Inflammatory bowel disease patients suffer from chronic and relapsing intestinal inflammation, initiated by aberrant responses of the innate immune system (Bouma and Strober, 2003; Xavier and Podolsky, 2007). Recently, a number of animal studies have demonstrated that innate immune responses are attenuated by stimulation of the efferent arm of the vagus nerve through its neurotransmitter ACh, that acts on nAChRs, in particular the α7 nAChR, on resident macrophages (Borovikova *et al.*, 2000; Wang *et al.*, 2003). In various mouse models of inflammatory disease, we (de Jonge

et al., 2005; The *et al*., 2007; van Maanen *et al*., 2009) and others (Wang *et al*., 2003; 2004) observed anti-inflammatory effects of vagus nerve stimulation, as well as pharmacological stimulation of the cholinergic system by administration of nicotine and α7 nAChR agonists. In the current study, we aimed to extend these findings by treating experimental colitis through targeting α7 nAChRs with nicotine, and two selective α7 nAChR agonists AR-R17779 and GSK1345038A. The agonists were tested in two mouse models of acute colitis induced by DSS or TNBS. *In vitro*, nicotine reduces macrophage NF-κB activity and cytokine release significantly. In addition, treatment of DSS-induced colitis with nicotine leads to a significant reduction in colonic oedema and colonic IL-6 and IL-17 production. However, this reduction was not marked enough to be reflected in clinical parameters and histopathology scores. The histopathology scores are the end point of the inflammatory reaction and contribute greatly to the functionality of the colon and thus have a large influence on clinical outcome. In addition, reduced IL-17 levels do not strictly imply reduced disease activity and, recently, data have been obtained indicating that IL-17 might act as an anti-inflammatory cytokine in the gut (Ogawa *et al*., 2004).

Ulcerative colitis patients with a history of smoking usually acquire their disease after they have stopped smoking (Pullan *et al*., 1994; Thomas *et al*., 1995; Ingram *et al*., 2004). Patients who smoke intermittently often experience an improvement in their colitis symptoms during the periods when they smoke (Pullan *et al*., 1994; Sandborn, 1999; Birrenbach and Bocker, 2004). Following this reasoning and given the previous reports on the positive effect of cholinergic activation in experimental models of DSS colitis (Ghia *et al*., 2006; Ghia *et al*., 2007), nicotine treatment may well be beneficial in UC. Indeed, in patient studies treatment with transdermal nicotine was effective at inducing disease remission in UC patients

Table 3 The effect of nicotine on colonic inflammation in dextran sodium sulphate-induced colitis

	Nicotine (μmol·kg^{-1})		
	Vehicle	*0.25*	*2.5*
Area involved	2.8 ± 0.30	2.9 ± 0.32	2.8 ± 0.40
Follicles	1.5 ± 0.55	1.5 ± 0.41	0.6 ± 0.22
Oedema	0.6 ± 0.20	0.7 ± 0.20	0.8 ± 0.21
Erosion/ulceration	1.0 ± 0.10	1.3 ± 0.19	1.0 ± 0.18
Crypt loss	2.4 ± 0.33	2.6 ± 0.35	2.5 ± 0.42
Granulocytes	1.4 ± 0.12	1.3 ± 0.19	1.4 ± 0.33
Monocytes	1.8 ± 0.19	1.4 ± 0.12	1.5 ± 0.18
Total score	11.6 ± 1.69	11.7 ± 1.58	10.5 ± 1.94

C57BL/6 mice were administered 1.5% dextran sodium sulphate in drinking water and killed at day 7. H&E stainings were performed on whole colons including rectum from groups treated with vehicle, 0.25 and 2.5 μmol·kg^{-1} nicotine and scored by an experienced pathologist. H&E stainings of colon and rectum showed inflammatory features including crypt damage, follicles, oedema, ulceration and influx of inflammatory cells. Mice per group: $n = 10$. Data represent mean ± SEM.

Table 5 The effect of α7 agonists AR-R17779 and GSK1345038A on colonic inflammation in TNBS-induced colitis

	GSK1345038A (μmol·kg^{-1})		
	Vehicle	*60*	*120*
Area involved	3.7 ± 0.19	3.7 ± 0.18	2.9 ± 0.26
Follicles	1.0 ± 0.24	1.1 ± 0.40	0.4 ± 0.20
Oedema	0	0.2 ± 0.14	0.3 ± 0.10
Erosion/ulceration	0.4 ± 0.19	0.6 ± 0.32	0.7 ± 0.36
fibrosis	0.7 ± 0.20	0.6 ± 0.30	0.7 ± 0.21
Hyperplasia	3.7 ± 0.20	2.6 ± 0.69	3.1 ± 0.34
Crypt loss	0.8 ± 0.15	1.4 ± 0.48	0.5 ± 0.20
Granulocytes	0	0.6 ± 0.17*	0.3 ± 0.14
Monocytes	0.8 ± 0.15	1.1 ± 0.13	0.9 ± 0.20
Total score	11.1 ± 1.3	11.9 ± 2.8	9.8 ± 2.0

Mice ($n = 7$ per group) received one dose of 2 mg TNBS in 30% ethanol intrarectally and were killed after 5 days. Vehicle, 60 or 120 μmol·kg^{-1} GSK1345038A was injected daily. H&E stainings were performed on whole colons including rectum and scored by an experienced pathologist. H&E stainings of colon and rectum showed inflammatory features including crypt damage, follicels, oedema, ulceration, hyperplasia and influx of inflammatory cells. Asterisks indicate significant differences (*$P < 0.05$) as compared with vehicle. Data represent mean ± SEM.
AR-R17779, (-)-spiro[1-azabicyclo[2.2.2] octane-3,5′-oxazolidin-2′-one; TNBS, 2,4,6-trinitrobenzene sulphonic acid.

Table 4 The effect of α7 agonists AR-R17779 and GSK1345038A on colonic inflammation in DSS-induced colitis

	AR-R17779 (μmol·kg^{-1})			*GSK1345038A (μmol·kg^{-1})*		
	Vehicle	*1.8*	*30*	*Vehicle*	*60*	*120*
Area involved	2.9 ± 0.10	2.6 ± 0.53	2.9 ± 0.1	3.6 ± 0.15	3.8 ± 0.15	3.0 ± 0.24
Follicles	1.1 ± 0.16	1.3 ± 0.45	0.8 ± 0.21	0.8 ± 0.24	0.3 ± 0.17	0.5 ± 0.16
Oedema	1.5 ± 0.33	1.4 ± 0.27	1.3 ± 0.14	1.7 ± 0.16	1.9 ± 0.15	1.4 ± 0.15
Erosion/ulceration	1.8 ± 0.36	1.4 ± 0.22	1.9 ± 0.11	0.9 ± 0.22	1.3 ± 0.24	1.5 ± 0.21
Crypt loss	2.0 ± 0.15	2.3 ± 0.09*	2.1 ± 0.99	1.6 ± 0.15	2.4 ± 0.24*	2.2 ± 0.19
Granulocytes	1.1 ± 0.06	0.8 ± 0.13	1.2 ± 0.07	1.6 ± 0.18	1.2 ± 0.17	1.1 ± 0.17
Monocytes	0.7 ± 0.11	0.9 ± 0.10	0.8 ± 0.12	1.3 ± 0.15	1.7 ± 0.14	1.6 ± 0.15
Total score	11.1 ± 0.6	10.7 ± 2.2	11.0 ± 0.3	11.4 ± 1.25	12.6 ± 1.26	11.3 ± 0.8

C57BL/6 mice were administered 1.5% DSS in drinking water and killed at day 7. H&E stainings were performed on whole colons including rectum from groups treated with vehicle, 1.8 and 30 μmol·kg^{-1} AR-R17779 or 60 and 90 μmol·kg^{-1} GSK1345038A and scored by an experienced pathologist. H&E stainings of colon and rectum showed inflammatory features including crypt damage, follicles, oedema, ulceration and influx of inflammatory cells. Asterisks indicate significant differences (*$P < 0.05$) as compared with vehicle. Mice per group: vehicle: $n = 18$; 1.8 μmol·kg^{-1} AR-R17779 and 60 μmol·kg^{-1} GSK1345038A $n = 10$; 120 μmol·kg^{-1} GSK1345038A: $n = 18$. Data represent mean ± SEM.
AR-R17779, (-)-spiro[1-azabicyclo[2.2.2] octane-3,5′-oxazolidin-2′-one; DSS, dextran sodium sulphate.

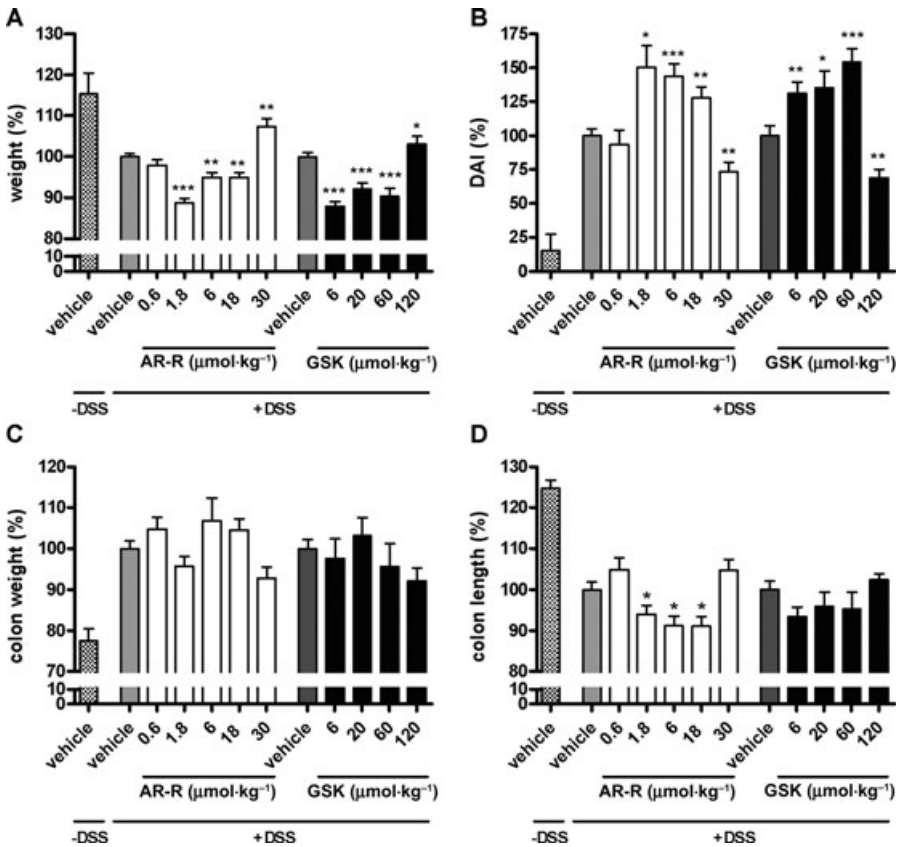

Figure 4 Effects of the α7 nicotinic acetylcholine receptor (nAChR) agonists AR-R17779 [(-)-spiro[1-azabicyclo[2.2.2] octane-3,5′-oxazolidin-2′-one] and GSK1345038A on dextran sodium sulphate (DSS)-induced colitis. (A) %Body weight on day 7 as compared with body weight on day 0 of the experiment. (B) Disease activity index (DAI) as described in *Methods*. (C) Colon weight per centimetre colon. (D) Colon length. Data are expressed as % of those values in DSS group treated with vehicle. Asterisks indicate significant difference (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) as compared with DSS group treated with vehicle. Mice per group: 0.6, 1.8, 6 and 18 μmol·kg^{-1} AR-R17779 and 6, 20 and 60 μmol·kg^{-1} GSK1345038A, $n = 10$; vehicle groups and 120 μmol·kg^{-1} GSK1345038A, $n = 18$. Columns indicate mean ± SEM.

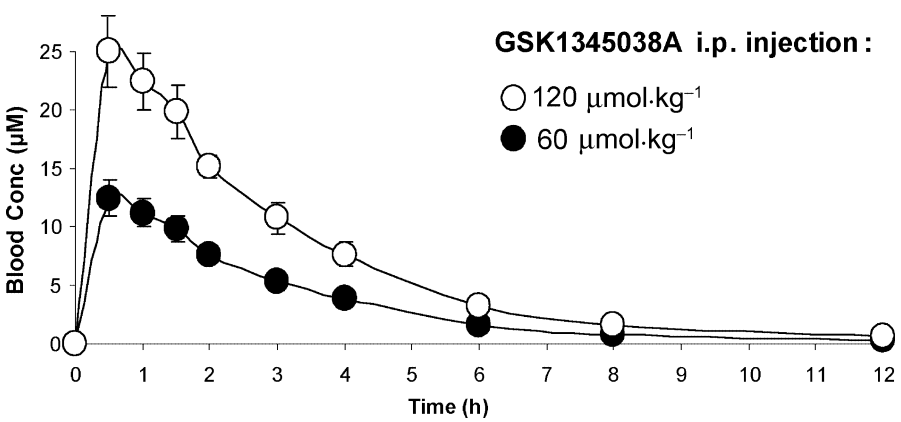

Figure 5 The time course for the concentrations of GSK1345038A in the blood. GSK1345038A, 60 or 120 μmol·kg^{-1}, was administered i.p. to C57Bl/6 mice and the concentrations of GSK1345038A in the blood were measured at the indicated time points. Data shown are the mean ± SEM of triplicate measurements from four mice.

(McGrath *et al*., 2004), but the number of patients that suffered from adverse effects was significantly higher in the nicotine-treated groups (McGrath *et al*., 2004) as compared with patients treated with standard therapy. It should be noted that smoking in Crohn's disease patients worsens the disease.

In the current study, besides nicotine, we tested the effects of two selective α7 nAChR agonists, AR-R17779 (Mullen *et al*., 2000) and GSK1345038A, in the mouse model of DSS and induced colitis. Treatment with both of the α7 nAChR agonists induced a bell-shaped dose–response curve; the highest doses of AR-R17779 and GSK1345038A significantly amelio-

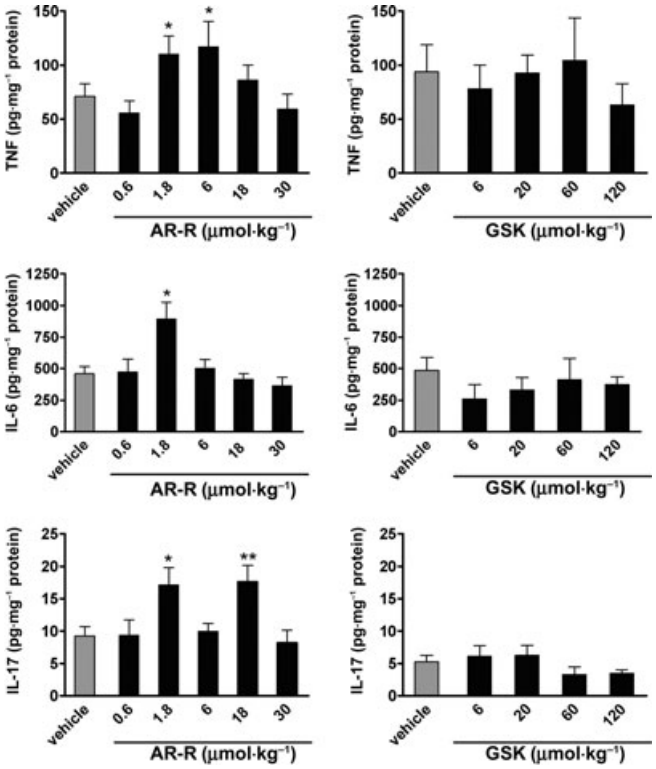

Figure 6 TNF, IL-6 and IL-17 production in the colon. The effects of treatment with AR-R17779 [(-)-spiro[1-azabicyclo[2.2.2] octane-3,5′-oxazolidin-2′-one] and GSK1345038A at the indicated doses on colonic cytokine production. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) as compared with vehicle. Agonist-treated groups: $n = 10$, vehicle groups and 120 μmol·kg^{-1} GSK1345038A, $n = 18$. Data are expressed as the mean ± SEM.

rated clinical parameters, whereas lower doses of both compounds worsened or did not affect clinical parameters. The highest doses used ameliorated clinical outcome but did not affect inflammatory parameters. Although our data confirm the capacity of AR-R17779 and GSK1345038A to reduce pro-inflammatory mediator release *in vitro* in macrophage cultures (The *et al*., 2007) and whole blood, the reduction in cytokines by nicotine as well as both α7 agonists was around 20–40%, which proved not to affect disease outcome in the colitis models used in this study.

However, it is possible that at the highest dose the α7 nAChR agonists might exhibit off-target activity and lose their selectivity for the α7 nAChR, thereby affecting disease in an α7 nAChR-independent manner. This is also indicated by the marked dose–response relationship observed between colonic IL-17 levels and AR-R17779 treatment, which may be explained by concentration-dependent off-target activity of AR-R17779. It should be borne in mind that nAChRs are expressed peripherally as well as centrally and that activation of nAChR on neurones can have analgesic effects, or modify mucus production, gut motility and blood flow to the gut (Birrenbach and Bocker, 2004; Thomas *et al*., 2005). In the DSS colitis model, these effects might control food intake and formation of stools thereby influencing disease activity parameters independently of the severity of colonic inflammation. Another effect of nAChR activation can be a change in muscle tone thereby reducing colon length. This might play a role in the significant reduction of colon length we observed on treatment with AR-R17779.

In addition, activation of nAChRs plays a role in regulating epithelial permeability (Thomas *et al*., 2005; McGilligan *et al*., 2007) and bacterial clearance (van Westerloo *et al*., 2005; van der Zanden *et al*., 2009b), important factors in the development of colitis that were not assessed in our experiments. Thus, nAChR activation can have a variety of effects on disease, independent of immune mediation, because of its widespread expression on different cell types as well as on different tissue types.

Although we report here that treatment with nicotine, or selective α7 nAChR agonists, is not effective in experimental colitis, enhanced vagus nerve output has been shown to reduce inflammation in various mouse models (Borovikova *et al*., 2000; Luyer *et al*., 2005; van Westerloo *et al*., 2005; de Jonge and Ulloa, 2007; The *et al*., 2007; van der Zanden *et al*., 2009a). This cholinergic anti-inflammatory effect seems to rely on the expression of the α7 nAChR on innate immune cells (Borovikova *et al*., 2000; Wang *et al*., 2003). Reciprocally, in mouse models of colitis, it has been shown that vagotomy worsens colitis, an effect that was shown to be counteracted by nicotine administration (Ghia *et al*., 2006; 2007; O'Mahony *et al*., 2009). Of note in the interpretation of these studies is that the vagus nerve only marginally innervates the distal colon, making direct effects of ACh on colonic immune cells unlikely. The vagus nerve probably relays its immune modulatory effects to the colon in an indirect fashion, that is, via postganglionic activation or by targeting alternative cell types. Of interest in this respect is a more recent study in which vagotomy was shown to worsen DSS colitis due to an impaired potential of antigen presenting cells to induce regulatory T-cells (O'Mahony *et al*., 2009). Notably, the physiological effects of vagus nerve stimulation or vagotomy as compared with pharmacological activation of ACh receptors differ greatly, especially when taking into account the changes in sympathetic output. In addition, vagus nerve stimulation or vagotomy will not only target nAChRs, but also influence the release of a number of neurotransmitters in the gut that regulate immune functions, and gut functions such as permeability and blood flow that possibly influence disease outcome.

Irrespectively, however, nicotine administration ameliorated disease in previous studies of experimental colitis (Sykes *et al*., 2000; Ghia *et al*., 2006). We cannot explain why the effectiveness of nicotine to reduce disease parameters was less pronounced in our study. The nicotine dose used in this study had been shown to be effective at reducing inflammation in other models of inflammation (The *et al*., 2007; van Maanen *et al*., 2009), but possibly, in our colitis experiments a higher dose is required. However, in mice, a higher dose would result in adverse effects because of activation of a broad range of receptors both peripherally and centrally (Matta *et al*., 2007). In addition, a large array of nAChRs subtypes are expressed (Wessler and Kirkpatrick, 2008), and previous studies point towards a role in modulation of intestinal inflammation for nAChRs containing α5 (Orr-Urtreger *et al*., 2005) or α4β2 (Kawashima *et al*., 2007). Thus, the nAChR subtype involved

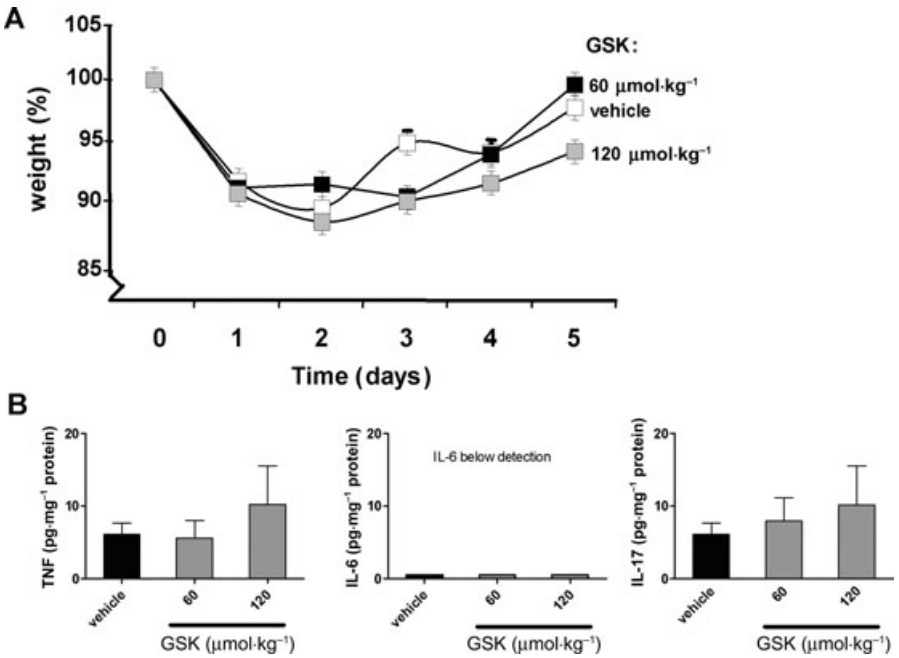

Figure 7 Effect of the α7 nicotinic acetylcholine receptor (nAChR) agonist GSK1345038A on 2,4,6-trinitrobenzene sulphonic acid-induced colitis. (A) Body weight is shown as a percentage of body weight on day 0 of the experiment. (B) Cytokine levels in colon homogenates. Columns indicate mean ± SEM, $n = 7$.

in the immunomodulatory properties of the vagus nerve remains to be established.

Alternatively, the outcome of animal experiments with nAChR agonists could be dependent on the model of inflammation studied, as expression of the nAChR might vary depending on tissues and cell types involved in disease development. Notably, in other studies, nicotine treatment worsened the course of jenunitis in rodent models (Eliakim *et al*., 2002) and TNBS colitis (Eliakim *et al*., 1998). There are notable differences among colitis models (Te Velde *et al*., 2007), which might be important in the effectiveness of the administered agents. Thus, the effects of nicotine and α7 nAChR agonists may depend on many experimental factors such as the dose used, administration method, disease severity and disease model.

We conclude that in developing a strategy for treating colitis using cholinoceptor agonists we should keep in mind that the expression of nAChRs is extremely widespread both centrally and peripherally. In addition, the expression of the various nAChRs subtypes on a particular target cell should be carefully investigated before evaluating the effectiveness of α7 nAChRs as a drug target in colitis patients.

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Conflict of interest

The authors state that there is no conflicting financial interest.

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