Title: Scopolamine Treatment and Muscarinic Receptor Subtype 3 Gene Ablation Augment Azoxyomethane-Induced Murine Liver Injury

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Muscarinic Receptors and Liver Injury

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Abstract

Previous work suggests that vagus nerve disruption reduces hepatocyte and oval cell expansion following liver injury. The role of post-neuronal receptor activation in response to liver injury has not been ascertained. We investigated the actions of scopolamine, a non-selective muscarinic receptor antagonist, and specific genetic ablation of a key cholinergic receptor, muscarinic subtype-3 (Chrm3), on azoxymethane (AOM)-induced liver injury in mice. Animal weights and survival were measured as was liver injury using both gross and microscopic examination. To assess hepatocyte proliferation and apoptosis, ductular hyperplasia and oval cell expansion, we used morphometric analysis of BrdU-, activated caspase-3-, H&E-, CK-19- and EpCAM-stained liver sections. Sirius red staining was used as a measure of collagen deposition and its association with oval cell reaction. In AOM-treated mice, both muscarinic receptor blockade with scopolamine and Chrm3 ablation attenuated hepatocyte proliferation and augmented gross liver nodularity, apoptosis, and fibrosis. Compared to control, scopolamine-treated and Chrm3−/− AOM-treated mice had augmented oval cell reaction with increased ductular hyperplasia and oval cell expansion. Oval cell reaction correlated robustly with liver fibrosis. No liver injury was observed in scopolamine-treated and Chrm3−/− mice that were not treated with AOM. Only AOM-treated Chrm3−/− mice developed ascites and had reduced survival compared to AOM-treated wild type (WT) controls. In AOM-induced liver injury, inhibiting post-neuronal cholinergic muscarinic receptor activation with either scopolamine treatment or Chrm3 gene ablation results in prominent oval cell reaction. We conclude that Chrm3 plays a critical role in the liver injury response by modulating hepatocyte proliferation and apoptosis.
Introduction

Acetylcholine activates two classes of cholinergic receptors: nicotinic (CHRN) which function as cation channels and muscarinic (CHRM) which mediate G-protein-coupled signaling. Five mammalian CHRM subtypes (CHRM1-5) are described; odd-numbered CHRM (CHRM1,3,5) preferentially activate Gq/11-proteins whereas even-numbered Chrm (CHRM2,4) activate Gi/o-proteins. Cell type-specific expression of CHRM subtypes mediates diverse signaling events. In the nervous system, muscarinic receptors play a major role in synaptic transmission and regulate sensory, motor, and autonomic functions. In non-neuronal tissue, muscarinic receptor activation regulates cell function (Shah et al., 2009). For example, muscarinic receptors mediate proliferation of lung and colon cancer cells (Raufman et al., 2008; Shah et al., 2009).

In the liver, stimulation of the vagus nerve induces proliferation of oval cells (designated intermediate hepatobiliary cells in humans) by release of acetylcholine and activation of muscarinic receptors (Cassiman et al., 2002). Oval cells are hepatic progenitor cells that can differentiate into either hepatocytes or cholangiocytes (Roskams et al., 2004). Following severe and chronic liver injury, oval cells play a critical role in hepatic parenchymal restoration (Forbes et al., 2002; Newsome et al., 2004). Oval cell expansion and ductular hyperplasia are observed in animal models of liver injury and in human chronic liver diseases (Roskams et al., 2004; Clouston et al., 2005). Oval cells are also thought to be precursors of hepatic cancer cells (Lee et al., 2009). In animal models of liver cancer, expansion of the oval cell compartment is observed before the development of frank neoplasia (Libbrecht et al., 2000).

Human and rodent hepatic oval cells, hepatocytes and cholangiocytes express CHRM3 subtype muscarinic receptors (Alvaro et al., 1997; Cassiman et al., 2002; Li et al., 2009). Based on several observations, it was suggested that liver injury responses to vagus nerve stimulation are mediated by activation of CHRM3 muscarinic receptors (Cassiman et al., 2002). Compared to innervated control livers, denervated livers (transplanted) have decreased numbers of bile ductules and intermediate hepatobiliary cells (Cassiman et al., 2002). In vagotomized rats, acute galactosamine-induced liver injury results in reduced oval cell proliferation compared to sham-
operated controls (Cassiman et al., 2002). Modulating cholinergic input by vagal nerve disruption or stimulation in other models of liver injury alters oval cell proliferation and hepatocyte restoration. Following partial hepatectomy, animals with disrupted hepatic vagus nerve branches have attenuated hepatocyte proliferation and ductular reaction (Ikeda et al., 2009). In a rat ischemia-reperfusion injury model, vagus nerve stimulation attenuates hepatic TNF synthesis and systemic shock (Bernik et al., 2002). In guinea pigs, bile duct ligation-induced chronic liver injury is associated with the formation and proliferation of acetylcholinesterase-positive nerve fibers that traverse bands of deposited collagen (Akiyoshi, 1989).

Collectively, these observations indicate that vagus nerve stimulation promotes ductular reaction and may regulate the liver injury response. Moreover, in animal models of liver injury and in human chronic liver disease these findings suggest that oval cell reaction plays a key role in parenchymal restoration and possibly hepatic carcinogenesis. Nonetheless, the importance of muscarinic receptors, specifically CHRM3, in modulating oval cell and ductular reaction, and the response to liver injury is not known.

In the course of evaluating the role of muscarinic receptors and ligands in colon cancer (Raufman et al., 2008), we observed unanticipated severe liver injury in azoxymethane (AOM)-treated mice with concurrent muscarinic receptor inactivation. AOM, an ingredient in cycad palms found on the island of Guam, was first identified, and subsequently used by investigators, as a selective intestinal carcinogen (Laqueur et al., 1963; Hirono, 1981). Subsequent work in rodents revealed that low-dose AOM treatment induces hepatotoxicity with ductular hyperplasia (Ward, 1975; Shan et al., 2008). At higher doses, AOM causes severe liver damage resulting in acute hepatic failure, encephalopathy, and increased rodent mortality (Matkowskyj et al., 1999; Belanger et al., 2006). AOM is now used by many investigators as a model of acute liver failure and hepatic encephalopathy (Chen et al., 2009).

Using AOM to induce chronic liver injury in mice, we conducted the current study to elucidate the role of muscarinic receptors in mediating ductular hyperplasia and oval cell proliferation. To
evaluate the overall role of muscarinic receptors in these processes, we used scopolamine butylbromide, a non-selective muscarinic receptor antagonist that does not cross the blood-brain-barrier. To determine more specifically the role of CHRM3 in modulating ductular hyperplasia and oval cell proliferation, we used knockout (Chrm3\(^{-/-}\)) mice. Our findings show that muscarinic receptor signaling mediated by CHRM3 plays a critical role in the liver injury response by modulating oval cell expansion, ductular reaction, and hepatocyte proliferation and apoptosis.
Methods

Experimental Design and Animal Procedures

All animal studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the U.S. National Academy of Sciences (National Institutes of Health publication 86-23, revised 1985). Mouse studies were approved by both the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine and the Research and Development Committee at the VA Maryland Health Care System. All mice (genetic background: 129S6/SvEv X CF1 (50%:50%), Taconic Labs, NY) were housed under identical conditions in a pathogen-free environment with a 12:12-hour light/dark cycle and free access to standard mouse chow and water. Mice were acclimatized for two weeks prior to any treatment. The study design is outlined in Figure 1. In study A, twenty-six 6-week-old male mice were treated with intraperitoneal (ip) AOM (10 mg/kg once each week for 6 weeks; Midwest Research Institute, Kansas City, MO) (Fig 1A). In addition, these mice were allocated to treatment with subcutaneous (sc) injection of scopolamine butylbromide, a quaternary ammonium compound (http://chem.sis.nlm.nih.gov/chemidplus) that does not cross the blood-brain-barrier (3 mg/kg/day in 2 divided doses, 5 days/week for 20 weeks, n=13; Butler Animal Health Supply, Dublin, OH) or PBS control administered sc using the same schedule (n=13). Four control mice that received ip PBS instead of AOM were also treated with sc scopolamine. Mice were euthanized 20 weeks after the first AOM dose.

The study design utilizing *Chrm3*-deficient (*Chrm3*−/−) and muscarinic receptor wild type (WT) control mice is shown in Figure 1B. Generation of *Chrm3*-deficient mice (*Chrm3*−/−) was described previously (Yamada et al., 2001). In study B, fifty-four 6-week-old male wild type (WT, N=25) and *Chrm3*−/− mice (N=29) were treated with ip AOM (10 mg/kg once each week for 6 weeks). As additional controls, 10 WT mice were treated with ip PBS instead of AOM and 4 *Chrm3*−/− mice received no treatment. Mice were euthanized 20 weeks after the first ip dose of AOM or PBS.
Mouse weight and mortality were recorded weekly. To measure cell proliferation, 2 hours before euthanasia, mice were injected with 50 mg/kg 5-bromo 2'-deoxyuridine (BrdU; Sigma, St. Louis). At euthanasia, gross liver appearance was graded by two investigators masked to study group: 0, normal; 1, mild liver surface nodularity; 2, intermediate liver surface nodularity; 3, marked nodularity with or without ascites. Livers were harvested after visual inspection, weighed and fixed in 4% para-formaldehyde.

**Liver Histology**

Five-micrometer sections obtained from formalin-fixed, paraffin-embedded liver tissue were stained with hematoxylin and eosin. The sections were analyzed by an expert pathologist blinded to study-groups. H&E sections were reviewed to assess liver fibrosis and scored using Batts-Ludwig criteria. Further, liver fibrosis was quantified using morphometric analysis of sirius red-stained sections. After dewaxing and hydration, liver sections were stained with picrosirius red solution for 1 hour followed by washing in acidified water. The resultant sections were dehydrated, mounted and examined under light microscopy using Nikon i80 photo-microscope at 100 x magnification. To minimize variation, all sections were examined and photographed with the same the microscope settings. At least five different areas were photographed from each section. Since the degree of sirius red staining measured by the saturation of the red channel correlates well with chemically-determined collagen content and morphometrically-determined fibrosis, fibrosis was expressed as the percentage of summed pixels per unit area of liver section (in arbitrary units) determined using Image Pro-plus software (version 5.0; Media Cybernetics, Silver Spring, MD).

**Immunohistochemistry (IHC)**

Hepatocyte proliferation was assessed using primary antibodies against BrdU (BD Bioscience, San Jose, California; dilution 1:10). Liver sections were blocked with normal serum for non-specific protein binding after treating with H₂O₂ for non-specific peroxidase activity and heat-induced antigen retrieval. They were incubated overnight with biotinylated anti-BrdU antibody at 4°C. Sections were incubated with streptavidin-HRP for 30 min at room temperature followed by staining with diaminobenzidine (DAB) and counterstaining with hematoxylin. At least 1000
hepatocyte nuclei were counted at 200X magnification and expressed as BrdU-stained nuclei/1000 cells.

IHC was performed for markers of apoptosis using anti-activated caspase-3 antibody (Cell Signaling Technology, Beverly, MA; dilution 1:100) and markers of bile ducts and oval cells using anti-CK-19 and anti-EpCAM antibodies (both from Abcam, Cambridge, MA; dilution 1:100). IHC was performed utilizing the avidin-biotin reaction with the vectastatin elite ABC kit (Vector Labs, Burlingame, CA) per manufacturer’s recommendations. After deparaffinization, hydration and endogenous peroxidase blockade (10% H<sub>2</sub>O<sub>2</sub>), heat-induced antigen retrieval was performed using citrate buffer, the sections were incubated at room temperature with 5% normal goat serum (20 min), avidin blocking reagent (15 min) and biotin blocking reagent (15 min). Sections were washed 3 times with 0.1% tween-20 in PBS between each step. Sections treated overnight at 4°C with primary antibody, were incubated with biotinylated goat anti-rabbit antibody (30 min) followed by incubation with streptavidin-HRP (30 min). They were stained with DAB (2 min) followed by counterstaining with hematoxylin (4 min). At least 1000 hepatocytes were counted to determine activated caspase-3 activity. Bile ductular cells were defined as CK-19- or EpCAM-stained cells forming ductular structure with lumens. Oval cells were defined as CK-19- or EpCAM-stained small cells with oval nuclei and scant cytoplasm not forming ductular structures. A specific marker for oval cells has not been identified. In liver injury, antigen expression profile of proliferating non-parenchymal cells overlaps with that of oval cells; this makes identification of oval cells difficult. Hence, morphometric analysis of liver sections with two recently established oval cell markers was used not only to identify and grade, but also to validate oval cell reaction (Yovchev et al., 2008; Okabe et al., 2009). At least 5 fields were examined at 200X total magnification and results expressed as the number of bile ducts/high-power field (HPF), bile duct size/HPF (number of cells forming bile ducts) and oval cells/HPF.

**Statistical Analysis**

All data are expressed as mean ± the standard error (SE). Data distribution was determined using the Anderson-Darling method. Based on distribution of data, Student’s unpaired t-test (normally distributed data) or the Mann-Whitney U test (nonparametric data) were used to determine
significance. Nominal data were analyzed using a \( \chi^2 \) test with Fisher’s test. Linear correlations were determined using Pearson’s \( r^2 \). Survival was analyzed using the Kaplan-Meier method. Analysis was performed using Stat-View (SAS, version 5.0.1; Cary, NC). Significance was defined as \( p < 0.05 \) and expressed in illustrations as \( ^* p < 0.05 \), \( ^{**} p < 0.01 \), and \( ^{***} p < 0.001 \).
Results

Effects of Scopolamine Treatment on Gross Liver Appearance in AOM- and PBS-Treated Mice. During the 20-week treatment period (Figure 1A), 1 AOM-treated mouse that received scopolamine died; there was no mortality in the PBS-treated group. Throughout the study, scopolamine- and PBS-treated mice did not differ in body weight (Figure 2A). Likewise, liver weights were similar in all study groups (Figure 2B). Figure 2C shows representative photographs of gross liver nodularity grades at euthanasia. Eighty-two percent of AOM-treated mice had gross liver nodularity (Figure 2D). In mice treated with scopolamine alone (no ip AOM), the liver surface was normal and there was no mortality (Figure 2E). AOM-treated mice that received scopolamine had a higher gross liver nodularity score compared to mice treated with PBS (1.3 ± 0.2 vs. 0.8 ± 0.1, p < 0.05, Figure 2E).

Histological Studies in Scopolamine-Treated Mice. Figure 2F shows that liver fibrosis (H&E) was not observed in mice treated with scopolamine alone (no AOM). In contrast, as predicted by the nodularity scores (Figure 2D,E), AOM-treated mice developed fibrosis; fibrosis scores were higher in scopolamine- compared to PBS-treated mice (Batts-Ludwig fibrosis score, 2.2 ± 0.2 vs. 1.2 ± 0.3, p < 0.05). These findings indicate that co-treatment with AOM plus scopolamine augments the fibrotic response to liver injury.

Previous work suggested that muscarinic receptors regulate oval cell reaction in response to galactosamine- and bile duct ligation-mediated liver injury(LeSag et al., 1999; Cassiman et al., 2002). To confirm the importance of muscarinic receptor activation in mediating oval cell reaction following AOM-induced liver injury, we examined the effects of the non-selective muscarinic receptor antagonist, scopolamine(van Koppen and Kaiser, 2003), on ductular hyperplasia and oval cell expansion measured by IHC for cytokeratin-19 (CK-19) and epithelial cell adhesion molecule (EpCAM). Cells forming the ductular phenotype express these ligands. We counted oval cells and bile ducts in five randomly-selected HPF (200X total magnification). As a surrogate marker for bile duct size, we counted the number of cells forming bile ducts. AOM treatment induced ductular hyperplasia and expansion of oval cells. In scopolamine-compared to PBS-treated mice, using CK-19 (Figure 3A,B) and EpCAM (Figure 3A,C) staining, ductule size and the numbers of ductules and oval cells were increased. These findings indicate
that blocking muscarinic receptor activation augments oval cell reaction following AOM-induced liver injury.

In chronic hepatitis C, and alcoholic and non-alcoholic fatty liver diseases, ductular reaction correlates strongly with the stage of fibrosis (Roskams et al., 2003; Clouston et al., 2005). To determine whether hepatic collagen deposition correlated with oval cell reaction, we used Sirius red staining to measure liver fibrosis. Mice that received scopolamine alone (no AOM) had no collagen deposition (Figure 4A). In AOM-treated mice, Sirius red staining was increased in scopolamine-treated compared to PBS-treated mice (10.3 ± 1.0 vs. 3.4 ± 0.8%, SCOP vs. PBS, p < 0.001, Figure 4 A,B). There was a significant correlation between oval cell reaction and liver fibrosis; CK-19-stained liver sections revealed that fibrosis correlated with bile ductular size and oval cell expansion but not with duct number. Ductular and oval cell proliferation detected by EpCAM staining also correlated with liver fibrosis (Sirius red staining) (Table 1). These data indicate that in AOM-treated mice, co-treatment with scopolamine promotes ductular reaction which correlates robustly with collagen deposition.

Hepatocyte restoration following liver injury results from a balance between hepatocyte proliferation and apoptosis. Cholinergic stimulation via the vagus nerve is reported to modulate hepatocyte proliferation (Ohtake et al., 1993; Kiba et al., 1994; Kiba, 2002). To determine the effects of scopolamine treatment on hepatocyte restoration, we measured cell proliferation (BrdU staining) and apoptosis (activated caspase-3 staining). In AOM-treated mice that received scopolamine compared to mice that received AOM plus PBS, BrdU staining was reduced (5.7 ± 1.3 vs. 10.7 ± 1.6 BrdU-stained cells/1000 hepatocytes, p < 0.05, Figure 5A,B) and apoptosis was augmented (27.8 ± 7.2 vs. 7.5 ± 2.5 activated caspase-3-stained cells/1000 hepatocytes, p < 0.05, Figure 5A,C). These data indicate that scopolamine alters AOM-induced ductular reaction by augmenting bile ductular proliferation and oval cell expansion, and reducing hepatocyte proliferation.
Gross Liver Injury in AOM-Treated Chrm3-Deficient Mice. Previously it was suggested in animal models of liver injury that vagus nerve stimulation augments ductular hyperplasia and oval cell proliferation by specific activation of Chrm3 (Cassiman et al., 2002). To focus on the role of Chrm3 in ductular reaction and to determine whether Chrm3 ablation mimics pharmacological blockade of muscarinic receptors we studied Chrm3<sup>−/−</sup> mice (Figure 1B). Figure 6A (left panel) shows representative Chrm3<sup>−/−</sup> mice treated with AOM (left mouse) or PBS (right mouse). The AOM-treated mouse had a distended abdomen and laparotomy revealed ascites (Figure 6A, middle panel) with grade 3 liver nodularity (Figure 6A, right panel). The liver of the PBS-treated mouse was normal. Over the course of these experiments, AOM-treated Chrm3<sup>−/−</sup> mice gained more weight compared to AOM-treated WT mice (41.7 ± 5.4% vs. 21.9 ± 3.9% initial body weight, \( p < 0.05 \), Figure 6B), a finding that we attribute to fluid retention and the development of ascites (Figure 6A). Moreover, whereas no PBS-treated mice died over the course of treatment, in the AOM treatment group, 3 WT and 13 Chrm3<sup>−/−</sup> mice died (mortality rate 12% vs. 45% for WT and Chrm3<sup>−/−</sup> mice treated with AOM, \( p < 0.01 \), Figure 6C).

At laparotomy, WT and Chrm3<sup>−/−</sup> mice treated with PBS (no AOM) had a normal liver appearance (Figure 6D). In contrast, 30% of WT and 88% of Chrm3<sup>−/−</sup> AOM-treated mice had liver nodularity (\( p < 0.05 \)). Compared to WT, AOM-treated Chrm3<sup>−/−</sup> mice had greater liver nodularity scores (Figure 6D,E). The highest grade, liver nodularity with ascites, was observed in approximately 25% of AOM-treated Chrm3<sup>−/−</sup> mice and in no AOM-treated WT mice (Figure 6E).

Histological Studies in Chrm3-Deficient Mice. To determine the effects of Chrm3 ablation on AOM-induced ductular reaction we measured CK-19 and EpCAM staining. AOM-treated mice had marked ductular reaction with bile ductular proliferation and oval cell expansion. In Chrm3<sup>−/−</sup> compared to WT mice, IHC for CK-19 (Figure 7A,B) and EpCAM (Figure 7A,C) revealed increased numbers of bile ductules, ductular cells and oval cells.
To determine the effect of *Chrm3* ablation on AOM-induced liver fibrosis, we analyzed H&E- and Sirius red-stained sections. Gross liver nodularity in AOM-treated mice correlated with the Batts-Ludwig fibrosis score (data not shown). Sirius red-stained liver sections revealed increased collagen deposition in *Chrm3*<sup>−/−</sup> compared to WT mice (21.4 ± 3.2% vs. 7.6 ± 1.0% Sirius red-stained area, *p* < 0.001, Figure 8A,B). A strong correlation was observed between liver nodularity on gross examination and the Sirius red-stained area (*r*<sup>2</sup> = 0.82, *p* < 0.001). As observed in scopolamine-treated animals, in AOM-treated mice oval cell and ductular hyperplasia were most prominent in areas of fibrosis; ductular hyperplasia and oval cell proliferation (CK-19 and EpCAM staining) correlated strongly with Sirius red staining (Table 1).

The data shown in Figure 5 indicate that muscarinic receptor blockade modulates hepatocyte restoration by reducing hepatocyte proliferation and increasing apoptosis. To determine whether *Chrm3* ablation mimicked the actions of scopolamine we used BrdU and activated-caspase-3 staining. In PBS-treated mice (no AOM), very few BrdU-stained nuclei and little activated caspase-3 staining were observed (Figure 9A). These findings confirm that, in the absence of injury, hepatocytes remain quiescent and do not undergo mitosis. In contrast, in liver sections from AOM-treated animals we observed increased hepatocyte proliferation. However, BrdU-stained hepatocytes were fewer in *Chrm3*<sup>−/−</sup> compared to WT mice (3.1 ± 0.8 vs. 9.2 ± 2.0 BrdU-stained cells/1000 hepatocytes, *p* < 0.05, Figure 9A,B). Moreover, in the AOM treatment group, *Chrm3*<sup>−/−</sup> mice had augmented hepatocyte apoptosis compared to WT mice (36.6 ± 13.7 vs. 17.3 ± 0.6 activated caspase-3-stained cells/1000 hepatocytes, *p* < 0.05, Figure 9A,C). These data indicate that hepatic parenchymal restoration following AOM-induced liver injury is modulated by both pharmacological inhibition of muscarinic receptor activation and by specific genetic ablation of *Chrm3* resulting in attenuated hepatocyte proliferation, augmented apoptosis, and increased ductular hyperplasia and oval cell expansion.
Discussion

Our findings are consistent with previous observations that AOM treatment induces chronic liver injury in murine liver (Ward, 1975; Shan et al., 2008). Mice treated with AOM developed liver surface nodularity, fibrosis and ductular hyperplasia. Additionally, ductular hyperplasia and oval cell expansion following AOM-treatment correlated strongly with liver fibrosis, a hallmark of chronic liver disease. Our results extend previous observations by showing that in addition to ductular hyperplasia (Shan et al., 2008), AOM treatment induces oval cell expansion. To investigate the role of muscarinic activation in mediating cellular responses to AOM-induced liver injury, we used pharmacological inhibition of muscarinic receptor activation and genetic ablation of *Chrm3*. Experiments using scopolamine-treated WT and *Chrm3*−/− mice revealed reproducible augmentation of ductular hyperplasia and oval cell expansion, with increased fibrosis compared to control mice. Also, attenuating muscarinic receptor activation with scopolamine treatment or *Chrm3* gene ablation modulated hepatocyte restoration following AOM injury by reducing hepatocyte proliferation (BrdU staining) and increasing apoptosis (activated caspase-3 staining). Collectively, these data indicate that in mice, *Chrm3* activation plays a key role in protecting the liver from AOM-induced toxicity and imply that *Chrm3* activation is likely to play an important role in hepatic regeneration in chronic liver disease.

Whereas scopolamine treatment increased apoptosis, oval cell reaction, and fibrosis, these effects were less robust than those observed in *Chrm3*−/− mice. Moreover, in the AOM treatment groups, only 1 mouse that received scopolamine died whereas 13 *Chrm3*−/− mice died (*p* < 0.01) and cirrhosis with ascites following AOM treatment was seen only in *Chrm3*−/− mice. Several factors are likely to account for the greater efficacy of *Chrm3* ablation compared to scopolamine treatment. To avoid systemic toxicity, we used a modest dose of scopolamine, administered the drug only twice daily, and mice had a 2-day treatment holiday each week. Hence, scopolamine treatment was not likely to achieve the same magnitude or duration of reduced-muscarinic receptor activation achieved by genetic ablation. We consider the possibility that scopolamine, a non-selective muscarinic receptor antagonist, modulated liver injury by blocking other muscarinic receptor subtypes. However, a recent study revealed that *Chrm3* is the only muscarinic receptor subtype expressed in mouse liver and other *Chrm* subtypes (1, 2, 4 and 5)
are not expressed (Li et al., 2009). Hence, it is unlikely that other Chrm subtypes are involved in regulating AOM-induced injury in mouse liver.

All AOM-treated mice developed ductular hyperplasia and oval cell expansion that was most prominent along collagen bands. Sirius red staining correlated strongly with all components of ductular reaction. Clouston et al. (Clouston et al., 2005) demonstrated in chronic hepatitis C that fibrosis correlates strongly with periportal ductular reaction. Oval cell proliferation (intermediate hepatobiliary cells in humans) is described in a variety of chronic liver diseases with a strong association between fibrosis, ductular proliferation and oval cell numbers in non-alcoholic and alcoholic fatty liver disease (Ray et al., 1993; Roskams et al., 2003). These findings are similar to those observed in AOM-treated mice. Also, it was shown recently that increased oval cell numbers correlates with more fibrosis (Fotiadu et al., 2004). Hence, our findings of strong correlations between CK-19/EpCAM staining and collagen deposition are consistent with observations in animal models of liver injury and in human chronic liver disease.

Various organ systems Chrm3 play an important role in mediating the actions of muscarinic ligands on cell proliferation, migration and wound healing (Shah et al., 2009). Branches of the vagus nerve provide parasympathetic innervation to the liver (Kiba, 2002). Previously, vagus nerve stimulation and transection were employed to investigate the cholinergic regulation of liver injury (LeSag et al., 1999; Bernik et al., 2002; Cassiman et al., 2002). The work described herein is the first to evaluate the role of post-neural activation of muscarinic receptors on liver injury. Expression of Chrm3 in bile ductules, oval cells and hepatocytes was confirmed by others (LeSag et al., 1999; Cassiman et al., 2002; Li et al., 2009). Their observations are consistent with our finding of increased hepatocyte apoptosis and reduced proliferation in AOM-treated Chrm3−/− and scopolamine co-treated mice. PBS-treated Chrm3−/− mice and mice that received scopolamine but not AOM had no gross or histological evidence of liver injury. Moreover, our finding of diminished hepatocyte proliferation in scopolamine-treated and Chrm3−/− mice are similar to results following partial hepatectomy, where vagotomy impairs hepatocyte proliferation and diminishes liver weight (Ohtake et al., 1993).
In contrast to our findings, two studies reported decreased ductular and oval cell proliferation following vagotomy (LeSag et al., 1999; Cassiman et al., 2002). We ascribe this discrepancy to different models of liver injury, a different method of inhibiting cholinergic signaling, and shorter time courses compared to our study. LeSage et al. (LeSag et al., 1999) used bile-duct ligation to induce liver injury and investigated cholangiocyte proliferation one week after surgery. Because bile duct ligation causes cholestasis, the consequent elevation in serum and liver bile acids may activate Chrm3, thereby stimulating cell proliferation (Raufman et al., 2002). Cassiman et al. (Cassiman et al., 2002) performed studies 48 hours after galactosamine-treatment, an acute hepatocyte injury model that triggers oval cell reaction. In contrast to these experimental models, we studied AOM-induced injury 20 weeks after treatment. Moreover, an AOM metabolite, methyl-azoxymethanol, inhibits hepatocyte proliferation (Zedeck and Swislocki, 1975). Hence, it is possible that the predominant effect in chronic AOM-induced toxicity is hepatocyte injury and that muscarinic receptor inactivation exacerbates liver injury by attenuating hepatocyte proliferation or by augmenting hepatocyte apoptosis, thus triggering compensatory ductular reaction (Figs 5 and 9). Alternatively, inhibiting Chrm3 activation may prevent differentiation of oval cells into hepatocytes, thus preventing oval cell-dependent parenchymal restoration. Also scopolamine treatment and Chrm3 ablation are systemic inhibitors of muscarinic receptor activation, whereas vagotomy results in a regional effect.

Vagotomy may also modulate liver injury by preventing nicotinic receptor activation in Kupffer cells. Vagotomy promoted anti-Fas antibody-induced hepatocyte apoptosis and mortality in mice; that was blocked by nicotine and an α7 nicotinic receptor agonist (Hiramoto et al., 2008). Studies utilizing vagotomy to investigate cholinergic regulation of liver injury may also create an imbalance between sympathetic and parasympathetic regulation of liver regeneration and oval cell proliferation. A growing body of evidence suggests that the sympathetic nervous system plays a major role in regulating liver injury. Specifically, sympathetic neural input inhibition reduces hepatocyte injury and promotes oval cell proliferation (Oben and Diehl, 2004). Finally, oval cells were shown to express vasoactive intestinal peptide (VIP) receptors and receive input from VIP-containing neurons (Miyazawa et al., 1988; Akiyoshi et al., 1998; Cassiman et al., 2007). Hence, in addition to acetylcholine, the vagus nerve stores and releases other neurotransmitters, including VIP, that may modulate liver injury responses.
In conclusion, our findings demonstrate convincingly that in mice, pharmacological inhibition or genetic ablation of Chrm3 augments AOM-induced chronic liver injury. In particular, we observed attenuated hepatocyte restoration and augmented ductular hyperplasia and oval cell expansion. Potential therapeutic implications of these findings include pharmacological or other approaches to modulating muscarinic receptor activity as a means of preventing liver injury or hastening repair. Future work will be directed at defining the role of muscarinic receptors in mediating responses to liver injury caused by other hepatotoxins (e.g. ethanol and acetaminophen) and determining whether stimulating muscarinic receptor activation attenuates hepatic injury.
References


Footnotes

*These authors contributed equally to this work and share first authorship.

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Legends for Figures

**Figure 1. Study Design.** (A) 129S6/SvEv X CF1 mice were treated with ip PBS (N=4) or AOM (N=26; 10 mg/kg) once each week for 6 weeks. Mice were also allocated to treatment with sc PBS (N=13) or scopolamine (SCOP, 3 mg/kg/day in 2 divided doses, N=17) 5 days/week for 20 weeks. (B) *Chrm3−/−* (N=29) and WT mice (N=25) were treated with ip AOM (10 mg/kg once each week for 6 weeks). Additional WT (N=10) and *Chrm3−/−* mice (N=4) treated with ip PBS only were used as controls.

**Figure 2.** Effects of scopolamine (SCOP) on azoxymethane (AOM)-induced chronic liver injury in mice. Livers were analyzed 20 weeks after the first injection of AOM. (A) Animal weights were recorded over 20 weeks in AOM-treated mice co-treated with PBS or SCOP. (B) Average liver weight expressed as percentage of body weight is shown for AOM-treated mice co-treated with PBS or SCOP; livers from four mice treated with scopolamine only (no AOM) were also analyzed. (C) Representative photographs show grades of liver nodularity determined by gross inspection: 0, normal; 1, mild liver surface nodularity; 2, intermediate liver surface nodularity; 3, marked nodularity. (D) Abnormal livers (≥ grade 1 nodularity) were observed only in AOM-treated mice. (E) Liver nodularity score was greater in mice treated with AOM plus scopolamine compared to those treated with AOM plus PBS. (F) H&E sections were examined to assess fibrosis using the Batts-Ludwig criteria. Compared to PBS, scopolamine co-treatment increased AOM-induced liver fibrosis. Livers from mice that were treated with scopolamine only (no AOM) appeared normal, without fibrosis. Bars represent mean values ± SE. *p < 0.05.

**Figure 3.** Effects of scopolamine (SCOP) on AOM-induced ductular reaction. (A) Ductules and oval cells (stained brown) are demonstrated using IHC for CK-19 and EpCAM in liver sections from AOM-treated mice co-treated with SCOP or PBS. Liver sections from mice treated with scopolamine only (no AOM) were also stained. Arrows indicate ductules and arrowheads indicate oval cells. Treatment with scopolamine increased AOM-induced oval cell reaction with proliferation of large ductules (demonstrated by the number of cells/ductule) and oval cell expansion that was confirmed by both CK-19 (B) and EpCAM (C) staining. Ductular cells were defined as CK-19- or EpCAM-stained cells forming ductular structures with lumens. Oval cells
were defined as CK-19- or EpCAM-stained small cells with oval nuclei and scant cytoplasm not forming ductular structures. Bars represent mean values ± SE. *p < 0.05 and ***p < 0.001.

Figure 4. Effects of scopolamine (SCOP) on AOM-induced liver fibrosis. (A) Collagen deposition was evaluated by Sirius red staining. (B) Co-treatment with scopolamine increased liver fibrosis in AOM-treated mice. Bars represent mean values ± SE. ***p < 0.001.

Figure 5. Effects of scopolamine (SCOP) on hepatocyte restoration in AOM-treated mice. (A) Representative sections show BrdU and activated caspase-3 staining in livers from mice treated with scopolamine alone (no AOM) and from mice treated with AOM plus PBS or scopolamine. Arrows indicate BrdU- and activated caspase-3-stained hepatocytes. In AOM-treated mice, treatment with scopolamine reduced the numbers of hepatocytes that stained for BrdU (B) and increased the number of hepatocytes that stained for activated caspase-3 (C). Bars represent mean values ± SE. *p < 0.05.

Figure 6. Effects of Chrm3 ablation on AOM-induced chronic liver injury. (A) Left panel: Chrm3−/− mice following treatment with AOM (left) and PBS (right) are shown. Middle and right panels: Necropsy of the AOM-treated Chrm3−/− mouse revealed ascites (blue arrow) and a grossly nodular liver. (B) At 20 weeks, AOM-treated Chrm3−/− mice had gained more weight than WT mice. (C) As shown by the Kaplan-Meier curve, following treatment with AOM, Chrm3−/− mice had reduced survival compared to WT mice. (D) Liver nodularity was not observed in WT or Chrm3−/− mice that were not treated with AOM. Liver nodularity scores were greater in AOM-treated Chrm3−/− compared to WT mice. (E) Chrm3−/− mice were more likely to have grossly abnormal livers than WT mice. Grade 3 liver nodularity was seen only in AOM-treated Chrm3−/− mice. Bars represent mean values ± SE. *p < 0.05, **p < 0.01, and ***p < 0.001.

Figure 7. Effects of Chrm3 ablation on AOM-induced ductular reaction. (A) Ductules and oval cells (stained brown) are demonstrated using IHC for CK-19 and EpCAM in liver sections from PBS- and AOM-treated Chrm3−/− and WT mice. Arrows indicate ductules and arrowheads indicate oval cells. As demonstrated by CK-19 (B) and EpCAM (C) staining, sections from Chrm3−/− mice revealed had robust AOM-induced ductular reaction with increased numbers of
large ductules (demonstrated by numbers of cells/ductule) and oval cell expansion compared to those from WT mice. Ductular cells were defined as CK-19- or EpCAM-stained cells forming ductular structures with lumens. Oval cells were defined as CK-19- or EpCAM-stained small cells with oval nuclei and scant cytoplasm not forming ductular structures. Bars represent mean values ± SE. * p < 0.05, ** p < 0.01, and *** p < 0.001.

**Figure 8.** Effects of *Chrm3* ablation on AOM-induced liver fibrosis. (A) Collagen deposition was evaluated by Sirius red staining in *Chrm3<sup>−/−</sup>* and WT mice treated with PBS or AOM. (B) AOM-treated *Chrm3<sup>−/−</sup>* mice had increased liver fibrosis compared to AOM-treated WT mice. Bars represent mean values ± SE. *** p < 0.001.

**Figure 9.** Effects of *Chrm3* ablation on hepatocyte restoration in AOM-treated mice. (A) Representative sections show BrdU and activated caspase-3 staining in livers from PBS- and AOM-treated WT and *Chrm3<sup>−/−</sup>* mice. Black arrows indicate BrdU-stained hepatocytes (top row) and activated caspase-3-stained hepatocytes (bottom row). Compared to WT mice, AOM-treated *Chrm3<sup>−/−</sup>* mice had reduced numbers of BrdU-stained hepatocytes (B) and increased numbers of activated caspase-3-stained hepatocytes (C). Bars represent mean values ± SE. * p < 0.05.
**TABLE 1.**
Correlation of fibrosis with oval cell reaction in liver sections from AOM-treated mice\(^a\)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sirius Red-Stained Area</th>
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<tbody>
<tr>
<td></td>
<td>(r^2) (Study A)</td>
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<tr>
<td>CK-19 Stained-</td>
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<tr>
<td>Ductules</td>
<td>0.31(^c)</td>
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<tr>
<td>Ductule Cells</td>
<td>0.44(^*)</td>
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<td>Oval Cells</td>
<td>0.49(^*)</td>
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<tr>
<td>EpCAM Stained-</td>
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<tr>
<td>Ductules</td>
<td>0.63(^{**})</td>
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<tr>
<td>Ductule Cells</td>
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<tr>
<td>Oval Cells</td>
<td>0.69(^{**})</td>
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</table>

\(^{a}\)See Figure 1 and Method Section for details.
\(^{b}\)\(r^2\) is Pearson Correlation Coefficient.
\(^{c}\)\(p = 0.06\).
\(^*\)\(p < 0.05\), \(^{**}\)\(p < 0.01\), \(^{***}\)\(p < 0.001\).
Figure 1

A

AOM or PBS ip

0 1 2 3 4 5 6 7 8 9 10 19 20

Euthanize

Daily subcutaneous PBS or Scopolamine

PBS ip + SCOP sc  N=4
AOM ip + SCOP sc  N=13
AOM ip + PBS sc   N=13

B

AOM or PBS ip

0 1 2 3 4 5 6 7 8 9 10 19 20

Euthanize

WT, AOM   N=25
Chrm3−/−, AOM N=29
WT, PBS    N=10
Chrm3−/−, PBS N=4
Figure 3

A

SCOP

AOM + PBS

AOM + SCOP

CK-19

EpCAM

B

Ductules/HPF

Ductular Cells/HPF

Oval Cells/HPF

AOM + PBS

AOM + SCOP

AOM + PBS

AOM + SCOP

AOM + PBS

AOM + SCOP

C

Ductules/HPF

Ductular Cells/HPF

Oval Cells/HPF

AOM + PBS

AOM + SCOP

AOM + PBS

AOM + SCOP

AOM + PBS

AOM + SCOP

***

***

***
Figure 4

A

<table>
<thead>
<tr>
<th>SCOP</th>
<th>AOM + PBS</th>
<th>AOM + SCOP</th>
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Sirius Red

B

Bar chart showing Sirius Red-stained area (% total) for SCOP, AOM + PBS, and AOM + SCOP.

- SCOP: 0%
- AOM + PBS: 2%
- AOM + SCOP: 12%

Significance: ***
Figure 7

A

<table>
<thead>
<tr>
<th>WT</th>
<th>Chrm3⁻⁻⁻⁻</th>
<th>WT-AOM</th>
<th>Chrm3⁻⁻⁻⁻⁻⁻⁻⁻</th>
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</thead>
<tbody>
<tr>
<td>CK-19</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>EpCAM</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

- **Ductules/HPF**
  - WT-AOM
  - Chrm3⁻⁻⁻⁻⁻⁻⁻⁻

- **Ductular Cells/HPF**
  - WT-AOM
  - Chrm3⁻⁻⁻⁻⁻⁻⁻⁻

- **Oval Cells/HPF**
  - WT-AOM
  - Chrm3⁻⁻⁻⁻⁻⁻⁻⁻

C

- **Ductules/HPF**
  - WT-AOM
  - Chrm3⁻⁻⁻⁻⁻⁻⁻⁻

- **Ductular Cells/HPF**
  - WT-AOM
  - Chrm3⁻⁻⁻⁻⁻⁻⁻⁻

- **Oval Cells/HPF**
  - WT-AOM
  - Chrm3⁻⁻⁻⁻⁻⁻⁻⁻
Figure 8

A

WT  Chrm3⁻⁻  WT-AOM  Chrm3⁻⁻-AOM

Sirius Red

B

Sirius Red-Stained Area (% total)

WT-AOM  Chrm3⁻⁻-AOM

***
Figure 9

A

WT  Chrm3\(^{-/-}\)  WT-AOM  Chrm3\(^{-/-}\)-AOM

BrdU

Activated Caspase-3

B

C

BrdU-Stained Cells/1000 Hepatocytes

WT-AOM  Chrm3\(^{-/-}\) AOM

Activated Caspase-3-Stained Cells/1000 Hepatocytes

WT-AOM  Chrm3\(^{-/-}\) AOM

*