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Role of Nicotinic Acetylcholine Receptors in Experimental Colitis

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Role of Nicotinic Acetylcholine Receptors in Experimental Colitis

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

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References
LIST OF ABBREVIATIONS

CC            Choline chloride
IL             interleukin
α-RgIA        α-conotoxin RgIA
TNF-α          Tumor necrosis factor-alpha
DAI            disease activity index
AUC            area under the curve
IBD            inflammatory bowel disease
UC             Ulcerative Colitis
CD             Crohn’s disease
IFN-γ          interferon γ
LPS            lipopolysaccharide
STAT3          signal transducer and activator of transcription 3
TFG-β          transforming growth factor β
TNBS           trinitrobenzene sulphonic acid
ACh            acetylcholine
i.p.           intraperitoneal
KO             knockout
WT             wild-type
MLA            methyllycaconitine
MP             mini pump
nAChR          nicotinic acetylcholine receptor
NIC            nicotine
SEM            standard error of the mean
s.c.           subcutaneous
DSS            dextran sodium sulfate
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Abstract
Role of Nicotinic Acetylcholine Receptors in Experimental Colitis

By Shakir Dakheelallah Zayed AlSharari, B.S.Pharm
A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University, 2012

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Professor, Pharmacology and Toxicology

Substantial evidence in the literature shows that tobacco smoking has complex and divergent effects on inflammatory bowel diseases (IBD). It ameliorates ulcerative colitis (UC); whereas it aggravates the risk of Crohn’s disease (CD) and affects the disease course and severity. Studies have shown that nicotine has a positive influence on symptoms of UC. Also, it is demonstrated that nicotinic acetylcholine receptor, especially $\alpha_7$ subunit plays an essential component in the vagus nerve-based cholinergic anti-inflammatory effects. In the present study, we explored the effect of nicotine and $\alpha_7$ nicotinic agonists treatment in the DSS colitis mouse model. We also investigated the effects of cotinine, a major metabolite of nicotine, in the model. **Methods:**

Different groups of C57BL6 mice, as well as $\alpha_7$, $\alpha_5$, and $\beta_2$ nicotinic receptor knock out mice, and their littermates wild-type nicotinic receptor male adult mice were given DSS solution freely in the drinking water for 7 consecutive days after which tap water was given on the 8th day. We measured a Disease Activity Index (DAI) that includes body weight loss, blood presence in stools, stool consistency, local rectal irritation and length of the colon. The mice were then sacrificed on day 8 to allow examination of the entire colon. Disease severity and colon tissue histology and inflammatory markers including colonic myeloperoxidase (MPO) and colonic tumor necrosis factor-$\alpha$ (TNF-$\alpha$) were evaluated. Levels of MPO and TNF-$\alpha$ were determined by enzyme-linked immunosorbent assay analysis of the homogenized colon samples. The effect of oral, subcutaneous, mini pump nicotine, and oral cotinine treatments were examined on
experimental colitis induced by 2.5% DSS in mice. In addition, we measured the plasma levels of the nicotine and cotinine in our treatment protocols. **Results:** The DSS 2.5% model of colitis is easily induced in mice. Administration of low doses of oral nicotine (12.5 and 25 µg/ml), but not high doses in DSS-treated mice displayed a significant decrease in disease activity index value, total histological damage scores, as well as colonic level of TNF-α compared to the control group. However, the anti-inflammatory effect of nicotine was not seen with chronic s.c., mini pump nicotine or oral cotinine administration. Differences in plasma levels of nicotine and cotinine do not seem to account for this lack of effect. Moreover, neither nicotine nor cotinine reversed colon length shortening in DSS-treated mice, except with the 0.5 mg/kg s.c. dose of nicotine. There was no change in MPO activity among the groups treated with oral or s.c. nicotine. Cotinine oral administration on its own failed to show a significant effect in the DSS model of colitis. α7 KO mice displayed a significantly increased in DAI value starting from day 4 till day 8, histological damage scores and TNF-α levels were increased significantly compared to their littermate WT mice. Moreover, pretreatments with PHA-543613 (8 mg/kg), a selective α7 agonist, and choline chloride (40 ug/ml), an α7 nAChR natural agonist, significantly reduced clinical parameters in DSS-treated mice; however, they slightly inhibited the increase in the colonic TNF-α levels compared with vehicle DSS-treated mice. Moreover, PNU-120596 (3 mg/kg), a positive allosteric modulator for α7 nAChRs, significantly reduced DAI value and total histological damage score in DSS-treated mice. **Conclusion:** Results obtained from this study highlight that dose and route of administration play a critical role in the protective effect of nicotine in the DSS mouse colitis model. Also, these data suggest that α7 nAChR has a protective role in colitis with narrower therapeutic index. Data obtained from this study further
understanding of the effect of nicotine in UC and may contribute in the development of new pharmaceutical designs for targeting nAChRs for the treatment of ulcerative colitis.
CHAPTER ONE

General Introduction

1.1. Ulcerative Colitis: definition, clinical presentation, and diagnosis

Ulcerative colitis (UC) is one of the main two entities of inflammatory bowel diseases (IBD), beside Crohn’s disease (CD). Differences between these two typical IBD disorders are related to the disease nature and location as a result of abnormal immune response, various genetic and environmental factors (Baumgart and Carding, 2007; Strober et al., 2007). Interestingly, cigarette smoking has a dual opposite influence on these two diseases; it exacerbates CD, but ameliorates the course of UC disease (Tobin et al., 1987). For that reason, in my dissertation, I pay special attention to UC. I also discuss the influence of nicotine and the involvement of nicotinic receptor subtypes in UC. Ulcerative colitis is a chronic inflammatory disease with recurrent symptoms and significant morbidity of unknown etiology, due partly to genetic susceptibility to environmental factors that misdirect the mucosal immune system response to attack the epithelial layer of the colon causing inflammation and was first described by Sir Wilks (Wilks S, 1859). UC is characterized by inflammation of the mucosa and occasionally the submucosa of the colon and it is the most common form of inflammatory bowel disease worldwide that results in excessive mononuclear cell and neutrophil infiltration into mucosa and submucosa vicinities that affects primarily the distal colon and rectum (Hanauer, 1996). The incidence of UC in North America ranges from 2.2 to 14.3 cases per 100,000 (Loftus, 2004) and the adult prevalence of UC in the United States has ranged between 37 and 238 per 100,000 (Loftus, 2004; Kappelman et al., 2007) with age range between 15 and 30 years. Northern Europe and North America populations have the highest incidence and prevalence of IBD (Ahuja and Tandon, 2010).
1.2. Abnormalities in Immune Responses in Ulcerative Colitis

UC is associated with alterations of innate or adaptive arms of the immune response. The increased levels of IgM, IgA, and IgG are common in IBD, with an increase in IgG1 antibodies in UC (Takahashi and Das, 1985). In addition, cytokines are up-regulated at the mucosa of patients with UC, and play central components of the inflammatory pathways in ulcerative colitis. Abnormalities of adaptive immunity in the UC are defined by mucosal CD4+ lymphocytes, the T helper 1 (Th1) and T helper type 2 cells (Th2) paradigm. Immune responses have been classified based on the prominent cytokine profile in different disease conditions. Although this traditional concept of mucosal immunology considered ulcerative colitis mainly as a Th2-mediated response, due to the lack of IFN-γ expression increase rather than IL-4 elevation (Fuss et al., 1996), recent research focuses on the alternative Th2 responses such as role of IL-13 in UC. This approach indicates that there is a significant increase of IL-13 (Fuss et al., 2004, Heller et al., 2005). It has been reported that both of IL-13 receptors, IL-4Rα and IL-13Rα2, were expressed in intestinal epithelial cells and that IL-13 has an important role on epithelial barrier function (Heller et al., 2005) which indicates the involvement of IL-13 in UC signaling pathogenesis (Fuss and Strober, 2008). It has been proposed that colonocytes are the main players in the UC pathogenesis since the inflammation in UC does not reach into the small intestine (for review see, Danese and Fiocchi, 2011). The defects in UC epithelial include impaired epithelial barrier and reduced peroxisome proliferator activated receptor γ (PPAR-γ) expression (Dubuquoy et al., 2003). In fact, PPARγ ligands treatment has been reported to reduce colonic inflammation in animal colitis models (Su et al., 1999; Dubuquoy et al., 2006) and reduce the pro-inflammatory cytokine including IL-1β and TNF-α (Dubuquoy et al., 2006).
Clinical Presentations. The diagnosis of colitis is generally established by assessment of inflammatory markers followed by colonoscopy, with biopsy of pathological lesions to determine specific histologic characteristics. The hallmark symptoms of UC are intermittent bloody diarrhea, rectal urgency, and tenesmus (straining at stool) (Kornbluth and Sachar, 2004). The ultimate diagnosis relies on a combination of history, endoscopic finding, histologic features. Patients typically present with bloody diarrhea and passage of pus and/or mucus that often persists for months and is accompanied by abdominal cramping during bowel movements. In mild ulcerative colitis, rectal mucosal inflammation (proctitis) is sometimes associated with fecal urgency and the presence of blood. In moderate disease, small erosions are present, however, in severe UC, ulcerations with bleeding are seen.

Histological Features. UC is not a static disease but is a disease that frequently changes as a result of active stage and remissions. In UC, inflammation is characteristically limited to the mucosal layer, with inflammatory cell infiltrates into lamina propria. Infiltrates consist primarily of lymphocytes, plasma cells, and granulocytes; neutrophils can invade crypt epithelium causing cryptitis and accumulate resulting in crypt abscesses (Loddenkemper, 2009). Other histological features include depletion of goblet cells, alteration of shapes and sizes of crypts, and colonic ulcerations.

1.3. Conventional Pharmacological Therapy

Management of ulcerative colitis involves acute treatment of all inflammatory symptoms, followed by maintenance of remission. Treatment of UC, generally depends on the disease severity (mild, moderate, or severe) (Stange et al., 2008; Travis et al., 2008; Kornbluth and Sachar, 2010), and most commonly requires sulfasalazine and 5-aminosalicylates given orally or
rectally (either suppository or enema), representing first-line therapy for UC, with approximate 50% remission rate (Nielsen and Munck, 2007). Many UC patients receive sub doses of drugs (mainly the aminosalicylates), continue to take glucocorticoids as a maintenance therapy, or may require biologic agents, such as immunosuppressive therapy, azathioprine, 6-mercaptopurine, or cyclosporine to control the symptoms. However, 5-aminosalicylic acid drugs and glucocorticoids have poor efficacy and frequency of adverse effects such as nausea, vomiting, fever, headache, and gastrointestinal distress for 5-ASA with the frequency rate of these adverse events between 20-30% and a 5-10% withdrawal rate (Moss and Peppercorn, 2007) and for steroids these side effects include weight gain, fluid retention, immune suppression, hypokalemia, hypertension (Ardizzone and Bianchi Porro, 2002) that limit their use (Rosenberg and Peppercorn, 2010). It is, therefore, highly desirable to develop more efficacious treatments that elicit fewer side events than presently used medications. Alternative medicine treatments include probiotics, nicotine patches and fish oil. In some severe cases, colectomy is a final option.

1.4. Cigarette Smoking and Ulcerative Colitis disease

UC has been linked to tobacco smoking for more than three decades. Smoking cigarettes is associated with less frequent exacerbations of ulcerative colitis (Cosnes, 2004). Moreover, tobacco smoking has been reported to often have a beneficial outcome on the course of activity of UC in smokers (Boyko et al., 1988; Mokbel et al., 1998; Gheorghe et al, 2004; Höie et al., 2007) and associated with fewer hospitalizations rate, decrease the clinical disease activity, and need for therapy (Birrenbach and Bocker, 2004) and to decrease the need for colon surgery (Odes et al., 2001; Cosnes, 2004). For example, smokers with UC who quit smoking experienced an increase in disease activity; however, mild disease activity and symptom improvements were seen in ex-smokers who returned to smoking (de Castella 1982; Motley et al., 1987; Ruda et al.,
The etiopathology of UC and underlying mechanism of smoking in UC is still unclear. Smoking and nicotine have multiple actions which can affect gut inflammation. It has been demonstrated that cigarette smoking decreased intestinal permeability in healthy volunteers (Prytz et al., 1989; Suenaert et al., 2000); however, UC disease in smokers did not demonstrate intestinal permeability decrease when compared with the nonsmokers (Benoni and Prytz, 1998) which is most likely to occur in UC patients, since smoking cigarette is protective in UC.

1.5. The Effectiveness of Nicotine Replacement Therapy in Ulcerative Colitis patients

Although cigarette smoke contains hundreds of substances, there is evidence that nicotine and its metabolites account for the beneficial effect of smoking. However, since nicotine is metabolized to cotinine (> 80%) and other metabolites (Benowitz, 1998; Gorrod and Jacob, 1999), it is possible that these latter metabolites account, at least in part, for the protective effect of smoking in UC. Nicotine has shown UC symptoms improvement in some clinical trials with different formulations including gum (43% of 7 patients, Lashner et al., 1990), transdermal patches (78% of 18 patients, Srivastava et al., 1991; 70% of 10 patients, Guslandi and Tittobello, 1996), and enema (71 and 73% of 7 and 22 patients, respectively; Sandborn et al., 1997; Green et al., 1997 respectively), although it has some few adverse effects which included nausea, headache and sleep disturbance (Pullan et al., 1994; McGilligan et al., 2007). Since most of the previous clinical studies showing nicotine replacement therapy in humans, including nicotine gum, transdermal and enemas, decreases the UC disease activity, but with inconsistent and conflicting results due to the different nicotine formulas, various doses of nicotine, duration of the studies, type of the studies, and number of subjects in these studies, and their interpretation has been confounded by the side
effects experienced by individuals as a result of the high systemic nicotine concentrations required (Pullan et al., 1994; Cosnes et al., 2004; McGilligan et al., 2007). In addition, nicotine in different formulations including gum, patches, and enema, was not effective in treating disease relapses and remission (Perera et al., 1984; Thomas et al., 1996; Ingram et al., 2005). Furthermore, clinical studies conducted by Green et al., (1997) and Sandborn et al. (1997) tested nicotine enemas, in which nicotine is applied directly to the colon, indicated that UC symptoms, endoscopic features, and histologically-revealed damage were improved with only a few of the patients reporting some side effects. These initial studies indicate that nicotine applied directly to the colon, perhaps in an enema formulation, may be beneficial while causing fewer side effects.

1.6. The Effectiveness of Nicotine treatment in Experimental Animal Colitis Model

Similar findings with nicotine have been reported in rodent models of colitis. For example, low doses of oral nicotine treatment (12.5 µg/ml) improved the macroscopic and inflammatory damage of experimental colitis in rats, but not with the high doses (250 µg/ml) (Eliakim et al., 1998, 2001). A similar observation has been reported by Qiu et al., (1997), in which they showed that low doses of oral nicotine (5-20 µg/ml) reduced colonic damage and MPO activity; whereas, at dose of 50 µg/ml increased ulceration area and MPO activity in rats. Ghia et al., (2006) reported also that 20 µg/ml of nicotine in drinking water decreased clinical signs of inflammation and reduced colonic myeloperoxidase (MPO) activity and TNF-α levels in the mouse DSS model. However, chronic intraperitoneal injection of nicotine (0.25 and 2.50 µmol/kg) in DSS-induced colitis in mice failed to decrease clinical signs of inflammation, colonic TNF levels and histological features of the disease (Snoek et al., 2010). Although experimental studies indicate that the beneficial effects of nicotine in UC is due to its anti-
inflammatory effect by decreased pro-inflammatory cytokines production in healthy subjects and active UC patients (Madretsma et al., 1996; Bhatti and Hodgson, 1997; Louvet et al., 1999; Eliakim and Karmeli, 2000; Sykes et al., 2000), nicotine alters adherent mucus thickness in rabbits colonic mucosa; reduced by low doses and increased by high doses of nicotine in control animals (Zijlstra et al., 1994). In experimental animal models of colitis, nicotine also has been shown to be effective in the treatment of intestinal inflammation (Sykes et al., 2000; Eliakim and Karmeli, 2003). Overall, both clinical and animal studies with nicotine treatment suggest the usefulness of nicotine in UC disease activity.

1.7. The Concept of “Cholinergic Anti-inflammatory Pathway” and involvement of α7 nicotinic Receptor

Many studies reported that the central nervous system (CNS) can regulate the innate immune responses through the peripheral nervous system (PNS) (Gallowitsch-Puerta and Tracey, 2005; Czura and Tracey, 2005; Pavlov and Tracey, 2006; Gallowitsch-Puerta and Pavlov, 2007). Since cholinergic fibers innervate many lymphoid organs, for example, the immune organ thymus, express α7 (Navaneetham et al., 1997), also α3 and α5 nicotinic receptors subunits (Mihovilovic and Roses, 1991 and 1993) which are expressed by thymocytes. Nicotinic receptors may mediate the immune-modulatory effect of nicotine (Conti-Fine et al., 2000), the presence of nAChRs on immune cells might play a role in the interaction between the nervous and immune systems (Battaglioli et al., 1998; Kawashima and Fujii 2000; 2003; Fujii, 2004).

The anti-inflammatory effect of nicotine is also supported by the finding that nicotinic receptors can regulate inflammation locally and systemically primarily through the vagus nerve so-called “cholinergic (nicotinic) anti-inflammatory pathway” which is a link between
parasympathetic and innate immune system and is one of the main endogenous regulatory
mechanism to control the innate immune responses and ameliorates systemic inflammation by
regulating the production of pro-inflammatory cytokines mainly through activation of $\alpha_7$
AChRs on macrophages surface (Borovikova et al., 2000; Tracey et al., 2001, 2002; Wang H et
al., 2003, 2004; Ulloa, 2005; de Jonge and Ulloa, 2007). It is reported that nicotinic acetylcholine
receptor, especially $\alpha_7$ subunit plays an essential role in the cholinergic anti-inflammatory effects
(Kelso et al., 2006; Hamano et al., 2006). Other studies reported that vagus nerve activation
attenuates intestinal inflammation via $\alpha_7$ nAChRs in animal model of postoperative ileus (de
Jonge et al., 2005) and in experimental colitis (Ghia et al., 2006; 2007). Moreover, $\alpha_7$ nAChR
agonists has shown anti-inflammatory effects in various animal models of inflammation (Damaj
et al., 2000; Giebelen et al., 2007), peritoneal macrophages and in human whole blood (Li J et al.,
2011), in a murine endotoxemia and severe sepsis model (Pavlov et al., 2007), and decreased
severity of experimental pancreatitis (van Westerloo et al., 2005). These anti-inflammatory
effects appear to be mediated by the activation of $\alpha_7$ nAChRs on immune cells such as
macrophages in human (Wang et al., 2003; 2004) and mice (Wang et al., 2003; 2004;
Kawashima et al., 2007).

Nicotine is a full agonist at $\alpha_7$ nAChRs (Gerzanich et al., 1995; and Feuerbach et al., 2005).
$\alpha_7$ nAChRs form homo-pentameric subtypes in the CNS and peripheral nervous system
(Couturier et al., 1990; Keyser et al., 1993; Sharples and Wonnacott, 2001). $\alpha_7$ subtype is a well-
characterized nAChR subunit (Sharma et al., 2008) and exhibits distinct physiological and
pharmacological profiles relative to other nAChR subtypes. It is characterized by its high
calcium permeability (Shen and Yakel, 2009), the rapid desensitization after agonist activation,
and the blockade by methyllycaconitine and $\alpha$-bungarotoxin (for reviews, Dajas-Bailador and
Previous studies demonstrated that $\alpha_7$ nAChR subunits express on macrophages can inhibit transcriptional activity of NF-$\kappa$B, hence decrease the release of pro-inflammatory cytokines (Nizri et al., 2006; Yoshikawa et al., 2006; Parrish et al., 2008). During inflammation, activation of $\alpha_7$ nAChRs is associated with calcium influx and inhibits activation of nuclear factor $\kappa$B (NF $\kappa$B) (Borovikova et al., 2000; Tracey et al., 2005; 2009). From our previous study (Abdrakhmanova et. al., 2010), we showed that in vitro nicotine suppressed firing of action potential in colonic sensory neurons from DSS-treated mice. Moreover, we showed that nicotine fails to suppress multiple-spike action potential firing in inflamed mouse neurons in the presence of the $\alpha_7$ competitive antagonist MLA, which was confirmed by using natural $\alpha_7$ agonist choline chloride which suppressed hyper excitability of inflamed colonic sensory neurons which support the essential role of $\alpha_7$ nAChRs in the mechanism of nicotine-induced suppression of action potential firing in inflamed colonic neurons. Since $\alpha_7$ nicotinic receptors involve in the cholinergic anti-inflammatory pathway (Wang et al., 2003; de Jonge et al., 2005; Ulloa, 2005) and earlier studies reveal the important role of $\alpha_7$ nAChRs in controlling gut inflammation, using selective $\alpha_7$ agonists and a positive allosteric modulator for $\alpha_7$ nAChRs could open a new therapeutic avenue and represent a promising pharmacological strategy for the treatment of inflammation in ulcerative colitis.

1.8. Nicotinic Receptors: Composition, Distribution, and Subtypes

Neuronal nicotine acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels that exist as homomeric or heteromeric complexes of $\alpha$ and $\beta$ subunits. To date, 12 neuronal subunits ($\alpha_2$-$\alpha_{10}$ and $\beta_2$-$\beta_4$) have been identified in mammals (see Gotti et al., 2007 for review). Nicotine exerts its effects by activating nicotine acetylcholine receptors. Nicotinic receptors are found in nerves of the central and peripheral nervous systems and in the autonomic
ganglia, colonic submucosal and myenteric plexuses. They are also present in non-neuronal cells including human immune cells. Human lymphocytes express mRNA for nAChR subunits for \(\alpha_3\), \(\alpha_4\) (Hiemke et al., 1996), \(\alpha_3\), \(\alpha_5\), and \(\beta_4\) (Mihovilovic et al., 1998; Mihovilovic and Roses, 1991, 1993). Human mononuclear leukocytes expressed mRNAs encoding the \(\alpha_2\), \(\alpha_5\), and \(\alpha_7\) subunits (Sato et al., 1999). They are expressed in the enteric nervous system, in enteric plexuses (Kirchgessner and Lu, 1998; Obaid et al., 2005). For example, many nAChR subunits mRNA for \(\alpha_3\), \(\alpha_5\), \(\alpha_7\), \(\beta_2\), and \(\beta_4\) have been detected in the intestine (Kirchgessner and Lu, 1998; Obaid et al., 1999a,b; Obaid and Lindstrom, 2000). Nicotinic receptors have been identified in colonic epithelial HT29 cells, including \(\alpha_3\), \(\alpha_5\), \(\alpha_7\) nAChR subunits (Summers et al., 2003). \(\alpha_7\) nAChRs form homo-pentameric subtypes in the central nervous system (CNS) and peripheral nervous system (PNS) (Couturier et al., 1990; Keyser et al., 1993; Sharples and Wonnacott, 2001). Although these receptors are predominantly expressed in neuronal tissues, several types of immune cells express \(\alpha_7\) nAChR mRNA, including macrophages (Tracey, 2002; Kawashima et al., 2007 and Rosas-Ballina et al., 2008), T-cells (Fujii et al., 1999), B-cells (Fujii et al., 1999; Sato et al., 1999), microglia (Shytle et al., 2004), monocytes (Matsunaga et al., 2001; Kawashima et al., 2007) and dendritic cells (Aicher et al., 2003; and Kawashima et al., 2007) and express on endothelial cells (Wang Y et al., 2001; Abbruscato et al., 2002; Moccia et al., 2004; Saeed et al., 2005; Kawashima et al., 2007). \(\alpha_9\) subunit can form functional homopentamer nicotinic receptors (Elgoyhen et al., 1994) and functional heteromeric with \(\alpha_{10}\) subunits (Elgoyhen et al., 2001; Sgard et al., 2002). \(\alpha_9\) and \(\alpha_{10}\) subunits have restricted anatomical tissue expression (Nashmi and Lester, 2006; Gotti et al., 2006). They have not been detected in the brain and spinal cord (Elgoyhen et al., 2001; Sgard F et al., 2002). Gene transcripts for \(\alpha_9\) or \(\alpha_{10}\) have been reported within hair cells of the inner ear (Elgoyhen et al., 1994; Elgoyhen et al.,
2001; and Vetter et al., 2007), skin keratinocytes (Arredondo J et al., 2002), in the sperm (Kumar and Meizel, 2005), dorsal root ganglion (DRG) neurons (Lips et al., 2002), the pars tuberalis of the pituitary (Sgard et al., 2002), and lymphocytes (Peng H et al., 2004). α10 is expressed in arteries (Bruggmann et al., 2002; 2003) and sympathetic ganglia (Lips et al., 2006). The functional significance of α9 and α10 in these tissues is not known. Finally, both α9 and α10 are present in many immune cells (Lustig et al., 2001, Peng et al., 2004; Galvis et al., 2006; Kawashim et al., 2007; Wessler and Kirkpatrick, 2008). The α9 and α10 nAChR subunits have been detected in human cultured T cells, blood lymphocytes, in monocytes, and macrophages (Lustig et al., 2001, Peng et al., 2004; Galvis et al., 2006; Kawashim et al., 2007). However, the functioning of α9α10 nAChRs in the immune cells is not clear.

1.9. Animal Experimental Models of Colitis

Inflammatory bowel disease (IBD), includes Crohn’s disease (CD) and ulcerative colitis (UC), results from chronic dysregulation of the mucosal immune system in the gastrointestinal tract, and it is widely accepted that genetic, environmental, and immunological factors are involved. Although the pathogenesis of IBD remains unclear, animal models of intestinal inflammation have provided critical perceptions and investigating various factors involve into the pathogenesis of colitis (Blumberg et al., 1999; Strober et al., 2002) and evaluating different therapeutic agents. Several models of experimental colitis that demonstrate various pathophysiological aspects of the human IBD have been described such as dextran sodium sulfate (DSS), trinitrobenzene sulfonic acid (TNBS), acetic acid, indomethacin, and oxazolone-induced colitis models. Here, we induced colitis in mice using dextran sulfate sodium (DSS) which is one of the best described models of UC and a widely used animal model of colitis. It has been used over 20 years (Ohkusa et al., 1985) due to its simplicity. In addition, it has some
advantages over other experimental colitis models; for instance, different stages of the disease conditions, acute, chronic, or relapsing condition can be induced by simply modulating DSS concentration in the drinking water (animals stress-free handling) and importantly shows some resemblance of human UC in both clinical (body weight loss, loose stool, bloody diarrhea or rectal irritation and hematochezia) and histopathologic findings (Okayasu et. al., 1990; Takizawa et. al., 1991; Cooper et al., 1993; Gaudio et. al., 1999), and is characterized by an excessive T helper 2 (Th2) cell response (Targan and Karp, 2005). The responsiveness of the DSS-induced colitis model to conventional therapeutic agents for human UC supports its suitability as a model of human UC and help to translate basic research to clinical practice (Böjrck et. al., 1997). However, using DSS-induced colitis model, various factors may affect the responsiveness to DSS; such as, concentration of DSS used in the experiment (Egger et al., 2000), duration of exposure, molecular weight of DSS (Kitajima et al., 2000; Hirono et al., 1983), animals strain and sex differences (Mahler et al., 1998). Thus, all these factors should be considered during study design before conducting the experiment.

1.10. Using Genetically Modified Animals: a complementary approach to study the role of nAChR subtypes in murine colitis model

The use of knock-out mice for different nicotinic receptor subunits is an attractive approach to overcome the limitation of the availability and/or selectivity of nicotinic receptor ligands. Importantly, this approach helps to elucidate the role of specific nicotinic receptor subtypes and provides a powerful tool to confirm results derived from pharmacological studies. Overall, the use of genetically modified animals and knockout technology offers many advantages that cannot be achieved through the use of pharmacological agents that can be utilized as complementary tools to insight the specific functional role of certain nicotinic receptor subunit.
The KO mouse is typically engineered using mouse embryonic stem (ES) cells from the 129/Sv inbred mouse. The gene of interest is inactivated by replacement of coding sequences essential for gene function with a neomycin cassette. Through homologous recombination, the normal gene on the chromosome is replaced with the targeted cassette, thus “knocking out” the gene function. These engineered stem cells from the 129/Sv mouse that contain the homologous recombination construct are then injected into C57Bl/6 (B6) mouse blastocysts, and implanted into a foster mother; thus, the pups born from this event are composed of cells from a B6 embryo and 129/Sv stem cells. The resulting chimeric mice that contain the mutated embryonic stem cells are then mated with B6 mice to allow the mutation of the KO allele to pass onto the next generation, the resulting mice will be heterozygote that can be interbred to generate homozygous mutant KO mice and ultimately generate KO mice in later generations. To date, several nAChR KO mice have been generated and the phenotypes have been characterized. Here in our studies we used α5 (Salas et al., 2003), α7 (Orr-Urtreger et al., 1997), β2 (Picciotto et al., 1995). One major limitation to KO studies is the compensatory mechanisms in knockout mice, and the occurrence of developmental requirements that may occur as a result of gene deletion, and can interfere with interpretation of results. It may be difficult to determine if the mouse phenotype is a result of the gene deletion. The backcrossed of these knockout mice for 10 generations with C57BL/6 mice could help reduce the possibility that mixed genetic of mutant mouse background.
1.11. Dissertation Objectives

Using DSS-induced experimental colitis model in male C57BL/6J mice:

- To characterize the dextran-sulphate sodium (DSS)-induced colitis model in mice.
- To investigate the effect of the main active substance of tobacco, nicotine and its main metabolites, cotinine, in a DSS-induced colitis model.
- To examine if dose and route of nicotine administration have a role in ameliorating DSS-induced colitis model.
- To examine the role of $\alpha_7$ nAChRs in mouse DSS-induced colitis model using pharmacological tools by examining the effect of chronic treatment of $\alpha_7$ agonists, PHA-543613, choline and the positive allosteric modulator for $\alpha_7$ nicotinic receptor, PNU 120596 and confirm our results using genetically modified animals, $\alpha_7$ KO mice.
- Finally, to assess the specificity of the involvement of $\alpha_7$ nAChRs by investigating the role of non-$\alpha_7$ nAChRs including $\alpha_5$, $\alpha_9$, $\alpha_{10}$ and $\beta_2$ nAChRs in DSS colitis model.
CHAPTER TWO

Materials and Methods

2.1. Animals

Male C57BL/6J, α5, α7 knockout mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice null for the β2 subunit were provided from Institut Pasteur (Paris, France). The null mice and their wild-type littermates were bred in an animal care facility at Virginia Commonwealth University (Richmond, VA, USA) and are maintained on a C57Bl/6 background and have been backcrossed to at least N10. For all experiments, mutants and wild type controls are obtained from crossing heterozygote mice. This breeding scheme allows us to rigorously control for any anomalies that may occur with crossing solely mutant animals. Male animals were 8-10 weeks of age at the start of the experiments, weighing 25-30 g and were group-housed in a 21°C humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care-approved animal care facility with ad libitum access to food and water. Mice were housed under standard conditions for a minimum of 1 week before experimentation. Experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

2.2. Drugs

(-)-Nicotine hydrogen tartrate salt [(-)-1-Methyl-2-(3-pyridyl) pyrrolidine (+)-bitartrate salt] and (-)-cotinine were purchased from Sigma-Aldrich Inc. (St. Louis, MO). DSS (molecular weight 36-50,000 kilodaltons) was obtained from ICN Biomedicals Inc. (Aurora, OH). Nicotine doses are expressed as the free base of the drug. PHA-543613,(N-[(3R)-1-Azabicyclo[2.2.2]oct-3-yl]furo[2,3-c]pyridine-5-carboxamide Dihydrochloride) and PNU-120596 (1-(5-chloro-2,4-
dimethoxy-phenyl)-3-(5-methyl-isoxan-3-yl)-urea) were obtained from the drug supply program of the National Institute on Drug Abuse (Rockville, MD). PHA-543613 was dissolved in physiological saline (0.9% sodium chloride) and administered by subcutaneously twice daily. PNU-120596 was suspended in a vehicle of absolute ethanol, Emulphor-620 (Rhône-Poulenc, Inc., Princeton, NJ), and saline at a ratio of 1:1:18 and administered by i.p. injection once daily at a volume of 10 ml/kg body weight unless noted otherwise. Doses are expressed as the free base of the drug. Choline chloride (Sigma-Aldrich) was dissolved in the drinking water and given orally. DSS at different concentrations was dissolved in the drinking water and given orally. The doses of PHA-543613, PNU 120596, and choline chloride were chosen based on previous data (Wishka et al., 2006; Krafft et al., 2012; Hurst et al., 2005; Ghia et al., 2009). α-Conotoxin-RgIA (α-RgIA), a selective α9* antagonist, (provided from Dr Michael McIntosh, University of Utah) (0.1, 0.2, and 0.02 mg/kg once daily) treatment started 3 days before and for 7 days after the induction of colitis. α-RgIA, was dissolved in physiological saline (0.9% sodium chloride) and administered by subcutaneous (s.c.) injection at a volume of 10 ml/kg body weight. Molecular mass of RgIA is 1572 daltons. In addition, we conducted a dose-response curve for each drug in DSS-induced colitis model in mice.

2.3. Dextran Sulphate Sodium (DSS)-induced colitis model:

DSS was added to the drinking water at different concentrations (in % w/v) for 7 days in most experiments. On day 8, mice received drinking water without DSS. Controls were all age- and time-matched and consisted of mice that received regular tap drinking water for the corresponding number of days. Each treatment group consisted of 8-10 mice.
2.4. Assessment of the Severity of Colitis: Disease Activity Index

Disease Activity Index (DAI) scores historically have correlated well with the pathologic findings in a DSS-induced model of IBD (Cooper et al., 1993). DAI is the combined score of four clinical parameters, including a) weight loss, b) stool consistency, c) rectal irritation, and d) blood in the stool. Scores were defined as follows: for weight: 0, no loss; 1, 5%–10%; 2, 10%–15%; 3, 15%–20%; and 4, 20% weight loss; for irritation around the anal area: 0, normal; 1, mild irritation; 2, moderate irritation; 3, severe irritation; for stool: 0, normal; 1, mild loose stool; 2, moderate loose stool and 3, diarrhea; and for bleeding: 0, no blood; 1, presence of blood (Hemoccult II positive; Beckman Coulter, Fullerton, CA); and 2, gross blood. DAI symptoms were recorded same time of every day and scored from days 0–8 in blinded manner to the treatment and mouse genotypes. Total DAI score ranged from 0-12. On day 8, after replacing the DSS with water, the mice were sacrificed and the abdominal cavity was opened, the entire colon was immediately removed and the colon length (cm) measured.

2.5. Colonic Histology Assessment

Seven days after the beginning of the DSS treatment, two-three mice of each group were sacrificed and the colon was removed. Formalin-fixed colon segments were paraffin-embedded and 3-µm sections were stained with haematoxylin-eosin (H&E). Colonic damage was scored based on a published scoring system that considers architectural derangements, epithelium changes, goblet cell depletion, ulceration, and degree of inflammatory cells infiltrate in a blinded fashion (Iba Y et al., 2003). The histological scoring system was used to evaluate the degree of colitis. The total histological score ranged from 0 to 12, which represented the sum of scores from 0 to 3 (0= none, 1= 0-5 %, 2= 5-10%, 3= > 10%) for loss of epithelium, (0= none, 1= 0-
10 %, 2= 10-20%, 3= > 20%) for crypt damage, (0= none, 1= mild, 2= moderate, and 3= severe) for each of depletion of goblet cells and infiltration of inflammatory cells. Each section was scored for each feature separately by establishing the product of the grade for that feature and the percentage involvement in the loss of epithelium and crypt damage features (in a range from 0 to 3 for each feature). The number of inflammatory cells infiltration in 10 randomly selected power fields (40X) was counted and the number per 10 fields was calculated. The scores were assigned by one experienced histopathologist with no knowledge of the group being examined in each analysis. The histological colitis score of individual mice represents the sum of the different histological subscores. Light microscope images were acquired with an Axioscope AX10 microscope and Axiovision 4.6 software (Carl Zeiss, Inc.). Mean ± SE, n=3 animals were used per group.

2.6. Assessment of Colonic Myeloperoxidase (MPO) Activity:

The colonic tissues were homogenized with a homogenizer in phosphate buffered saline (PBS) for the assessment of MPO. Tissue samples were homogenized in 100 mM sodium acetate (pH 6.0) containing 0.5% hexadecyl trimethyl ammonium bromide and 5 mM EDTA. The homogenate was briefly sonicated, and then centrifuged at 13000 rpm for 10 min at 4° C. The supernatant was then added to a homogenizer solution in 96 well plate. 75 µl of 3,3’5,5’-tetramethylbenzidine (TMB) substrate were added to each well and the plates were incubated for 2 min; 50 µl of stop solution (2N H2SO4) were then added. The plates were read at 450 nm within 30 min. For each mouse, a semilog curve of sample dilution vs. optical density (O.D.) was plotted to obtain a midpoint titer. Mean ± SE, n=5-8 animals were used per group.
2.7. Tumor necrosis factor-alpha (TNF-α) Levels:

The colonic sample was homogenized in 1 mL of Tris-HCl buffer containing protease inhibitors (Sigma-Aldrich Inc., St. Louis, MO, USA). Samples were centrifuged then centrifuged (1811 x g, 5 min) at a temperature of 4°C, and the supernatant was frozen at -80°C until assay. The protein concentration was determined by the Bradford assay (Bradford, 1976). Cytokine level (TNF-α) was determined using an enzyme-linked immunosorbent assay commercial kit (Quantikine M murine; R&D Systems, Minneapolis, MN).

2.8. Total Protein Quantification

The Bradford protein assay was used to determine the total protein content in the supernatant. We have chosen the most widely used protein as our standard - Bovine Serum Albumin (BSA). The assay is based on the proportional binding of the dye Coomassie to proteins. 2 µl of supernatant was added to 750 µl of Bradford reagent (Sigma) at room temperature. The absorbance at 595 nm was measured and the protein concentration determined using a standard curve.

2.9. Nicotine and Cotinine Plasma Levels:

Blood samples were taken from mice receiving different protocols of nicotine: 10 days after oral (-)-nicotine or (-)-cotinine administration in the drinking water, 2 weeks after continuous minipump (-)-nicotine infusion (2.5 and 25 mg/kg) and 5 min after the last s.c. injection of chronic nicotine injection (0.5 and 2 mg/kg) (Tables 4 and 5). Animals were anesthetized with CO₂, blood samples were taken by intra-cardiac puncture just before death and blood was kept in sodium heparin blood collection tubes, and centrifuged (1400 x g, 10 min). The serum then was
stored at - 4°C. Nicotine and cotinine serum levels were measured using HPLC/MS/MS analysis. At least 6 animals were used per group.

2.10. Nicotine and metabolites HPLC/MS/MS analysis:

*Specimen extraction:* To a 200 µL aliquot of whole blood, 50 µL of internal standard (ISTD) containing 50 ng of nicotine-d4 and cotinine-d3 in methanol was added with mixing. Then 100 µL of 5M ammonium hydroxide was added to each sample followed by 2ml methylene chloride. The samples were mixed for 2 minutes and then centrifuged (1811 x g, 5 min) at a temperature of 4°C. The organic layer was transferred to a clean test tube. The aqueous phase was extracted twice more with 2 mL of methylene chloride. The organic phases were combined and 500 µL of 25mM HCl in methanol was added. Samples then were evaporated to dryness under a gentle stream of nitrogen. They were reconstituted with 100 µL of mobile phase and placed in auto-sample (HPLC/MS/MS) vials for analysis.

*Instrumental Analysis:* The HPLC/MS/MS system used was an Applied Bio systems 3200 Qtrap with a turbo V source for TurbolonSpray with a Shimadzu SCL HPLC system controlled by Analyst 1.4.2 software. The chromatographic separation was performed using a Hypersil Gold, 3mm X 50 mm, 5 micron (Thermo Scientific, USA). The mobile phase contained 10 mM ammonium formate; methanol (10:90 V/V) and was delivered at a flow rate of 0.5 mL/min. The acquisition mode used was multiple reaction monitoring (MRM) in a positive mode. Transition ions monitored for nicotine (163>130; 163>117), nicotine-d4 (167>134), cotinine (177>80; 177>98) and cotinine-d3 (180>80). The total chromatographic separation time for each extract injection was 2 minutes. A calibration curve ranging from 12.5 ng/ml to 500 ng/ml was
constructed for each compound based on linear regression using the peak area ratios of the drug to its deuterated ISTD. Cotinine-d3 also was used as the ISTD for 3-hydroxycotinidine.

2.11. Statistical Analysis. Statistical analysis of all studies was performed with ANOVA. Two-way repeated-measures ANOVAs were used at the different time points. Significant overall ANOVA were followed by Tukey’s test post hoc test when appropriate. All differences were considered significant at *p<0.05. The GraphPad Prism® program was used for data manipulations, graphical representations, and statistical analysis (GraphPad Software Inc., San Diego, CA). Clinical, histological, cytokine data are presented as mean ± SE at different treatments and time points (n=6-8 animals for clinical DSS results, n=3 per group for histological results, n=5-8 animals for MPO and cytokine measurements).
CHAPTER THREE

The Impact of Nicotine Route of Administration in Murine DSS-induced Colitis

Background and purpose. Studies have shown that nicotine has a positive influence on symptoms of ulcerative colitis (UC). In the present study, we explored the effect of nicotine treatment using different route of administrations in the DSS colitis mouse model. We also investigated the effects of cotinine, a major metabolite of nicotine, in the model.

Experimental approach. C57BL/6 male adult mice were given DSS solution at different concentrations freely in the drinking water for 7 consecutive days after which tap water was given on the 8\textsuperscript{th} day. Disease severity, length of the colon, colon tissue histology, inflammatory markers including colonic myeloperoxidase (MPO) activity and colonic tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) levels were then evaluated. The effect of oral, subcutaneous, infusion of nicotine, and oral cotinine treatments were examined the DSS model. In addition, we measured the plasma levels of the nicotine and cotinine in our treatment protocols.

Key Results. Administration of low, but not high, doses of oral nicotine in DSS-treated mice produces a significant decreases in DAI severity, histological damage scores, as well as colonic level of TNF-\(\alpha\). However, the anti-inflammatory effect of nicotine was not seen after chronic s.c. and infusion of the drug. Differences in plasma levels of nicotine and cotinine do not seem to account for this lack of effect. Finally, cotinine oral administration on its own failed to show a significant effect in the DSS model of colitis.

Conclusions and Implications. Results obtained from this study highlight that dose and route of administration play a critical role in the protective effect of nicotine in the DSS mouse colitis model.
3.1. Introduction

Ulcerative colitis (UC) is a chronic inflammatory bowel disease of unknown etiology, due partly to genetic susceptibility to environmental factors that misdirect the mucosal immune system response to attack the epithelial layer of the colon causing inflammation. UC is a remitting and relapsing inflammatory condition that results in excessive mononuclear cell and neutrophil infiltration into mucosa and submucosa vicinities that affects primarily the distal colon and rectum (Hanauer, 1996). Patients typically present with bloody diarrhea and passage of pus and/or mucus that often persists for months and is accompanied by abdominal cramping during bowel movements. Current pharmacotherapies, which include 5-aminosalicylic acid drugs and glucocorticoids, have poor efficacy and many adverse effects that limit their use (Rosenberg and Peppercorn, 2010). It is, therefore, highly desirable to develop more efficacious treatments that elicit fewer side effects than presently used medications.

UC has been linked to tobacco smoking for more than three decades. For example, smokers with UC who quit smoking experienced an increase in disease activity; however, mild disease activity and symptom improvements were seen in ex-smokers who returned to smoking (de Castella 1982; Motley et al., 1987; Ruda et al., 1989; Kuisma et al., 2004). Moreover, tobacco smoking has been reported to often have a beneficial outcome on the course of activity of UC in smokers (Boyko et al., 1988; Mokbel et al., 1998; Gheorghe et al, 2004; Höie et al., 2007) and to decrease the need for colon surgery (Odes et al., 2001; Cosnes J, 2004). Although cigarette smoke contains hundreds of substances, there is evidence that nicotine accounts for the beneficial effect of smoking. However, since nicotine is metabolized to cotinine (> 80%) and other metabolites, it is possible that these latter metabolites account, at least in part, for the protective effect of smoking in UC.
A number of clinical studies has been carried out in which nicotine was administered in different formulations using patches and gums to UC patients. Nicotine has shown positive results on disease symptomology in some trials. Nevertheless, results have been conflicting and interpretation has been confounded by side effects experienced by individuals as a result of the high systemic nicotine concentrations required (Pullan et al., 1994; Cosnes et al., 2004; McGilligan et al., 2007). In addition, it has been indicated that nicotine lacks efficacy in treating disease relapses and remission (Perera et al., 1984; Thomas et al., 1995; Ingram et al., 2005). Thus, collectively these results indicate that, while transdermal nicotine may be effective for UC especially in ex-smokers, its use is limited by its adverse event profile. However, it is not clear if the delivery of nicotine through patches and gum formulations, which do not mimic the intermittent delivery of nicotine from cigarettes, plays a role in the efficacy of the drug. Furthermore, clinical studies conducted by Green et al., (1997) and Sandborn et al. (1997) tested nicotine enemas, in which nicotine is applied directly to the colon, indicated that UC symptoms, endoscopic features, and histologically-revealed damage were improved with only a few of the patients reporting some side effects. These initial studies indicate that nicotine applied directly to the colon, perhaps in an enema formulation, may be beneficial while causing fewer side effects.

Similar findings with nicotine have been reported in rodent models of colitis. For example, oral nicotine treatment improved the macroscopic damage of experimental colitis (Eliakim et al., 2001). Ghia et al., (2006) reported also that 20 µg/ml of nicotine in drinking water decreased clinical signs of inflammation and reduced colonic MPO activity and TNF-α levels in the mouse DSS model. However, chronic intraperitoneal injection of nicotine (0.25 and 2.50 µmol/kg) in DSS-induced colitis in mice failed to decrease clinical signs of inflammation,
colonic TNF levels and histological features of the disease (Snoek et al., 2010).

In the present study, we used the DSS mouse model, one of the best described and widely used animal models of UC since it shows some resemblance to human UC in both clinical (body weight loss, loose stool, bloody diarrhea or rectal irritation and hematochezia) and histopathologic findings (Okayasu et. al., 1990; Takizawa et. al., 1991; Cooper et al., 1993; Gaudio et. al., 1999). Furthermore, the model is characterized by an excessive T helper cell response (Targan and Karp, 2005). The responsiveness of the DSS-induced colitis mouse model to conventional therapeutic agents for human UC supports its suitability as a model of human UC (Böjrck et. al., 1997). We examined the effect of chronic nicotine administration using various doses and routes of administration (subcutaneous, infusion using osmotic minipump, and oral) in order to define the optimal route and dose that resulted in ablation of DSS-induced colitis model in the mouse. We also tested the effect of one of the main nicotine metabolites, cotinine, in order to garner insight as to whether it contributes to the beneficial effect of cigarette smoking in UC patients.

3.2. Materials and Methods

See chapter 2

3.2.1. Drugs

(-)-Nicotine hydrogen tartrate salt [(−)-1-Methyl-2-(3-pyridyl) pyrrolidine (+)-bitartrate salt] and (-)-cotinine were purchased from Sigma-Aldrich Inc. (St. Louis, MO). DSS (molecular weight 36-50,000 kilodaltons) was obtained from ICN Biomedicals Inc. (Aurora, OH). Doses are expressed as the free base of the drug.
3.3. Chronic nicotine treatment

*DSS-induced colitis model:* DSS was added to the drinking water at 1%, 2.5% and 5% concentrations (in % w/v) for 7 days. On day 8, mice received drinking water without DSS. Controls were all age- and time-matched and consisted of mice that received regular tap drinking water for the corresponding number of days. Each treatment group consisted of 8-10 mice.

3.3.1. Study 1. *Dextran sulfate sodium (DSS) dose response curve.* Separate group of mice were allocated to the following treatments: controls (water only), 1% DSS, 2.5% DSS and 5% DSS for 7 days.

3.3.2. Study 2. *Effect of Oral (-)-nicotine administration.* Different doses of (-)-nicotine (6, 12.5, 25, 50, and 100 µg/ml) were added to the drinking water 3 days before and for 7 days along with the induction of colitis with DSS 2.5%.

3.3.3. Study 3. *Effect of s.c. (-)-nicotine administration.* Saline or (-)-nicotine treatment (0.1, 0.5, 2 mg/kg) was given s.c. twice daily for 10 days. (-)-nicotine was dissolved in physiological saline (0.9% sodium chloride) and administered by s.c. injection at a volume of 10 ml/kg body weight. The s.c. nicotine or saline injection was administered 3 days before induction of DSS colitis and was continued along with DSS treatment period.

3.3.4. Study 4. *Effect of (-)-nicotine administration through mini pump.* Nicotine was infused for 14 days though osmotic mini pump at doses of 2.5 and 25 mg/kg/day. Mice were implanted with Alzet osmotic mini pumps [model 2002 (14 days), Durect Corporation, Cupertino, CA] filled with (-)-nicotine or saline solution. The concentration of nicotine was adjusted according to animal weight and the mini pump flow rate, resulting in 2.5 and 25 mg/kg/day for 14 days. The mini pumps were surgically implanted s.c. under sterile conditions with sodium pentobarbital anesthesia (45 mg/ml, i.p.). An incision was made in the back of the animal, and a pump was
inserted. The wound was closed with wound clips, and the animal was allowed to recover before being returned to its home cage. DSS was added to the drinking water for 7 days in the second week of saline or (-)-nicotine minipump implantation.

3.3.5. Study 5. Effect of oral cotinine administration. Two doses of (-)-cotinine (25 and 250 µg/ml) were added to the drinking water 3 days before and for 7 days along with the induction of colitis with DSS 2.5%.

3.4. Results

3.4.1. Characterization DSS dose response curve on colitis severity in the mouse

We first evaluated the colitis severity in the mouse DSS model. For that, we examined the effects of 1, 2.5 and 5% of DSS concentrations in C57BL/6J mice on the disease activity scores, colon length, colonic histological damage, colonic MPO activity and colonic TNF-α levels.

Oral DSS administration for 7 days induced signs of colitis in C57BL/6J mice in a dose-related manner \([F (3, 30) =46.12, p<0.0001]\) as measured by the disease activity scores (Figure 1 A-C). Mice treated with 1% DSS did not show significant differences in any of the colitis parameters including rectal irritation, loose stool, bloody diarrhea and loss in body weight, compared with the water-treated control group. However, both 2.5 and 5% DSS concentrations significantly increased all clinical signs of colitis severity. The loss of body weight was significant on day 5 and exhibited a gradual increase in mice receiving 2.5% DSS and 5% DSS with a - 15% loss on day 8 (Figure 1 C). Similar to disease activity, the decrease in the colon length was dose related with all DSS concentrations significantly resulting in reduction in length compared to the water-treated control group (Figure 1 D).
In general, the increase in the inflammatory markers MPO and the pro-inflammatory cytokine TNF-α correlated with the severity of colitis. MPO activity was undetectable in the control water-treated animals and was higher in mice-treated with DSS 2.5 and 5% respectively [F (3, 14) = 7.668, p=0.0029] (Figure 2 A). The level of TNF-α, an acute inflammatory marker, was increased significantly in mice treated with 2.5 and 5% DSS compared to the control group [F (3, 16) = 49.46, p<0.0001] (Figure 2 B).

The histology of the colon after 7 days of DSS treatment was characterized by multifocal changes in crypts with some areas showing focal lesions and inflammatory cell infiltration that included neutrophils and lymphocytes. The histological damage was significantly higher in the 2.5 and 5% DSS group when compared with the control group [F(3, 36) = 36.23, p<0.05] (Figure 3 E). There were focal changes suggesting a discontinuous appearance of histological features with varying severity of epithelial loss (Figure 3 A), shortening of crypts (Figure 3 B), depletion of goblet cells (Figure 3 C), and inflammatory cells infiltration (Figure 3 D). The extent of the inflammatory response also was assessed by histology of mucosal tissue using haematoxylin-eosin staining (Figure 4). DSS-treated mice showed extensive ulceration of the mucosa with destruction and inflammation mainly in the mucosa; however, some inflammation to the submucosa was seen with 5% DSS as well as shortening of the crypts, and inflammatory cell infiltration (Figure 4 D). Since the 7-day 2.5% DSS administration protocol showed a reliable inflammatory colitis with a low rate of mortality compared to the 5% DSS group [30% vs 75% for 2.5% and 5%, respectively] after 7 days recovery period post-DSS (Figure 5), it was chosen for subsequent studies with nicotine and nicotine ligands.
3. 4. 2. Oral nicotine administration attenuates DSS-induced colitis

Oral nicotine treatment at doses of 12.5, 25 and 50 µg/ml significantly decreased disease activity scores during the last three days of DSS disease course \[F(3,48) = 6.615, \ p=0.0021\] (Figure 6 A). In contrast, nicotine at the highest dose (100 µg/ml) significantly enhanced disease activity scores at day 7 and day 8 compared with the DSS-treated group as seen in the dose-response curves and area under the curve values \[F(2, 19) = 5.593, \ p=0.0123\] (Figure 6 A & B). A similar profile was seen with the body weight loss where the effect of nicotine was lost at higher doses (Figure 6 C). All tested doses of oral nicotine treatments did not significantly reverse the effect of DSS on the colon length shortening (Figure 6 D).

Oral nicotine at 25 µg/ml, a dose that attenuated DSS-induced colitis signs, reduced also the increase in colonic TNF-α levels seen with DSS-treated mice \[F(3,27) = 4.60, \ p=0.010\] (Figure 7 A). However, this effect was lost at the high dose of 100 µg/ml of the drug. In line with the disease activity scores, DSS-treated mice showed a significant decrease in histological scores after treatment with the low dose of 12.5 µg/ml of nicotine. However, this effect started to gradually decrease with increasing doses (25 and 100 µg/ml) of nicotine (Figure 7 B). Furthermore, nicotine oral administration at doses of 12.5 and 25 µg/ml normalized the appearance of epithelial architectures of colonic slices and reversed the DSS-induced colonic damage (Figure 7 E & F).

To determine if nicotine is bioavailable in our treatment protocols, the plasma levels of the drug and its main metabolite cotinine, were measured using the lowest active dose and highest dose of oral nicotine administration in DSS-treated mice. Oral nicotine administration resulted in a dose-related increase in the level of plasma levels of nicotine and cotinine (Table 4) in DSS-treated mice.
3.4.3. Effect of subcutaneous and mini pump nicotine treatments on DSS-induced colitis

Subcutaneous nicotine treatment (0.1, 0.5 and 2 mg/kg s.c. twice daily for 10 days) was evaluated in the DSS model. Overall, nicotine given s.c. failed to alter significantly the intensity of disease activity in the DSS-treated animals. Only the low dose of 0.1 mg/kg s.c. resulted in a significant decrease in the disease activity on days 7 and 8 (Figure 8 A). A decrease in the area under the curve at the same dose was observed, but it failed to reach statistical significance (Figure 8 B) [$F(3, 36) = 2.604, p=0.0668$]. Similarly, only mice injected with 0.5 mg/kg s.c. showed a significant attenuation in the colon length shortening when compared with the vehicle DSS-treated group [$F(4, 42) = 20.76, p<0.05$] (Figure 8 C). However, none of the nicotine doses tested after chronic s.c. injection showed a significant decrease in total histological damage score compared with DSS-treated mice (Figure 8 D). Similarly, nicotine did not reverse colonic TNF-α levels in DSS-treated mice (Table 2). Finally, chronic administration of 0.5 and 0.1 mg/kg of nicotine increased the plasma levels of nicotine and cotinine in mice. Drug plasma levels after nicotine administration of 0.1 mg/kg were below the level of detection (Table 4).

Chronic exposure of mice to nicotine via mini pumps infusion at doses of 2.5 and 25 mg/kg/day was assessed. Overall, nicotine given via infusion failed to alter significantly the colitis severity of the DSS-treated animals. Only the low dose of 2.5 mg/kg/day exerted a significant decrease in the disease activity index on day five compared with the control group (Figure 9 A). However, no significant decrease in the area under the curve of disease activity in nicotine-treated mice was seen at both infused doses of the drug (Figure 9 B). Similarly, nicotine treatment failed to significantly reverse the decrease in colon length in the DSS-treated mice (Figure 9 C). However, the low dose of nicotine infusion (2.5 mg/kg/day) showed a significant decrease in total histological damage score compared with DSS-treated mice [$F(2, 31) = 4.531$,
p=0.0188] (Figure 9 D). This effect was not observed at the high dose of 25 mg/kg/day of nicotine. In contrast, neither dose of nicotine resulted in lowering the increase in colonic TNF-α levels observed for DSS-treated mice [F(3, 46)=16.66, p<0.05] (Table 3). Finally, chronic infusion administration of nicotine induced a dose-related manner an increase in the plasma levels of nicotine and cotinine in mice (Table 4).

3.4.4. Effect of cotinine, the main metabolite of nicotine, in the DSS-induced colitis model

To determine whether cotinine, the principle metabolite of nicotine, possessed anti-inflammatory effects in the colitis model, DSS-treated animals were subjected to chronic cotinine given orally at two different doses (25 and 250 µg/ml). As shown in Figure 10, cotinine administration neither had a significant effect on colitis severity as measured by disease activity scores (Figure 10 A & B) nor resulted in a shortening of the colon length (Figure 10 C). Cotinine was bioavailable since the plasma levels of the drug and its metabolite (3-OHCotinine) (Table 5) were shown to be increased in a dose-related manner after oral administration of the drug at 25 and 250 µg/ml.
Figure 1. Colonic inflammation was aggravated after DSS exposure to mice in a dose-related manner.

(A) Disease activity index (DAI) changes among groups exposed to different doses of DSS. (B) AUC of DAI for mice in (A). (C) Percentage of body weight change for mice in (A). (D) Mean colon length (cm) for the mice in (A). C57BL/6 mice were given different doses of DSS (1%, 2.5% and 5%) in the drinking water for 7 days. All clinical signs were assessed on daily basis for each mouse and were averaged per day for each group. Results are expressed as mean ± SE, n=6-8, *p<0.05. DAI = disease activity index, AUC = area under the curve, Ctrl = water-treated animals.
Figure 2. Aggravation of inflammatory markers after 7 days of exposure to different doses of DSS in mice.

(A) Colonic MPO activity (midpoint titer/g wet tissue) in groups exposed to different doses of DSS (0, 1, 2.5, 5%). (B) Colonic TNF α level (pg/mg protein) for mice in (A). Results are expressed as mean ± SE, n=6-8. *p<0.05 Ctrl = water treated group. Ctrl = water-treated mice, MPO = myeloperoxidase activity.
Figure 3. Histological scores from colons after 7 days of exposure to water or different doses of DSS.

(A) Loss of epithelium. (B) Crypt damage. (C) Depletion of goblet cells. (D) Infiltration of inflammatory cells. (E) Total histological damage score. Histological scores were blindly evaluated using H&E stain of the colonic tissue sections after 7 days of water-treated control group and DSS-treated animals. Results are expressed as mean ± SE, n=3, *p<0.05. Ctrl = water treated group.
Figure 4. Histological analysis of representative colons from mice after 7 days of exposure to water or different doses of DSS.

(A) Appearance of control water-treated control. (B) Appearance of colon treated with 1% DSS. (C) Appearance of colon treated with 2.5% DSS. (D) Appearance of colon treated with 5% DSS (Haematoxylin-eosin staining of colonic tissue sections, magnification: X40, Scale bar 20µm).
Figure 5: Survival rate of C57Bl/6 mice treated with DSS at 2.5 and 5% concentrations for 7 days then followed 7 days without DSS (recovery phase).

Figure 5. The 5% DSS-treated mice did not survive the recovery phase.

Mice received DSS (2.5 or 5%) during the acute phase (day 1-7), and replaced with tap drinking water during the recovery phase (day 8-14). On day 11: only 33% survived of 5% DSS group, while more than 70% survived from mice treated with 2.5% DSS.
Figure 6. Oral nicotine treatment suppressed the severity of DSS-induced colitis in mice.

Effects of chronic oral nicotine treatment with various doses (6-100 µg/ml) on (A) the time-course of DAI. (B) AUC of DAI in mice. (C) Percentage of body weight change. (D) Mean colon length (cm). Results are expressed as mean ± SE, n=6-8, *p<0.05 compared to control (vehicle-treated mice). Nic = nicotine.
Figure 7. Oral nicotine treatment (25 and 100 µg/ml) affects colonic TNF-α levels, histological damage score and appearance in DSS-induced colitis.

(A) Colonic TNF-α levels (pg/mg protein) in the homogenized colonic tissue samples. Results are expressed as mean ± SE, n=6-8, *p<0.05 vs. DSS group and #p<0.05 vs Ctrl group. (B) Histological colonic damage score in DSS-induced colitis. Representative colon sections at 7 days after induction of colitis of a control colon (C), DSS-treated mice (D), nicotine (12.5 µg/ml) (E), nicotine (25 µg/ml) (F) and nicotine (100 µg/ml) (G). *p<0.05 vs. DSS group, #p<0.05 vs. water control group. (Mean ± SE, n=3-5 per group). Nic = nicotine, Ctrl = water-treated group. (Haematoxylin-eosin staining of colonic tissue sections, magnification: X40, Scale bar 20µm).
Figure 8. Effect of chronic s.c. nicotine administration on the severity of DSS-induced colitis in mice.

Effects of chronic subcutaneous nicotine treatment (0.1, 0.5 and 2 mg/kg) on (A) the time-course of DAI. (B) AUC of DAI. (C) Mean colon length (cm). (D) Histological colonic damage score. Results are expressed as mean ± SE, n=6-8, *p<0.05 compared to control (vehicle-treated mice). Veh = vehicle, Nic = nicotine, s.c.= subcutaneous.
Figure 9. Influence of nicotine infusion via mini pump in DSS-treated mice on DAI, histological damage and colon length.

Effects of chronic mini pump infusion nicotine (2.5 and 25 mg/kg/day) on (A) the time-course of DAI. (B) AUC of DAI. (C) Mean colon length (cm). (D) Histological colonic damage score. Results are expressed as mean ± SEM of n=6-8. *p<0.05 compared to control (vehicle-treated mice). Veh = vehicle, Nic = nicotine, MP = mini pump.
Figure 10

Figure 10. Lack of suppression of the severity of colitis by oral cotinine.

Effects of oral cotinine (25 and 250 µg/ml) on (A) the time-course and (B) AUC of DAI in mice. (C) Mean colon length (cm). Results are expressed as mean ± SEM of n=6-8. *p<0.05 compared to control (water-treated mice).
Table 1. Daily fluid volume consumption.

There was no difference in the daily fluid volume consumption between the water (control) and the DSS 2.5% group.

<table>
<thead>
<tr>
<th>Volume consumption per day (ml)</th>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
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<td>23</td>
<td>18</td>
<td>23</td>
<td>21</td>
<td>20</td>
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<td>DSS 2.5%</td>
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<td>18</td>
<td>25</td>
<td>16</td>
<td>27</td>
<td>11</td>
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</tbody>
</table>
Table 2. Effect of subcutaneous nicotine treatment on colonic TNF-α levels in DSS-treated mice. Colonic TNF-α levels [pg/mg protein] were expressed as mean ± SE (n = 6-8) after treatment with s.c. nicotine (0.1, 0.5 and 2 mg/kg) or vehicle. *p<0.05 compared to water group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Water group</th>
<th>Vehicle</th>
<th>Nicotine (0.1 mg/kg)</th>
<th>Nicotine (0.5 mg/kg)</th>
<th>Nicotine (2 mg/kg)</th>
</tr>
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<tbody>
<tr>
<td>TNF-α levels (pg/mg protein)</td>
<td>20 ± 3.7</td>
<td>78.6 ± 10.7*</td>
<td>82.1 ± 8.3*</td>
<td>68.2 ± 10.6*</td>
<td>89.7 ± 9.7*</td>
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</tbody>
</table>
Table 3. Effect of chronic nicotine infusion on colonic TNF-α levels in DSS-treated mice.

Colonic TNF-α levels [pg/mg protein] were expressed as mean ± SE (n=6-8) after treatment with chronic infusion of nicotine (2.5 and 25 mg/kg/day) or vehicle. *p<0.05 compared to water group. The nicotine treatment at dose of 2.5 or 25 mg/kg/day were not significantly different compared to the vehicle treated group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Water group</th>
<th>Vehicle</th>
<th>Nicotine (2.5 mg/kg/day)</th>
<th>Nicotine (25 mg/kg/day)</th>
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<tbody>
<tr>
<td>TNF-α levels (pg/mg protein)</td>
<td>20 ± 3.7</td>
<td>73 ± 12.2*</td>
<td>103 ± 8.3*</td>
<td>69.2 ± 9.4*</td>
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</table>
Table 4. Summary of plasma levels of nicotine and its metabolite cotinine after chronic nicotine treatment using s.c. injection, infusion (mini pumps) or oral routes of administration. Results are expressed as mean ± SEM of plasma concentrations in ng/ml.

<table>
<thead>
<tr>
<th></th>
<th>Injection (mg/kg)</th>
<th>Infusion (mg/kg/day)</th>
<th>Oral (µg/ml)</th>
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<tbody>
<tr>
<td>0.5</td>
<td>2</td>
<td>2.5</td>
<td>25</td>
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<tr>
<td>Nicotine (ng/ml)</td>
<td>51 ± 4.7</td>
<td>163 ± 12</td>
<td>13 ± 1.5</td>
</tr>
<tr>
<td>Cotinine (ng/ml)</td>
<td>45 ± 6.5</td>
<td>97 ± 7</td>
<td>23 ± 5</td>
</tr>
</tbody>
</table>
Table 5. Summary of plasma levels of cotinine after chronic oral cotinine treatment. Results are expressed as mean ± SEM of plasma concentrations in ng/ml.

<table>
<thead>
<tr>
<th>Dose (μg/ml)</th>
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<th>250</th>
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</thead>
<tbody>
<tr>
<td>Concentration (ng/ml)</td>
<td>89 ± 33</td>
<td>1336 ± 356</td>
</tr>
</tbody>
</table>
CHAPTER FOUR

THE ROLE OF α7 NICOTINIC RECEPTORS IN MOUSE DSS COLITIS MODEL

4.1. Introduction

Ulcerative colitis (UC) is an idiopathic, chronic, autoimmune disease characterized by mucosal inflammation primarily affects the colon and the rectum (Hanaue, 1996). Curiously, various studies have reported that cigarette smoking may have beneficial effects on UC (Harries et al., 1982; Boyko et al., 1988; Van Dijk et al., 1995; 1998; Gheorghe et al, 2004; Höie et al., 2007). In addition, nicotine patches and enemas have shown positive results on disease symptomology of UC in some clinical studies (Srivastava et al., 1991; Guslandi and Tittobello, 1998; Green et al., 1997; Sandborn et al., 1997). In experimental animal models of colitis, nicotine has been shown to be effective after oral administration in the treatment of intestinal inflammation (Qiu et al., 1997; Eliakim et al., 1998; Sykes et al., 2000; Ghia et al., 2006). Several in vivo and in vitro evidence suggest that α7 nicotinic acetylcholine receptors (nAChRs) may mediate the anti-inflammatory effects of nicotine (Wang et al., 2003; de Jonge et al., 2005; Ghia et al., 2009).

Recent studies shown that α7 nAChRs can regulate inflammation primarily through the vagus nerve acting as an endogenous “cholinergic (nicotinic) anti-inflammatory pathway”. This system ameliorates inflammation by regulating the production of pro-inflammatory cytokines mainly through activation of α7 nAChRs on macrophages surface (Borovikova et al., 2000; Tracey et al., 2001; Wang H et al., 2003, 2004; de Jonge et al., 2005; Ulloa, 2005). Studies reported that vagus nerve activation attenuates intestinal inflammation via α7 nAChRs in animal models of postoperative ileus and experimental colitis (de Jonge et al., 2005; Ghia et al., 2006; 2007). Abdrakhmanova et al., (2010) showed that α7 nAChRs is critical in suppressing hyper excitability of inflamed colonic sensory neurons from DSS-colitis mice.
In the view of this evidence for an important role of α7 nAChRs in inflammation, it was expected that α7 nAChR agonists would ameliorate colonic inflammatory diseases. However, Snoek et al., (2010) recently reported that although selective α7 nAChR agonists reduced cytokine responses in vitro, they worsened the effects of DSS-induced colitis or were ineffective in those of TNBS-induced colitis. These surprising results prompt us to reevaluate the role of α7 nAChR in colonic inflammation in mice. Many experimental factors such as the dose used, type and receptor efficacy of α7 nAChR agonist tested, administration method, disease severity and disease model, may explain this unexpected results. We therefore explored the role of some of these factors on the efficacy of α7 nAChR agonists in a mouse colitis model. We first assessed the role of α7 nAChR in the development of colitis using α7-knockout mice in the DSS model. We then tested the effects of α7 nAChR activation on colitis using selective α7 full agonists including PHA-543613 given systemically (Wishka et al., 2006) and choline given orally (Alkondon et al., 1997; Albuquerque et al., 1998; Fayuk and Yakel, 2004). We also evaluated the effects of PNU-120596, a type II α7 nAChRs positive allosteric modulator (PAM) in the DSS model. PAMs, in principle, can enhance endogenous α7 nAChR functions without altering the temporal integrity of neurotransmission or interacting directly with the α7 nAChR binding site. This property would allow enhancing a potential endogenous α7-mediated anti-inflammatory tone activated in the colitis model. The use of PNU-120596 would also help investigating the role of receptor desensitization since type II PAMs cause a dramatic slowing of receptor desensitization (Hurst et al., 2005; Bertrand and Gopalakrishnan, 2007; Sitzia et al., 2011).

4.2. Materials and Methods

For more details see Chapter 2.
4.2.1. Study 1: DSS-induced colitis model in α7 KO and WT nAChR mice.

DSS 2.5% (wt/vol) was added to the drinking water of wild-type and α7 KO nAChR mice for 7 days. On day 8, DSS was replaced with normal drinking water. Controls were all age- and time-matched and consisted of mice that received regular tap drinking water only for the corresponding number of days. The experimenter was blinded to the mouse genotype.

4.2.2. Study 2: Effect of chronic PHA-543613 and choline treatment in DSS-induced colitis model.

Male C57BL/6J mice pre-treated with either PHA-543613 (2, 8, and 20 mg/kg s.c. twice daily) or vehicle. A separate group of mice were treated with oral administration of choline (10, 40, and 80 µg/ml) in the drinking water 3 days before adding the DSS, and for 7 days along with the induction of colitis with DSS 2.5%. On day 8, DSS was replaced with normal drinking water.

4.2.3. Study 3: Effect of chronic PNU-120596 treatment in DSS-induced colitis model.

Male C57BL/6J mice pre-treated with either PNU-120596 (1, 3, and 6 mg/kg i.p. once daily), or vehicle, started 3 days pre-DSS treatment and for 7 days along with the induction of colitis with DSS 2.5%. On day 8, DSS was replaced with normal drinking water.

4.3. Results

4.3.1. DSS-induced colitis in α7 KO mice

In this experiment we assessed the progress of the clinical parameters during the DSS treatment in mice lacking α7 nicotinic receptors and their littermates’ wild type. α7 KO mice treated with 2.5% DSS displayed a significant increase in inflammation as expressed in the disease activity index value starting from day 5 till day 8 of the experiment [t(8)=2.85, p=0.0213] (Figure 11 A) and in the area under the curve [t(9)=5.788, p=0.003] (Figure 11 B) compared with the WT DSS-treated mice. Both α7 KO and WT DSS-treated mice displayed a significant loss of body weight, especially α7 KO from the control water-treated mice only (Figure 11 C). The decrease in
the colon length was significantly different between α7 KO and WT DSS-treated mice, even more significant in α7 KO against water-treated control group [F(2, 19)= 117.6, p<0.0001] (Figure 11 D). To assess the extent of inflammation in the colon, we evaluated the histological damage score and TNF-α colonic levels. The representative of colon histological damage changes were expressed as the total histological change score (Figure 12 A). α7 KO mice treated with DSS showed a significant increase in colonic total histological damage score versus the WT DSS and the water-treated control group, [F(2, 47) = 39.24, p<0.001] (Figure 12 A). The histological appearance of the colon after 7 days of DSS treatment in the WT mice was characterized by multifocal changes in the crypts and some areas showed focal lesions, depletion of goblet cells and inflammatory cell infiltrates, including neutrophils and lymphocytes; however, these changes with epithelial destruction were seen more shortening of the crypts, inflammatory cell infiltrates, extensive ulceration and the inflammation was mainly in the mucosa, and submucosal in colons from the α7 KO DSS-treated mice (Figure 12 B).

Next, we measured colonic TNF-α level to observe the influence of α7 nicotinic receptor subunit on colonic inflammation in DSS-induced colitis. In the line with the worsen in the clinical parameters and histological damage score in α7 KO DSS-treated mice. The α7 KO DSS-mice showed significant elevated colonic TNF-α levels, approximately 1 fold increased compared to WT DSS-treated mice [F(2, 39) = 32.64, p<0.001] (Figure 12 C).

4.3.2. Effect of α7 nAChR agonists and PAM in DSS colitis model:

4.3.2.1. Effect of PHA-543613 on DSS-induced colitis model in mice

Treatment with twice daily of s.c. PHA-543613, a selective α7 nAChRs agonist. At low dose of 2 mg/kg does not affect either clinical parameters of colitis severity (Figure 13 A and B) or the
colon length (Figure 13 C); however a higher dose of 8 mg/kg significantly decreased disease activity index scores on day 6, 7, and 8 compared to DSS-treated group \[F(2, 16) = 4.051, p=0.0377\] (Figure 13 A), and in the area under the curve value as well \[F(3,30) = 5.403, p=0.0043\] (Figure 13 B). In addition, at that dose, PHA-543613 was able to reverse the shortening of the colon length of DSS-treated group \[F(3, 19) = 7.495, p=0.0017\] (Figure 13 C). As the dose increased to 20 mg/kg, PHA-543613 worsened the disease activity and shortened the colon length in the DSS-treated mice. PHA-543613 at 8 and 20 mg/kg did not reduce the increase in colonic TNF-α levels seen with DSS-treated mice (Figure 13 D).

In this experiment we also assessed the effects of PHA-543613 on the colonic histological damage in the DSS-treated mice. PHA-543613 at both doses (8 and 20 mg/kg) has no effect on the total histological damage score induced by DSS treatment (Figure 14 A). Figure 14 B, showed histological appearance of the colon sections with H & E stain after PHA-543613 treatment in DSS-treated mice. The DSS-treated animals were showed ulceration and destruction of the mucosal architecture with inflammatory cells infiltrate of polymorphonuclear leukocytes including numbers of macrophages and lymphocytes were the inflammation was mainly in the mucosa, and more shortening of the crypts (Figure 14 B, b). However, PHA-543613 treatment at 8 and 20 mg/kg had no effect on the total histological damage score compared with vehicle DSS-treated animals, at high dose of 20 mg/kg, PHA-543613 produced extensive epithelial and architecture destruction mainly in the mucosa and submucosa with inflammatory infiltrates (Figure 14 B, c and d).
4.3.2.2. Effect of Choline on DSS-induced colitis model in mice

We then tested the effect of another α7 nAChRs agonist, choline given in the drinking water. As seen in Figure 15 A & B, low dose of oral choline treatment (10 µg/ml) has no significant effect on the severity of clinical parameters of DSS-induced colitis model (Figure 15 A & B) or the shortening of the colon length (Figure 15 C). However, choline at higher dose of 40 µg/ml, significantly decreased disease activity index values on day 7 and 8 compared to the DSS-treated group [F(3, 24) = 4.818, p=0.0092] (Figure 15 A), and in the area under the curve as well [F(3, 23) = 3.15, p=0.044] (Figure 15 B). In addition, that dose was able to reverse the shortening of the colon length of DSS-treated group [F(3, 20) = 7.812, p=0.0012]. As the dose increased to 80 µg/ml, the protective effect of choline on disease activity and colon length disappeared in the DSS-treated mice (Figure 15 A, B & C). Both doses (40 and 80 µg/ml) of choline did not reduce the increase in colonic TNF-α levels seen with DSS-treated mice (Figure 15 D).

Interestingly, choline treatment at 40 and 80 µg/ml significantly decreased the total histological damage score of the colon compared to the vehicle DSS-treated group [F(2, 97) = 11.43, p<0.001] (Figure 16 A). As seen in Figure 16 B, the DSS-treated animals showed destruction of the mucosa, and extensive inflammatory cell infiltrates (b) as compared to the water-control (a) group. Choline at 40 and 80 µg/ml reversed the histological damage and normalized the epithelial architecture of the colon (Figure 16 B; c and d, respectively).

4.3.2.3. Effect of α7 nAChR positive allosteric modulator (PAM) in DSS colitis model:

As with PHA-543613 and choline, low dose of PNU-120596 treatment (1 mg/kg, i.p.) did not reverse the DSS-induced colitis as measured by the disease activity index scores (Figure 17 A & B). However, at dose of 3 mg/kg, PNU-120596 significantly decreased disease activity index
scores on day 5, 6, 7 and 8 compared to DSS-treated group [F(3, 24) = 8.01, p=0.0007] (Figure 17 A). In addition, at this dose, it significantly reduced the area under the curve of disease activity index score compared with vehicle DSS-treated mice [F(3, 18) = 3.341, p=0.0425] (Figure 17 B). As the higher dose of 6 mg/kg, the protective effect of PNU-120596 on disease activity disappeared in the DSS-treated mice (Figure 17 A & B).

None of the PNU-120596 doses tested did reduce the increase in colonic TNF-α levels seen with DSS-treated mice (Figure 17 D). However, PNU-120596 treatment at 3 mg/kg significantly decreased the total histological damage score in DSS-treated mice [F(2, 67) = 3.88, p=0.0254] (Figure 18 A). At a higher dose of 6 mg/kg, PNU 120596’s effects on the total histological score failed to reach statistical significance. As shown in Figure 18 B, the extensive ulceration of the mucosa and increase in inflammatory cell infiltrates induced by DSS was reversed by PNU-120596 treatment (c).
Figure 11. Colonic inflammation was aggravated after DSS exposure in α7-deficient mice.

(A) Clinical evaluation of disease activity index of α7 wild type and Knock-out mice treated with 2.5% DSS in time-course manner. The DAI values were computed as described in detail in Methods. (B) Area under the curve of DAI. (C) Percentage of body weight change during DSS treatment period. (D) Colon length (cm) after DSS treatment. All clinical signs were assessed on daily basis for each mouse and were averaged per day for each group. Results are expressed as mean ± SE, n=6-8, *p<0.05. DAI = disease activity index, AUC = area under the curve, Ctrl = water-treated animals.
Figure 12. Histopathologic changes in colonic tissues and colonic TNF α level following 1 week of dextran sulphate sodium administration in α7 deficient mice.

(A) Histological damage score significantly increased in colon from α7 KO mice. (B) The histological appearance of the colon after DSS treatment in α7 WT and KO mice. Some epithelial architectural destruction with minimal submucosal inflammation in colonic tissue of wild type mice. In the α7 KO mice colon, shown extensive mucosal and submucosal injury with inflammatory infiltrate in the mucosa and submucosa. Histology alteration in mucosal tissue assessed using haematoxylin-eosin staining, (H&E staining; magnification X20). (Mean ± SE, n=6-8, p<0.05). (C) TNF α level [pg/mg protein], an acute inflammatory marker, was measured by enzyme-linked immunosorbent assay (ELISA). DSS induced colitis significantly increased the TNF α level in the homogenized colonic tissue especially in α7 deficient mice samples. (Mean ± SE, n=3 per group, p<0.05). Ctrl = water-treated animals.
Figure 13. Effect of PHA-543613 on DAI and inflammatory markers in C57Bl/6 mice with DSS colitis. Effect of chronic PHA-543613 treatment on (A) the time-course of Disease activity index. (B) Area under the curve of disease activity index in mice. (C) Mean colon length (cm). (D) Colonic TNF-α levels (pg/mg protein) in the homogenized colonic tissue samples. Results are expressed as mean ± SE, n=6-8, *p<0.05 compared to Veh. #p<0.05 vs. Ctrl in (C) and vs. Veh in (D). Ctrl = water-treated animals, Veh = vehicle DSS-treated mice, s.c.= subcutaneous.
Figure 14

Figure 14. Histological analysis of representative colons from C57Bl/6 mice after 7 days of exposure to water, vehicle, or PHA-543613 treatment.

(A) The total histological colon damage score after PHA-543613 in C57Bl/6 mice. (B) Appearance of a control colon (a), DSS-treated mice (b), and colon from PHA-543613 treatments (8 and 20 mg/kg; c and d respectively) in DSS-treated mice. (Haematoxylin-eosin staining of colonic tissue sections, magnification: X40, Scale bar 20µm). Results are expressed as mean ± SE, n=3, #p<0.05 vs. Ctrl. Ctrl = water-treated animals, Veh = vehicle DSS-treated mice.
Figure 15. Chronic oral choline treatment suppressed the severity of DSS-induced colitis in mice.

Effect of oral choline treatment on (A) the time-course of disease activity index, (B) Area under the curve of disease activity index, (C) Mean of colon length (cm). (D) Colonic TNF-α levels [pg/mg protein] in the homogenized colonic tissue samples. Results are expressed as mean ± SE, n=6-8, *p<0.05 vs. DSS group and #p<0.05 vs Ctrl group. Ctrl = water-treated animals, Veh = vehicle DSS-treated mice, po = oral.
Figure 16

Figure 16. Histological analysis of representative colons from C57Bl/6 mice after 7 days of exposure to water, vehicle, or choline treatment in C57Bl/6 mice with DSS colitis.

(A) The total histological colon damage score after oral choline treatment in mice. (B) Histological appearance of a control colon (a), DSS-treated mice (b), oral choline (40 µg/mL) in DSS-treated mice (c), oral choline (80 µg/mL) treatment in DSS-treated mice (d). (Haematoxylin-eosin staining of colonic tissue sections, magnification: X20). Results are expressed as mean ± SE, n=3, #p<0.05 vs. Ctrl. Ctrl = water-treated animals, Veh = vehicle DSS-treated mice.
Figure 17. Effect of PNU-120596 on DAI, colonic TNF-α level and colon length in C57Bl/6 mice with DSS colitis.

Effect of PNU-120596 treatment on (A) the time-course of disease activity index in DSS-treated mice. (B) Area under the curve of disease activity index, (C) Mean of colon length (cm). (D) Colonic TNF α level [pg/mg protein] in the homogenized colonic tissue samples. Results are expressed as mean ± SE, n=6-8, *p<0.05 vs. Veh. #p<0.05 vs. Ctrl. Ctrl = water-treated animals, Veh = vehicle DSS-treated mice.
Figure 18. Influence of PNU-120596 on histological damage score in C57Bl/6 mice with DSS colitis (A) The total histological colon damage score after chronic PNU-120596 treatment in DSS-treated mice. (B) The histological appearance of normal colon (a), vehicle DSS-treated mice (b), PNU-120596 (3 mg/kg) in DSS-treated mice (c), PNU-120596 treatment (6 mg/kg) in DSS-treated mice (d). Results are expressed as mean ± SE, n=3 per group, p<0.05. (Haematoxylin-eosin staining of colonic tissue sections, magnification: X40, Scale bar 20µm). #p<0.05 vs. Ctrl group, and *p<0.05 vs. Veh group. Ctrl = water-treated animals, Veh = vehicle DSS-treated mice.
CHAPTER FIVE

Role of non-α7 Nicotinic Receptors in Experimental Colitis

5.1. Overview

Nicotine exerts its effect through acting on various subtypes of nicotinic receptors in multiple sites, centrally and peripherally. As described before in Chapter 1, these nicotinic receptors are express in the CNS, PNS, and non-neuronal tissues, such as immune cells. Human lymphocytes express mRNA for α3, α4, α5, and β4 nAChR subunits (Hiemke et al., 1996; Mihovilovic et al., 1998; Mihovilovic and Roses, 1991, 1993). Human mononuclear leukocytes express mRNAs encoding α2 and α5 nicotinic subunits (Sato et al., 1999). α9 and α10 nicotinic subunits have restricted anatomical tissue distribution profile among the nicotinic receptors. While they have not been detected in the brain and spinal cord (Elgoyhen et al., 2001; Sgard et al., 2002), they are expressed in human T cells, blood lymphocytes, monocytes, macrophages (Lustig et al., 2001, Peng et al., 2004; Galvis et al., 2006; Kawashim et al., 2007), and in dorsal root ganglion (DRG) neurons (Lips et al., 2002).

Nicotinic receptor subunits are also expressed in the ENS. For example, mRNA for α3, α5, β2 and β4 have been detected in the myenteric plexus of large intestine and α3, α5 and β4 expression in some ganglia of the submucosal plexus of large intestine in rat; however, there was no evidence for expression of α4, α6 and β3 mRNA in these tissues (Garza et al., 2009). Furthermore, nicotinic receptors have been identified in colonic epithelial HT29 cells, including α3, α5 nAChR subunits (Summers et al., 2003).

The distribution pattern of these various non-α7 nicotinic receptors suggests a possible role for them in the process of colitis.
One promising target is \( \alpha_9 \) and \( \alpha_{10} \) nicotinic acetylcholine receptor especially after the discovery of a novel selective \( \alpha_9\alpha_{10} \) conotoxin antagonist. \( \alpha \)-Conotoxins are small neurotoxic disulfide rich peptides derived from the marine cone snails venom of Conus species (Terlau and Olivera, 2004). Alpha-conotoxin RgIA (\( \alpha \)-RgIA) is one of three reported \( \alpha \)-conotoxins that block the \( \alpha_9\alpha_{10} \) nicotinic receptors and is the most potent \( \alpha_9\alpha_{10} \) nAChR subtypes antagonist with an \( IC_{50} \) 5.2 nM in Xenopus oocytes (Ellison et al., 2006; Vincler et al., 2006; Nevin et al., 2007). Interestingly, nicotine acts as an antagonist of rats \( \alpha_9 \) and \( \alpha_9\alpha_{10} \) nAChRs (Elgoyhen et al., 1994, 2001; Sgard et al., 2002; Verbitsky et al., 2000). Recent studies have provided evidence that administration of \( \alpha_9\alpha_{10} \) selective \( \alpha \)-conotoxin antagonists produce antinociceptive and anti-inflammatory effects in neuropathic and tonic inflammatory pain animal models (McIntosh et al., 2009; Vincler et al., 2006; Vincler and McIntosh, 2007). Immune cells seem to mediate the anti-inflammatory effect of \( \alpha_9\alpha_{10} \) nAChRs antagonist. Indeed, blockade of \( \alpha_9\alpha_{10} \) nAChRs in chronic constriction injury animals showed a decrease of lymphocytes and macrophages at the site of nerve injury (Vincler and McIntosh, 2007; Vincler et al., 2006). The activation of the functional cholinergic receptors in leukocytes might mediate the nicotinic anti-inflammatory pathway through these immune cells. While the function of \( \alpha_9\alpha_{10} \) nAChRs in the auditory system has been well characterized (Elgoyhen AB et al., 2001), little is known regarding the function of \( \alpha_9\alpha_{10} \) nAChRs in immune cells and inflammation. The \( \alpha_9\alpha_{10} \) nAChR selective antagonist, \( \alpha \)-conotoxin RgIA, enabled us to explore the role for \( \alpha^* \) nAChRs in animal DSS colitis model.

Here, because of the expression and distribution of non-\( \alpha_7 \) nicotinic receptors in peripheral nervous system and non-neuronal tissues; we examined the role of \( \alpha_5 \) and \( \beta_2 \) nAChR subunits using \( \alpha_5 \)- and \( \beta_2 \)-deficient nicotinic subunits KO mice using DSS-induced colitis model. In
addition, we explored the role of α9 nAChR subtypes by testing the effect of α-conotoxin RgIA, a α9α10 nAChR selective antagonist, in C57Bl/6 DSS-treated mice.

5.2. Materials and Methods.

See Chapter 2 for more details.

5.2.1. Study: Role of non-α7 nicotinic receptor (α5, β2, and α9) subtypes in DSS-induced colitis model in mice. We induced colitis in α5 and β2 nicotinic receptor KO mice, and their littermates wild-type nicotinic receptor male adult mice were given DSS solution freely in the drinking water for 7 consecutive days after which tap water was given on the 8th day. α-RgIA was administered s.c. once daily for 10 days in C57Bl/6 male mice, started 3 days pre-DSS and 7 days with DSS treatment. α-RgIA doses range (0.02, 0.1, 0.2 mg/kg). We measured the clinical parameters every day over the time-course of the study and the colon length at the end of the study.

5.3. Results: There were no significant differences in the disease activity scores and the area under the curve of disease activity seen between the β2, and α5 deficient mice and their littermates WT DSS-mice (Figure 19 A-D). In addition, colon shortening was not affected in β2, and α5 KO subunits DSS-treated mice (Figure 19 E and F).

Repeated subcutaneous treatment with a low dose of 0.02 mg/kg of α-RgIA once daily produced a significant decrease of disease activity score on day 8 and in the area under the curve (Figure 20 A & B). However, this effect was lost at higher doses of α-RgIA (0.1 and 0.2 mg/kg). Both high doses had no effect on the disease activity score. α-RgIA doses of 0.02 and 0.1 mg/kg in DSS-treated mice did not have a significant loss of body weight compared with the vehicle DSS-treated mice (Figure 20 C); however, at higher dose of α-RgIA (0.2 mg/kg) mice displayed
a significant percentage of body weight loss from the DSS-treated mice on day 8. α-RgIA at any of the tested doses had no effect on the shortening of the colon length of DSS-treated mice (Figure 20 D).

Chronic subcutaneous α-RgIA administration at dose of 0.02 mg/kg had no effect on reducing the total histological colonic damage score; interestingly, at higher dose of 0.2 mg/kg, α-RgIA showed a significant decrease in total histological damage score in a dose-dependent manner (Figure 21 A). At all tested doses, α-RgIA did not reduce the colonic TNF-α pro-inflammatory cytokine levels; interestingly, at low dose of 0.02 mg/kg, RgIA did not increased or decreased TNF-α levels (Figure 21 B).
Figure 19. Clinical evaluation of non-α7 nicotinic receptor subtypes, particularly α5 and β2 in DSS-induce colitis model in mice.

(A, C) The time-course of Disease activity index (DAI). (B, D) Area under the curve of DAI. (E, F) Colon length (cm). All clinical signs were assessed on daily basis for each mouse and were averaged per day for each group. Results are expressed as mean ± SE, n=6-8, *p<0.05. DAI = disease activity index, AUC = area under the curve.
Figure 20. Effect of repeated subcutaneous α-RgIA administration on the severity of DSS-treated C57BL/6J mice

Effects of chronic subcutaneous α-RgIA treatment (0.02, 0.1 and 0.2 mg/kg) on (A) the time-course of DAI. (B) AUC of DAI. (C) loss of body weight, and (D) Mean colon length (cm). Results are expressed as mean ± SE, n=6-8, *p<0.05 vs. Veh group. Ctrl = water-treated animals, Veh = vehicle DSS-treated mice.
Figure 21. Effect of repeated subcutaneous α-RgIA treatment in DSS-treated C57BL/6J mice on histological damage, and inflammatory marker.

(A) Histological colonic damage score in DSS-induced colitis, (B) Colonic TNF-α levels (pg/mg protein) in the homogenized colonic tissue samples. Results are expressed as mean ± SE, n=6-8, #p<0.05 vs. Ctrl group, and *p<0.05 vs. Veh group. Ctrl = water-treated animals, Veh = vehicle DSS-treated mice.
CHAPTER SIX

General discussion, summary and future studies.

The identification of nicotinic acetylcholine receptors (nAChRs), their distributions, structures, and functions increased understanding of the complexity of these receptors. The expression of nAChRs in the enteric nervous system (ENS) and immune cells suggest that they may play an important role in the gut regulation and inflammatory processes. Several clinical trials have tested nicotine efficacy in UC patients in various pharmaceutical formulations, including gum, transdermal patches, enemas (Perera et al., 1984; Lashner et al., 1990; Srivastava et al., 1991; Guslandi and Tittobello, 1994, 1996; Pullan et al., 1994; Thomas et al., 1995, 1996; Sandborn et al., 1997; Green et al., 1997; Ingram et al., 2005). It has been thought that nicotine alleviates UC through acting on nicotinic receptors in the gut immune cells (Thomas et al., 2005). The expression of nAChRs on lymphocytes, such as T-cells play a role in immunocytes differentiation, development, and function (Fujii et al., 2008). In addition, an earlier study showed that CD4 T cells have a role in experimental animal model of colitis (Elson et al., 1998).

Nicotine may have possible effects on various immunocytes of the immune system. In fact, immune cells express different nAChR subtypes. For example, α7 nicotinic receptors are present in intestinal lamina propria lymphocytes (Kikuchi et al., 2008) which may have important role in the pathogenesis and signaling pathway of intestinal inflammation.

6.1. DSS-induced colitis model dose-response curve in mice

Previous studies used different concentrations of DSS solution in animals (Ahmad et al., 2000; Araki et al., 2000; Egger et al., 2000). Furthermore, in the present study we demonstrated that as the DSS concentration increased, the severity of disease increased, including bloody diarrhea,
rectal irritation, body weight loss, and colonic histolopathology of mucosal injury (Egger et al., 2000). In the present study, we showed that 7 days of 2.5% DSS administration in the drinking water resulted in an acute inflammation of the colon with low rate of mortality. We showed all of the clinical signs of disease activity manifested by the loss of body weight, bloody diarrhea, rectal irritation, shortening of the colon, ulceration in the colonic mucosa which allows the infiltration of inflammatory cells into the lamina propria, including neutrophils and lymphocytes. The migration of neutrophils into the mucosa and the epithelial surface cause intestinal inflammation. To further examine the extent of the inflammation in the colon, we measured two inflammatory markers, colonic MPO activity and TNF α level. MPO is an enzyme in neutrophils used as a marker of inflammation and an index of neutrophil infiltration (Bradley et al., 1982). A reduction in the activity of this enzyme can be considered as an anti-inflammatory activity of the given treatment. TNF α is an early pro-inflammatory mediator, and plays a critical pathological role in the colonic inflammation. Both inflammatory markers were significantly increased after 7 days of DSS treatment in a dose-related fashion.

6.2. Influence of nicotinic dose and route of administration in mouse DSS colitis model

One of the objectives of this study was to determine the pharmacology of protective effects of nicotine in the DSS-induced colitis mouse model. In addition, we assessed whether cotinine, the main metabolite of nicotine, was effective in this model. The results suggest that low doses of nicotine delivered orally, but not cotinine, are most effective in rendering a protective effect in the mouse DSS colitis model. Our results demonstrated that the dose and route of administration play a pivotal role in nicotine’s in vivo anti-inflammatory efficacy in the DSS model of colitis. While the continuous infusion of a low dose of nicotine (2.5 mg/kg/day) through mini pumps resulted in a modest and short improvement of clinical signs and
histological damage, the effect was not observed at the higher dose of nicotine (25 mg/kg/day). Similarly, chronic s.c. injection of low dose of nicotine at 0.1 mg/kg resulted slightly improvement in clinical signs; however failed to reach statistical significant and at 0.5 mg/kg resulted in a reversal of the shortening in colonic length. This effect was not observed at the high dose of 2 mg/kg of nicotine. However, nicotine treatment in both routes of administration did not effect a reversal in DSS-induced increase in colonic TNF-α levels. In contrast, oral nicotine treatment attenuated dose-dependently DSS-induced colitis. The low doses of nicotine (12.5-50 µg/ml) were shown to exert a protective effect; they resulted in a reduction in DAI and histological architectural damage and in a reduction in colonic TNF-α levels. However, the protective effects of nicotine in the DSS colitis model dissipated at the high dose of 100 µg/ml. Interestingly, nicotine plasma levels increased only 1.5-fold between the doses of 25 and 100 µg/ml (Table 4) suggesting a narrow concentration-effect profile for oral nicotine in the DSS model. Curiously, oral nicotine treatment at any of the doses tested failed to reverse significantly the shortening in the colon length in the DSS-treated group.

The biphasic effect of chronic nicotine treatment on clinical parameters in DSS-induced colitis in mice was not unexpected. The low doses of oral nicotine (12.5–25 µg/ml) were shown to be protective while the higher doses were not. The low doses administered in drinking water resulted in a reduction of DAI, histology architectural damage and colonic TNF-α levels, whereas the high dose (100 µg/ml) resulted in an exacerbation of DSS colitis including the DAI and the AUC. Exacerbation of DAI at high doses of nicotine was not observed in s.c. or minipump nicotine-treated animals, suggesting a differential effect of nicotine dose and delivery on colonic inflammation. The results indicative of protective effects of low dose of nicotine in the drinking water were similar to those reported previously in various experimental colitis
models in rats and mice (Qiu et al., 1997; Eliakim et al., 1998; 2001; Sykes et al., 2000; Ghia et al., 2006). Qiu et al., (1997) demonstrated that nicotine treatment at a dose of 5 ug/ml in the drinking water in DNBS-treated rats resulted in a significant decrease in the intestinal inflammation; however, the inflammation was shown to increase at a dose of 50 ug/ml. Similarly, Eliakim et al., (1998) and Sykes et al., (2000) reported that nicotine had a dual effect on colitis in mice with low doses of nicotine being more effective at reducing the inflammation than higher doses. This dual anti-inflammatory effect of nicotine in the form of an “inverted-U,” has been described previously in behavioral effects of nicotine (Picciotto, 2003).

Overall, our results suggest that the route of administration of nicotine is an important factor to consider in the treatment of colitis. The lack of a clear anti-inflammatory effect of nicotine after s.c. and minipump infusion in the DSS model, could explain some of the inconsistent results that have been reported previously in rodent (Snoek et al., 2010) and human studies. Notably, the marginal effects observed after infusion of a constant dose of nicotine through minipumps is consistent with lack of effects of nicotine using transdermal patches in some UC studies (Pullan et al., 1994; Cosnes et al., 2004; McGilligan et al., 2007), suggesting that a route of administration that mimics nicotine intake profile seen in smokers, could result in an increase in the efficacy of treatment. Indeed, these and other (Eliakim et al., 1998; 2001; 2002; Sykes et al., 2000; Ghia et. al., 2006) results suggest that administering nicotine orally is a very efficacious route in reversing colitis in rodent models. This outcome is consistent with that obtained through UC studies using rectal administration of nicotine. A possible explanation for these results is that nicotine targets and affects the colon locally when administered orally with marginal therapeutic index. Other possible mechanisms of nicotine effects have been proposed. For example, the beneficial effects of oral nicotine treatment could be due to nicotine exerting
anti-inflammatory effects partially through changes in colonic TNF-α levels or through inhibition of release of TNF-α and other pro-inflammatory cytokines from cytokine-producing cells. In the mouse colitis model used in this study, colonic TNF-α levels were reduced significantly by the low dose of oral nicotine treatment, which is most likely mediated via suppression of pro-inflammatory cytokine release from macrophages and other cytokine-releasing immune cells as part of the initial innate immune response to DSS.

Although the colon may be an important site of action for nicotine following oral administration, the drug likely acts on many peripheral and central inflammatory pathways. Indeed, nicotine is absorbed in the small intestine with low bioavailability (up to 45%) because of a hepatic first pass effect (Benowitz et al., 1991; Compton et al., 1997; Zins et al., 1997). In the present study, oral administration of nicotine in the drinking water yielded measurable levels of nicotine in the plasma. For example, a plasma nicotine concentration of 18 ng/ml was found after an oral administration of an active dose of 25 µg/ml of the drug.

It has been proposed that some nicotine metabolites contribute to the beneficial effect of nicotine in UC patients, since the majority of nicotine is converted to cotinine (about 80%) through the cotinine pathway and is present in smokers in blood at much higher concentrations (about 250 to 300 ng/ml) than nicotine (Benowitz et al., 1983; Gori and Lynch, 1985; Benowitz and Jacob, 1994). In the present study, this hypothesis was tested by administering cotinine directly in the drinking water. The results show that oral cotinine administration has no protective effect in the mouse colitis model based on clinical parameters or measurement of colon length. Plasma concentrations after 10 days of cotinine oral administration of 25 and 250 µg/ml were 90 and 1336 ng/ml, respectively. These plasma levels cover well and above the concentration range of cotinine found after nicotine administration protocols used in our various
studies. The lack of effect of cotinine suggests that this major metabolite does not mediate the anti-inflammatory effects of nicotine reported in rodents and human studies. However, other metabolites such as nornicotine, which is a component in tobacco as well as a minor systemic metabolite of nicotine and is a nAChRs agonist (Papke et al. 2007), cannot be ruled out of the effects of nicotine.

Overall, our results with nicotine suggest that the protective effects of the drug are influenced by the route of administration and the dose of exposure. While nicotine is a non-selective agonist at the various nAChRs subtypes, several in vitro and in vivo evidence suggest that α7 nAChRs subtypes mediate its anti-inflammatory effects. In addition, it has been reported that α7 nicotinic receptor subunit may play an important role in experimental colitis (Ghia et al., 2006; 2007) and its effect mediated through activation of the cholinergic anti-inflammatory pathway (Wang et al., 2003; de Jonge et al., 2005; Kelso et al., 2006; Hamano et al., 2006). We therefore investigated the role of α7 nAChRs in the development of experimental colitis in mice.

6.3. Influence α7 nicotinic receptors in mouse DSS colitis model

The results presented in our study show that mice lacking α7 nicotinic receptor develop a marked increase in clinical colitis scores and colonic inflammation markers in the mouse DSS model. In contrast, α7 nAChRs agonists and PAM reduced the severity of the disease at a narrow range of doses. Taken together, these results strongly suggest an important role for a cholinergic anti-inflammatory pathway in the development of colitis, which at least in part is mediated by α7 nAChRs.

In the present study, we showed that α7 KO mice treated with 2.5% DSS have a significant increase in the disease activity index scores and decrease in the colon length; however, there were
no body weight difference between the α7 deficient and WT mice treated with DSS. This was accompanied by an increase in colonic histological damage and elevated pro-inflammatory cytokine levels of TNF-α. TNF-α is an early pro-inflammatory mediator that plays an important role in immuno-inflammatory responses and in the pathogenesis of UC (Yokota et al., 1988; and Anand and Adya, 1999). These results are consistent with recent data from various animal models of inflammation that point towards the α7 nAChR subtypes as an important player in cholinergic modulation of inflammation (Wang et al., 2003; Wang et al 2004; Ulloa, 2005; de Jonge et al., 2005; van Maanen et al., 2010). They are also in agreement with Ghia et al., (2009) study that showed that α7-deficient mice treated with DSS for 5 days showed an exaggeration of the colitis severity with an increase in the colonic inflammation including histological damage score, pro-inflammatory cytokine, IL-1β colonic levels and MPO activity compared with the WT mice. In addition, nicotine pretreatment counteracted the colitis inflammation in WT and lacks its effect in the α7 KO mice.

In line with our α7 KO observations, α7 nAChR agonists and PAM (PHA-543613, PNU-120596 and choline) were effective in reversing the clinical parameters of colitis and shortening of the colon induced by DSS in mice; however, PAM did not reverse the shortening of the colon length. This reduction was less pronounced with histopathology damage scores and levels of colonic TNF-α. The antinflammatory effects of all three α7 nAChR ligands occur at narrow dose ranges with U-shape dose-response relationships, with choline being relatively the most effective in that regard. Choline is an essential nutrient, a cell membrane constituent, a precursor in the biosynthesis of acetylcholine, and a selective natural full α7 nAChR agonist (Papke et al., 1996 and Alkondon et al., 1997). Interestingly, choline level is low in the colonic tissues from UC patients (Bjerrum et al., 2010). When choline administered orally, it is absorbed in the intestine
(Lekim and Betzing, 1976; Pelech and Vance, 1984; Tian et al., 2012) and its uptake is localized in the neural part of the intact intestine of the guinea pig small intestine (Snyder et al., 1973; Pert and Snyder, 1974). Recently, the anti-inflammatory efficacy of choline in murine endotoxemia and sepsis and its modulation of TNF release require α7 nAChR-mediated signaling (Parrish et al., 2008). Possibly, the lack of choline protective effect could contribute, at least in part in the development of colitis in active UC.

Our data with PNU-120596, a type II α7 nAChR PAM, suggest the presence of a pro-“anticolitis” endogenous tone mediated by α7 nAChRs. Generally speaking, PAMs are compounds that facilitate endogenous neurotransmission and/or enhance the efficacy and potency of agonists without directly stimulating the agonist binding sites. PNU-120596 may be acting in the DSS test through the enhancement of sub-threshold concentrations of endogenous α7 agonists, choline and/or ACh (Sarter and Parikh, 2005; Parikh and Sarter, 2006). Supporting the possibility of choline, PNU-120596 was recently reported to enhance the effects of sub-threshold, physiological concentrations of choline on native α7 nAChR in hypothalamic neurons (Gusev and Uteshev, 2010).

Our results are at odds with the recent study of Snoek et al., (2010) who reported that while AR-R17779 and GSK1345038A, two selective α7 nAChR agonists, reduced cytokine responses in vitro, they worsened the effects of DSS-induced colitis at low doses. The highest doses of these α7 nAChR agonists ameliorated clinical parameters, without affecting colonic inflammation. Many factors could account for the differences between the two studies. The route of administration does not seem to play a major role since the protective effects in the DSS model in our study was seen with α7 nAChR agonists after both systemic (via s.c. and i.p) and oral (via drinking water) routes of administration. The efficacy at α7 nAChRs of the agonists used in the treatment is
another possible factor. While no information on the pharmacological profile at nAChRs was available for GSK1345038A, AR-R17779 is a potent and selective partial (approximately 70% efficacy) α7 nAChR agonist (Papke et al., 2004). Both of our α7 nAChR agonists used are full α7 nAChR agonists. However, two major differences could have played an important role: the sex of the mice used and the disease severity.

In our study, we used male mice to investigate the role of α7 nAChRs in experimental colitis, while α7 nAChR agonists were tested in female mice in Snoek et al., (2010) report. This is an important difference that could account for the lack of effects seen in the later study. Indeed, sex differences to nicotine and nicotinic agonists in mice and rats were reported in pain (Craft and Milholland, 1998; Damaj, 2001) and dependence measures and behaviors (Isiegas et al., 2009; Lopez et al., 2003). Finally, Snoek et al., (2010) used a relatively low concentration of DSS (1.5%) to induce experimental colitis in C57 female mice compared to 2.5% in male C57 mice used in our study. The differences in severity of the disease may have affected susceptibility to DSS-induced lesions and modified the efficacy of α7 nAChR agonists.

The narrow window of anti-inflammatory effects of α7 nAChR agonists and PAM as shown by their U-shaped dose-effect curves in our results with the DSS test, was surprising. While drug distribution and metabolism factors could account for such dose-response profile, the same phenomenon was seen with three structurally different α7 nAChR agonists. This narrow window could have been related to the desensitization properties of α7 nAChRs agonists reported in vitro. However, a similar profile was observed with PNU-120596, a type II PAM known to cause a dramatic slowing of receptor desensitization (Bertrand and Gopalakrishnan, 2007). Importantly, this narrow U-shaped dose-effect curve was not seen with α7 nAChRs agonists and PAMs in
animal models of cognition and pain (Rowley et al., 2010; Feuerbach et al., 2008; Timmermann et al., 2007). Thus, making this phenomenon unique to the DSS experimental colitis in mice.

In conclusion, in the DSS colitis model, we found that $\alpha_7$ nAChR signaling may, at least in part play an important role in the protection against colitis development in mice. $\alpha_7$ nAChR agonists and PAM affected the clinical features of the DSS colitis disease and produced a U-shaped dose-response curve. Our results suggest that targeting $\alpha_7$ nAChRs is a viable therapeutic approach for intestinal inflammation diseases such as ulcerative colitis treatment.

Even though it has been established that $\alpha_7$ nAChR is an essential component of the vagus nerve activity in regulating cytokine production in the periphery (Tracey, 2007), nicotine can have multiple nAChR-stimulated pathways. It has been reported that nicotine can mediate its anti-inflammatory effect in immune cells such as macrophages through a non-$\alpha_7$ nicotinic receptors manner (Matsunaga et al., 2001), for example, van der Zanden et al., (2009) demonstrated that $\alpha_4\beta_2$ nicotinic receptors mediate cholinergic anti-inflammatory effects in intestinal macrophages. Recently, it has been reported that $\alpha_4\beta_2$ receptors partially mediated the nicotine anti-inflammatory effect through JAK2-STAT3 signaling pathway (Hosur and Loring, 2011).

6.4. Involvement of non-$\alpha_7$ nicotinic receptors in inflammation and experimental colitis model

In our study, $\beta_2$, and $\alpha_5$ nAChR subunits appear to not play a crucial role in the development and expression of colitis severity since there were no differences seen in either of the clinical parameters of disease activity score or the colon length in the $\beta_2$, and $\alpha_5$ deficient mice and their littermates WT DSS-treated mice. However, Orr-Urtreger et al., (2005) reported the involvement
of α5, but not β4 nAChRs, in experimental colitis. In which, they reported that the percentage of mice body weight loss and the disease activity score after DSS treatment were higher in α5 KO but not β4 KO nAChRs mice. The reason why α5 KO mice in their study displayed a pronounced effect in increasing the disease severity, shortening of the colon length, colonic MPO activity, and in histological damage score in DSS colitis model is unclear. The different adapted scoring systems for disease severity and histopathological damage from different experimental colitis models, may explain the varying observations. Here, we used similar DSS concentration, but possibly their α5 null mice background generation differ from our α5 KO mice. In addition, they did not report a mouse sex and strain they used in their study, since mouse strains have different susceptibility to DSS-induced colitis model (Mahler et al., 1998). Moreover, their adapted disease activity index scoring system (Rachmilewitz et al., 2002) included only two clinical parameters, body weight loss and rectal bleeding; however, our combined score has four clinical features of colitis for disease activity index (Cooper et al., 1993). In addition, their scoring system for the histology adapted from different model of colitis (Karmeli et al., 2000); however, we evaluated the total histological damage degree (Iba Y et al., 2003) for more details see Chapter 2, materials and methods section. Furthermore, in their study, nicotine reduced disease activity score in α5 null mice, but not in WT, noteworthy, they used similar dose and route of nicotine administration that we used and also has been reported to be effective in several experimental colitis models (Eliakim et al., 1998, 2001; Qiu et al., 1997; Ghia et al., 2006). Moreover, nicotine in their study did not reduce colonic MPO activity in the WT mice; however, Qiu et al. (1997) reported that oral nicotine (5-20 ug/ml) reduced MPO activity and colonic damage score. Similar observation reported by Ghia et al. (2006) after oral nicotine administration (20 ug/ml).
In our study, \( \alpha 9 \) and \( \alpha 10 \) nicotinic receptor subtype seems to play a role in colitis severity and inflammation since chronic administration of \( \alpha \)-RgIA, a \( \alpha 9\alpha 10 \) selective antagonist, at low dose of 0.02 mg/kg attenuated the clinical parameters in DSS colitis mice. However, this dose did not affect colonic inflammation including total histology damage score or colonic TNF-\( \alpha \) levels. On the other hand, at high dose (0.2 mg/kg), \( \alpha \)-conotoxin RgIA had neither effect on the colitis severity nor colonic TNF-\( \alpha \) level. However, it significantly decreased the total colon histological damage score in a dose-dependant fashion. \( \alpha \)-conotoxin RgIA at all tested doses (0.02, 0.1, 0.2 mg/kg) was unable to reverse the shortening of the colon length of the DSS-treated mice. DSS can cause colonic inflammation which may be due to the increase of the cholinergic cells number at the site of inflammation. It has been reported that \( \alpha 9\alpha 10 \) nicotinic acetylcholine receptor antagonists decreased the number of immune cells such as macrophages and lymphocytes, and positive choline acetyltransferase cells at the site of nerve injury (Vincler et al., 2006). RgIA may possibly act by a similar mechanism in the DSS-induced colitis model, that is, through antagonism of \( \alpha 9\alpha 10 \) nicotinic acetylcholine receptors on immune cells to reduce the inflammation development and disease activity. \( \alpha \)-RgIA possibly mediate its anti-inflammatory effect through immune system mechanisms; however, it not clear yet.

6.5. Summary

In summary, we demonstrated that seven days of DSS treatment induces acute colitis in C57BL6 mice. Chronic oral administration of low doses of nicotine in the drinking water was protective in DSS-induced colitis in mice as shown in the clinical parameters, inflammation markers and histological features. However, modest effects were seen after intermittent s.c injection or continuous infusion of nicotine. Collectively, these results highlight that dose and route of administration play a critical role in the protective effect of nicotine in intestinal
inflammation. Developing novel pharmaceutical formulation regimens such as topical nicotine administration to the colon (enema) could lead to decreased adverse effects seen with systemic nicotine use and ameliorate the clinical efficacy of nicotine in UC conditions. Also, the lack of effect of cotinine suggests that this major metabolite does not mediate the anti-inflammatory effects of nicotine reported in rodents and human studies.

In DSS colitis model, we found that the lack of α7 nAChR subtype is probably, at least in part a source of colitis development in α7 deficient mice. Our findings supported by using pharmacological evidence, where we found that selective α7 nAChR agonists PHA-543613 and choline reduced clinical parameters in DSS-treated mice and inhibited the increase in the colonic TNF-α levels. A similar profile was observed with of PNU-120596, a positive allosteric modulator for α7 nAChRs. Our results showed that these α7 nAChR agonists were protective in the DSS model at a narrow dose range with a U-shaped dose-response curve.

For the non-α7 nAChRs; our results suggest that both β2 and α5 nAChR subunits do not seem to play a role in colitis severity. Importantly, our data demonstrated for the first time that α9 nAChR subtypes may play an important role in experimental colitis. The α9 nAChR selective antagonist α-RgIA reduced the clinical parameters in the area under the curve of disease activity scores. Similarly to α7 nAChRs agonists, a U-shaped dose-response curve was observed with α-RgIA treatment.

To our knowledge, we are the first to report the importance of the dose and route of nicotine administration and the effect of cotinine, the α9α10 nicotinic acetylcholine receptor antagonist, and the positive allosteric modulator for α7 nAChR in the DSS-induced colitis model in mice.
6.6. Future Directions

This research opens up new avenues and strategy for experimental and clinical colitis research. Our data clearly showed that α7 nAChRs agonists and PAMs are good therapeutic candidates for treatment of colonic inflammation diseases such as UC. Indeed, the current UC conventional therapy has poor efficacies and associated with undesirable adverse effects, or contraindicated in some UC patients. However, further research is needed to understand the underlying mechanisms of the role of α7 nAChRs in colitis. For instance, nicotine has multiple targets, actions, and mechanisms to produce its anti-inflammatory effects. Investigating the site of action of the anti-inflammatory effects of nicotine, α7 nAChRs agonists and PAMs can clarify the immune and non-immune targets of these drugs. For example, isolation of lamina propria and spleen mononuclear cells by gradient centrifugation or whole blood from C57BL/6 mice or peritoneal macrophages isolation from α7 WT/KO and measure some inflammatory mediators could add new insight whether their anti-inflammatory effect mediated systemically or peripheral in the immune cells from colonic, or peritoneal cavity during DSS-induced colitis period. Notably, nicotine treatment reversed colitis severity in vagotomized mice (Ghia et al. 2006, 2007). Although the vagus nerve innervates only the proximal two-thirds of the colon, some studies the involvement vagus nerve in experimental colitis based on the cholinergic anti-inflammatory pathway concept in rodents. In fact, recent study by Vida et al., (2011), reported that systemic anti-inflammatory effect of the released acetylcholine of the celiac-mesenteric ganglia from vagus nerve activation is mediated through the α7 nAChR of the splenic nerve. de Jonge et al., (2005) demonstrated that macrophages inhibited after vagus nerve activation through Jak2-STAT3 signaling pathway activation. Thus, the idea that α7 nAChR mediated reduced inflammation through cholinergic vagus nerve activation and release of acetylcholine
seems to be indirect and more complicated. The intracellular mechanisms that induce nicotinic inhibition of macrophage activation involve the activation of anti-inflammatory Jak2/Stat3/Socs3 signaling pathways and inhibition of NF-κB signaling (Tracey, 2002; Gallowitsch-Puerta and Tracey, 2005; de Jonge et al., 2005; Arredondo et al., 2006). The effects of nicotine on NF-κB activity are significantly blocked by specific JAK2 inhibitor (AG-490) and STAT3 inhibitor (NSC74859) (Hosur and Loring, 2011). It would be of a great interest to utilize these selective inhibitors for JAK2 and/or STAT3 in vivo in DSS-treated mice and investigate the relevance of these pathways in the effects of α7 nAChR selective agonists to clarify some of the intracellular mechanisms.

The limited anatomical expression of α9α10 nAChRs might be an advantage in target these nicotinic receptor which also, could decrease the possibility of undesirable effects to occur especially the CNS side effects since the α9α10 subtype is neither express in brain nor in spinal cord. Therefore, the availablility of α9 KO mice and the new selective antagonist for these receptors could help to understand the role of α9α10 subtype in inflammation and colitis.


Ahuja V, Tandon RK. Inflammatory bowel disease in the Asia-Pacific area: a comparison with developed countries and regional differences. J Dig Dis 2010;11:134-147


Birrenbach T, Bocker U. Inflammatory bowel disease and smoking: a review of epidemiology, pathophysiology, and therapeutic implications. *Inflamm Bowel Dis* 2004;10:848-859


bowel disease. Crohn’s disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol* 157(3):1261-1270


Gusev, A.G., Uteshev, V.V., 2010. Physiological concentrations of choline activate native alpha7-containing nicotinic acetylcholine receptors in the presence of PNU-120596 [1-(5-chloro-2,4-dimethoxyphenyl)-3-(5-methylisoxazol-3-yl)-urea]. J Pharmacol Exp Ther. 332(2), 588-98.


Krafft, P.R., Altay, O., Rolland, W.B., Duris, K., Lekic, T., Tang, J., Zhang, J.H., 2012. α7 nicotinic acetylcholine receptor agonism confers neuroprotection through GSK-3β inhibition in a mouse model of intracerebral hemorrhage. Stroke. 43(3), 844-50


Takahashi F, Das KM. Isolation and characterization of a colonic autoantigen specifically recognized by colon tissue-bound immunoglobulin G from idiopathic ulcerative colitis. J Clin Invest 1985;76:311-318


Tracey, K.J. Fat meets the cholinergic antiinflammatory pathway. *J. Exp. Med.* 2005, 202,


Vincler M, McIntosh JM. Targeting the a9a10 nicotinic acetylcholine receptor to treat severe pain. Expert Opin Ther Targets 2007; 11:891-7.


Wilks S. Morbid appearances in the intestines of Miss Bankes. Med Times Gazette 1859;2:264-265


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