HHS Public Access

Author manuscript

Hepatology. Author manuscript; available in PMC 2021 March 01.

Published in final edited form as:

Hepatology. 2020 March ; 71(3): 990–1008. doi:10.1002/hep.30880.

Modulation of the Tryptophan Hydroxylase 1/Monoamine Oxidase-A/5-Hydroxytryptamine/5-Hydroxytryptamine Receptor 2A/2B/2C Axis Regulates Biliary Proliferation and Liver Fibrosis During Cholestasis

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Abstract

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Author Contributions: Thanks to all the authors for their contribution in this manuscript. Investigation: KK, LC, AO, JV, NQ LC, TZ, DZ, FM, HF, CW, LF, AG, PI; methodology: KK; visualization: KK, NQ, LC; formal analysis: KK, writing-original draft: KK; data curation: LC, AO, TZ, writing-review and editing: TH, DZ, FM, HF, CW, LF, AGPI, SL, RS, NJS, GA, SG; resources: SL, RS, NJS; funding acquisition, project administration, supervision, validation: GA, SG.

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Potential conflict of interest: Dr. Invernizzi received grants from Intercept, Gilead, and Bruschettini. Dr. Liangpunsakul consults for Durect.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.30880/suppinfo.

BACKGROUND AND AIMS: Serotonin (5HT) is a neuroendocrine hormone synthetized in the central nervous system (CNS) as well as enterochromaffin cells of the gastrointestinal tract. Tryptophan hydroxylase (TPH1) and monoamine oxidase (MAO-A) are the key enzymes for the synthesis and catabolism of 5HT, respectively. Previous studies demonstrated that 5-hydroxytryptamine receptor (5HTR)1A/1B receptor agonists inhibit biliary hyperplasia in bile-duct ligated (BDL) rats, whereas 5HTR2B receptor antagonists attenuate liver fibrosis (LF) in mice. Our aim was to evaluate the role of 5HTR2A/2B/2C agonists/antagonists in cholestatic models.

APPROACH AND RESULTS: While *in vivo* studies were performed in BDL rats and the multidrug resistance gene 2 knockout (*Mdr2*$^{-/-}$) mouse model of PSC, *in vitro* studies were performed in cell lines of cholangiocytes and hepatic stellate cells (HSCs). 5HTR2A/2B/2C and MAO-A/TPH1 are expressed in cholangiocytes and HSCs from BDL rats and *Mdr2*$^{-/-}$ mice. Ductular reaction, LF, as well as the mRNA expression of proinflammatory genes increased in normal, BDL rats, and *Mdr2*$^{-/-}$ mice following treatment 5HTR2A/2B/2C agonists, but decreased when BDL rats and *Mdr2*$^{-/-}$ mice were treated with 5HTR2A/2B/2C antagonists compared to BDL rats and *Mdr2*$^{-/-}$ mice, respectively. 5HT levels increase in *Mdr2*$^{-/-}$ mice and in PSC human patients compared to their controls and decrease in serum of *Mdr2*$^{-/-}$ mice treated with 5HTR2A/2B/2C antagonists compared to untreated *Mdr2*$^{-/-}$ mice. *In vitro*, cell lines of murine cholangiocytes and human HSCs express 5HTR2A/2B/2C and MAO-A/TPH1; treatment of these cell lines with 5HTR2A/2B/2C antagonists or TPH1 inhibitor decreased 5HT levels as well as expression of fibrosis and inflammation genes compared to controls.

CONCLUSIONS: Modulation of the TPH1/MAO-A/5HT/5HTR2A/2B/2C axis may represent a therapeutic approach for management of cholangiopathies, including PSC.

In addition to regulating ductal bicarbonate secretion[1] cholangiocytes are the target of cholangiopathies, which are liver diseases characterized by ductular reaction, enhanced biliary senescence (with increased secretion of senescence-associated secretory phenotypes [SASPs]), and subsequent increased collagen deposition[2,3] Changes in ductular reaction are modulated by a number of neuroendocrine factors, such as agonists for serotonin receptor subtypes, melatonin, secretin, somatostatin, neurotransmitters, and sex hormones.[4]

Serotonin or 5-hydroxytryptamine (5HT) is a indolamine monoamine neurotransmitter of the central nervous system (CNS) and the autonomic nervous system (ANS).[5] In the CNS, 5HT controls sleep, learning, feeding, weight regulation, alcoholism, anxiety, and psychiatric disorders,[5] whereas in the ANS it modulates cell proliferation, apoptosis, and platelet aggregation.[5] 5HT is synthesized, from the amino acid, L-tryptophan, in the serotonergic neurons of the CNS[6] and in the gastrointestinal tract in enterochromaffin cells as well as cholangiocytes.[6,7] The main enzyme regulating 5HT biosynthesis is tryptophan hydroxylase (TPH), which, in vertebrates, is expressed in two different isoforms: TPH1 (predominantly expressed in the periphery) and TPH2 (predominantly expressed in the brain). The enzyme, monoamine oxidase A (MAO-A), is also a key player for the maintenance of serotonin levels given that it regulates 5HT catabolism.[8] 5HT exerts its functions through 14 known types of 5HT receptor (5HTR) subtypes that have been grouped into seven broad families (5HT1, 5HT2, 5HT3, 5HT4, 5HT5, 5HT6, and 5HT7).[9] Whereas the 5HT3 receptors are ligandgated Na^+/K^+ ion channels, the other 5HT receptors are G-

protein-coupled transmembrane receptors, which activate different intracellular secondary messenger systems, including inositol 1,4,5-trisphosphate (IP_3), Ca^{2+} and cAMP.[9, 10]

In the liver, 5HT regulates blood flow though the portal vein.[5] 5HT is also involved in the pathogenesis of many liver diseases, such as steatohepatitis, liver fibrosis (LF), and cholangiocarcinoma (CCA).[7,11–13] Cholangiocyte 5HT secretion is enhanced in proliferating cholangiocytes from bile-duct ligated (BDL) rats.[7] Also, there is increased 5HT synthesis in CCA evidenced by enhanced TPH1, but reduced expression of MAO-A leading to increased CCA growth.[13] Another study has shown that ketanserin (a 5HTR2A antagonist) ameliorates ischemia-related biliary fibrosis in rats with donation after cardiac death liver transplantation.[14] Antagonists for 5HTR2A/2B also inhibit liver regeneration by reduced platelet-derived serotonin synthesis.[15]

To study the role of 5HTR2 in the modulation of biliary damage/senescence and LF, we evaluated the autocrine/paracrine role of the TPH1/MAO-A/5HTR/2A/2B/2C axis in regulation of biliary homeostasis and liver inflammation and fibrosis in BDL rats and the multidrug resistance gene 2 knockout (*Mdr2*$^{-/-}$) mouse model, which mimics some phenotypes of primary sclerosing cholangitis (PSC).[16,17]

Materials and Methods

MATERIALS

Reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO), unless otherwise indicated. The rat antibody against cytokeratin-19 (CK-19) was obtained from Developmental Studies Hybridoma Bank (Iowa City, IA). The mouse antibody against CK-19 was purchased from Leica Biosystems (Newcastle, UK). The goat antibody against GFAP (glial fibrillary acidic protein; a marker of hepatic stellate cells [HSCs])[18] was purchased from Abcam Systems (Minneapolis, MN); the anti-GFAP antibody, [Y66] (Alexa Fluor 488), was purchased from Abcam (Cambridge, MA). The antirat antibody (Cy^{TM3}), the antirabbit antibodies (Cy^{TM2} and Cy^{TM3}), and the anti-mouse antibody (Cy^{TM2}) were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The rabbit antibodies for serotonin receptor subtypes 2A, 2B, and 2C (5HTR2A, 5HTR2B, and 5HTR2C) were purchased from Bioss Antibodies (Boston, MA). Rabbit monoamine oxidase A and tryptophan hydroxylase antibodies were purchased from Abcam. The Nova Ultra Sirius Red Stain kit to detect interstitial collagen deposition was purchased from IHC World (Woodstock, MD). Antibodies for albumin (a marker of hepatocytes) against mouse and rat tissues were purchased from Novus Biologicals (Centennial, CO) and Santa Cruz Biotechnology Inc. (Dallas, TX), respectively.

The ImmPRESS Reagents kit for immunohistochemical (IHC) staining of 5HTR2A/2B/2C and TPH1 in human samples from healthy controls and PSC patients were purchased from Vector Laboratories (Burlingame, CA). The following chemicals: (1) 4-bromo-3,6-dimethoxybenzocyclobuten-1-yl) methylamine hydrobromide (TCB2; 5HTR2A agonist); (2) α-methyl-5-(2-thienylmethoxy)-1*H*-indole-3-ethanamine hydrochloride (BW723C86; 5HTR2B agonist); (3) 8,9-dichloro-2,3,4,4a-tetrahydro-1.W-pyrazino[1,2-*a*]quinoxalin-5(6*H*)-one hydrochloride (WAY 161503 hydrochloride; 5HTR2C agonist); (4)

spiperone hydrochloride (5HTR2A antagonist); (5) *N*-(1-methyl-1*H*-indolyl-5-yl)-*N*″-(3-methyl-5-isothiazolyl)urea (SB204741; 5HTR2B antagonist); (6) N-desmethylclozapine (5HTR2C antagonist); and (7) the TPH1 inhibitor, p-chlorophenylalanine (p-CPA), were purchased from TOCRIS (Minneapolis, MN). The serotonin enzyme-linked immunosorbent assay (ELISA) kit was purchased from Abcam. The Rat Cytokine ELISA Plate Array I (Colorimetric) and the mouse Cytokine ELISA Plate Array I (Colorimetric) were purchased from Signosis (Santa Clara, CA). The mirVANA miRNA Isolation kit for RNA isolation was purchased from Thermo Fisher Scientific (Waltham, MA).

We used the following rat primers: fibronectin 1 (Fn-1; NM_019143), smooth muscle α-actin (α-SMA; NM_031004), collagen type I a 1 (Col1a1; NM_0533504), tissue inhibitor of metalloproteinase 1 (TIMP1; NM_053819), TIMP2 (NM_0219890), interleukin (IL)-4 (NM_201270),IL-6 (NM_0.12589), IL-17α (NM_001106897), IL-1β (NM__012762), IL-10 (NM 012854), tumor necrosis factor (TNFα; NM_012675), TPH1 (NM_001100634), MAO-A (XM_001058993), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NM_017008, housekeeping); mouse primers: 5HTR2A (NM_172812), 5HTR2B (NM_008311), 5HTR2C (NM_0083120), TPH1 (NM_001136084), MAO-A (NM_173740), Fn-1 (NM_010233), Col1a1 (NM_007742), transforming growth factor-β1 (TGF-β1; NM_011577), TIMP1 (NM_001044384), TIMP2 (NM_011594), TIMP3 (NM_0.11595), IL-6 (NM_031168), IL-17α (NM_010522), IL-1β (NM_008361), TNFα (NM_013693), IL-4 (NM_021283), IL-33 (NM_ 001164724), and GAPDH (NM_008084); and the human primers: α-SMA (NM_ 001141945), Col1a1 (NM_000088), IL-1α (NM_000575), IL-8 (NM_01168298), and GAPDH (NM_01256799) were purchased from Qiagen (Valencia, CA). For the PCR assays, we used SYBR Green PCR Master Mix (SABiosciences, Frederick, MD) in the real-time thermal cycler (ABI Prism 7900HT sequence detection system). Data analysis of mRNA expression was performed with an online software program provided by Qiagen (Valencia, CA).

ANIMAL MODELS

Male Sprague-Dawley 344 rats (200-225 g) were purchased from Charles River (Wilmington, MA) and maintained in a temperature-controlled environment (20-22°C) with 12:12-hour light-dark cycles. Animals were fed standard rat chow and had access to drinking water *ad libitum*. Normal rats (NRs; sham) and BDL (immediately after surgery)[19] were treated with saline or the selected 5HTR2A/2B/2C agonists/antagonists (see above, at the dose of 100 nmoles/kg body weight [BW]/day) for 1 week by intraperitoneally implanted Alzet osmotic minipumps (Cupertino, CA). In separate experiments, male FVB/NJ wild-type (WT) and *Mdr2*$^{-/-}$ mice (25-30 g, 12 weeks old) were purchased from Jackson Laboratories (Sacramento, CA), housed in a temperature-controlled environment (22°C), and fed standard mice chow with access to drinking water *ad libitum*;, *Mdr2*$^{-/-}$ mice are from our breeding colony. WT and *Mdr2*$^{-/-}$ mice were treated with saline or the selected agonists/antagonists for 5HTR2A/2B/2C at the dose of 100 nmoles/kg BW/day by intraperitoneal implanted Alzet osmotic minipumps for 1 week. Before each experimental procedure, animals were treated with euthasol (200-250 mg/kg BW), following the regulations of the panel on euthanasia of the American Veterinary Medical Association. We collected serum, total liver, and cholangiocytes from the selected groups of animals. All

animal experiments were performed in accord with protocols approved by the Baylor Scott & White Research Institute CTX Institutional Animal Care and Use Committee.

ISOLATED CHOLANGIOCYTES, IMMORTALIZED MURINE CHOLANGIOCYTE CELLS, HUMAN H69, AND HEPATIC STELLATE CELL LINES

Cholangiocytes were isolated by immunoaffinity separation[20,21] using a monoclonal antibody (immunoglobulin M, a gift from Dr. R.A. Faris, Brown University, Providence, RI) expressed by all intrahepatic cholangiocytes. Cell number and viability were assessed by Trypan Blue exclusion. The *in vitro* studies were performed in the following cell lines: (1) immortalized murine cholangiocyte cells (IMCLs)[18], (2) immortalized normal human cholangiocyte cells, H69 (a gift of Dr. G.J. Gores, Mayo Clinic, MN)[22], and (3) human H69 and hepatic stellate (HHSTeCs) cells[18] purchased from ScienCell (Carlsbad, CA).

EXPRESSION OF 5HTR2A/2B2/C RECEPTORS, MAO-A, AND TPH1

We evaluated, by immunofluorescence (IF) and IHC, the immunoreactivity of 5HTR2A/2B/2C receptors, MAO-A, and TPH1 in frozen liver sections (4-5 μm thick) costained with CK-19 or GFAP from normal and BDL rats and WT and *Mdr2*$^{-/-}$ mice. By IF, we evaluated the immunoreactivity of MAO-A and TPH1 in frozen liver sections (4-5 μm thick) costained with albumin. Sections, costained with 5HTR2A/2B/2C/MAO-A/TPH1 and CK-19, were analyzed by a confocal microscope (Leica TCS SP5 II, equipped with LASX software, Leica Mircrosystems, Wetslar, Germany). Sections costained with 5HTR2A/2B/2C/MAO-A/TPH1 and GFAP, and sections costained with MAO-A/TPH1 and albumin, were analyzed with a Zeiss LSM 700 confocal laser scanning microscope (Carl Zeiss, Jena, Germany). Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA). Following staining, sections were analyzed in a coded fashion using an Olympus microscope (Olympus, Newport Beach, CA), equipped with an Olympus DP25 5-mega Pixel Digital Color Microscope Camera (Olympus, Melville, NY). Images were captured with cellSens Standard Life Science imaging software (Olympus). Positive staining intensity was analyzed by Adobe Photoshop CC 2015 software (Adobe, San Jose, CA). By real-time PCR (qPCR), mRNA expression of 5HTR2A/2B/2C was evaluated in total liver from WT and *Mdr2*$^{-/-}$ mice, and MAO-A and TPH1 in isolated cholangiocytes from both mouse and rat models.

EFFECT OF AGONISTS/ANTAGONISTS OF 5HTR2A/2B/2C RECEPTORS ON INTRAHEPATIC BILE DUCT MASS

Intrahepatic bile duct mass (IBDM) was measured in frozen liver sections (4-5 μm thick) from the selected groups of animals by semi-quantitative IHC measuring the area occupied by CK-19–positive bile ducts/total area × 100.[23] Sections were examined using the Olympus Image Pro-Analyzer software (Olympus, Tokyo, Japan). CK-19–positive staining was analyzed by Visiopharm software (Visiopharm North America, Broomfield, CO).

EFFECT OF AGONISTS/ANTAGONISTS OF 5HTR2A/2B/2C RECEPTORS ON LF, SASPs, AND INFLAMMATION

Extent of hepatic fibrosis was evaluated by Sirius Red staining in paraffin-embedded liver sections (4-5 μm thick). Slides were scanned by a digital scanner (SCN400; Leica Microsystems, Buffalo Grove, IL) and quantified using Image-Pro Premier software (version 9.1; Media Cybernetics, Rockville, MD). We also measured SASPs in cholangiocyte supernatant obtained from selected groups of rats. We evaluated by qPCR the mRNA expression of selected fibrosis markers as well as proinflammatory cytokines in cholangiocytes of mice and rats.

MEASUREMENT OF SEROTONIN SERUM LEVELS IN *Mdr2*$^{-/-}$ MICE AND HUMAN PSC SAMPLES AND EXPRESSION OF 5HTR2A/2B/2C AND TPH1 IN HUMAN SAMPLES

Serotonin levels in serum from the selected groups of animals and healthy control and PSC patients were measured by the Serotonin ELISA kit (Abcam). We evaluated by IHC the immunoreactivity of 5HTR2A/2B/2C and TPH1 in human samples from healthy controls and PSC patients. Stained slides were analyzed by Olympus microscope (Olympus, Newport Beach, CA), equipped with an Olympus DP25 5-mega Pixel Digital Color Microscope Camera (Olympus, Melville, NY), and images were captured with cellSens Standard Life Science imaging software (Olympus). The collection and use of coded, unidentified human samples (Supporting Table S1) from Dr. Pietro Invernizzi (Humanitas Research Hospital, Rozzano, Italy) and Dr. Suthat Liangpunsakul (Roudebush Medical Center and Indiana University, Indianapolis, IN) were approved under separate protocols approved by the Ethics Committee of the Humanitas Research Hospital and Indiana University Purdue University Indianapolis, respectively. The protocol related to the samples of Dr. Invernizzi was reviewed by the Central Texas Veteran's Health Care System Institutional Review Board and Research and Development Committee and approved by the Texas A&M HSC College of Medicine Institutional Review Board. Collection of serum as well as liver tissues from Dr. Liangpunsakul were approved by the Indiana University Purdue University Indianapolis Institutional Review Board. Briefly, coded human liver specimens for mRNA analysis were obtained through the Liver Tissue Procurement and Distribution System (Minneapolis, MN), as described[24] Serum specimens were obtained from healthy controls and patients with PSC. Liver specimens from patients with PSC were obtained from the explant during liver transplantation. Control liver samples were from patients with no known history of chronic liver diseases and collected during abdominal surgeries for various causes. Written informed consent was received from participants before inclusion in the study.

IN VITRO STUDIES IN IMCLs, H69, AND HHSTeCs CELL LINES

We first evaluated by IF the immunoreactivity of 5HTR/2A/2B/2C receptors, MAO-A and TPH1, in IMCLs, H69, and HHSTeCs cell lines. We next treated IMCLs and HHSTeCs cells with 5HTR2A/2B/2C receptor antagonists and/or p-CPA (TPH1 inhibitor) before measuring the mRNA expression of selected fibrosis and inflammation genes by *q*PCR as well as cellular senescence. After inducing senescence in IMCLs by stimulation with lipopolysaccharide (LPS; 10 ng/mL for 48 hours; Sigma-Aldrich), we measured SASP levels in supernatant from IMCLs by the mouse cytokine ELISA Plate Array I. By serotonin

ELISA kits, we measured 5HT levels in supernatant from IMCLs and HHSTeCs treated with 5HTR2A/2B/2C antagonists (10 μM).

STATISTICAL ANALYSIS

All data are expressed as mean ± SD. Differences between groups were analyzed by the Student unpaired *t* test when two groups were analyzed and analysis of variance when more than two groups were analyzed, followed by an appropriate post hoc test.

Results

EXPRESSION OF 5HTR2A/2B/2C RECEPTORS

By IF and semiquantitative immunohistochemistry in liver sections from normal and BDL rats, we demonstrated: (1) immunoreactivity for 5HTR2A/2B/2C in intrahepatic bile ducts and HSCs (costained with CK-19 and GFAP, respectively); (2) enhanced biliary immunoreactivity for 5HTR2A/2B/2C in liver sections (Fig. 1A–C).

IMMUNOREACTIVITY/EXPRESSION OF MAO-A AND TPH1

By both IF and IHC in liver sections from normal and BDL rats, we demonstrated immunoreactivity for MAO-A and TPH1 in intrahepatic cholangiocytes, HSCs, and hepatocytes (costained with CK-19, GFAP, and albumin, respectively; Fig. 2A,B). By qPCR, we demonstrated decreased expression of MAO-A, but enhanced expression of TPH1, in cholangiocytes from BDL compared to normal rats (Fig. 2A,B).

EFFECT OF AGONISTS/ANTAGONISTS OF 5HTR2A/2B/2C ON IBDM IN NORMAL AND BDL RATS

We demonstrated: (1) enhanced IBDM in BDL compared to normal rats; (2) increased IBDM in both normal and BDL rats treated with 5HTR2A/2B/2C agonists compared to saline-treated rats; and (3) reduced IBDM in both normal and BDL rats treated with antagonists for 5HTR2A/2B/2C compared to normal and BDL rats treated with saline (Fig. 3A–C).

EFFECT OF AGONISTS/ANTAGONISTS OF 5HTR2A/2B/2C ON LF, SASPs, AND INFLAMMATION IN NORMAL AND BDL RATS

There was enhanced LF and levels of SASPs in BDL compared to normal rats (Fig. 4A–C). There was: (1) increased collagen deposition in both normal and BDL rats treated with 5HTR2A/2B/2C agonists compared to respective saline-treated rats; (2) reduced LF in both normal and BDL rats treated with antagonists for 5HTR2A/2B/2C compared to respective saline-treated rats (Fig. 4A–C). By rat cytokine ELISA Plate Array I, the following SASP levels increased in cholangiocyte supernatant from BDL compared to control rats: TNFα, vascular endothelial growth factor (VEGF), basic fibroblast growth factor beta (FGFβ), interferon gamma (INFγ), leptin, monocyte chemoattractant protein-1 (MCP-1), stem cell factor (SCF), IL-1β, IL-5, IL-6, IL-15 and chemokine (C-C) motif ligand 5 (CCL-5), or regulated on activation normal T-cell expressed and secreted (Rantes) decreased in supernatant from cholangiocytes from BDL rats treated with 5HTR2A antagonist; MCP-1,

SCF, IL-1β, IL-5, IL-6, and IL-15 decreased in supernatant of cholangiocytes from BDL rats treated with 5HTR2B antagonist; and TNFα, FGFβ, INFγ, leptin, MCP-1, SCF, and IL-15 decreased in supernatant of cholangiocytes from BDL rats treated with 5HTR2C antagonist compared to BDL control rats (Fig. 4A–C). mRNA expression of fibrotic and proinflammatory cytokines increased in cholangiocytes from BDL compared to NRs, but decreased in cholangiocytes from BDL rats treated with antagonists for 5HTR2A/2B/2C compared to control BDL rats (Fig. 4A–C).

EXPRESSION OF 5HTR2A/2B/2C AND MAO-A AND TPH1 IN WT AND *Mdr2*$^{-/-}$ MICE

Similar to the findings in rats, by IF and IHC in liver sections, there was: (1) immunoreactivity for 5HTR2A/2B/2C in intrahepatic bile ducts and HSCs (costained with CK-19 and GFAP, respectively) in both WT and *Mdr2*$^{-/-}$ mice; (2) enhanced immunoreactivity for 5HTR2A/2B/2C (in liver sections) and expression (by qPCR in total liver) in *Mdr2*$^{-/-}$ compared to WT mice (Supporting Fig. S1A–C). We also demonstrated immunoreactivity of MAO-A and TPH1 in hepatocytes costained with albumin. By IF and IHC in liver sections from WT and *Mdr2*$^{-/-}$ mice, we demonstrated immunoreactivity for MAO-A and TPH1 in bile ducts, HSCs and hepatocytes (costained with CK-19, GFAP, and albumin, respectively; Fig. 5A,B). By qPCR, there was decreased expression of MAO-A, but enhanced expression of TPH1 in cholangiocytes from *Mdr2*$^{-/-}$ compared to WT mice (Fig. 5A,B).

EFFECT OF AGONISTS/ANTAGONISTS FOR 5HTR2A/2B/2C ON IBDM, LF, SASPs, AND INFLAMMATION IN WT AND *MDR2*$^{-/-}$ MICE

There was increased IBDM and collagen deposition in *Mdr2*$^{-/-}$ compared to WT mice, which both were further enhanced by administration of 5HTR2A/2B/2C agonists to WT and *Mdr2*$^{-/-}$ mice compared to their respective controls. Administration of 5HTR2A/2B/2C antagonists to WT and *Mdr2*$^{-/-}$ mice decreased IBDM and collagen deposition compared to *Mdr2*$^{-/-}$ mice (Figs. 6 and 7A). Similarly, the increase in the mRNA expression of fibrosis genes and proinflammatory cytokines (observed in *Mdr2*$^{-/-}$ mice compared to WT mice) was reduced in cholangiocytes from *Mdr2*$^{-/-}$ mice treated with the antagonists of 5HTR2A/2B/2C compared to vehicle-treated *Mdr2*$^{-/-}$ mice (Fig. 7B).

SEROTONIN LEVELS IN SERUM OF *Mdr2*$^{-/-}$ MICE AND PSC PATIENTS AND IMMUNOREACTIVITY OF 5HTR2A/2B/2C AND TPH1 IN HUMAN HEALTHY CONTROL AND pSC SAMpLES

By enzyme immunoassay, there was increased serotonin levels in serum samples from *Mdr2* and early- and late-stage PSC patient samples compared to their respective controls (Supporting Fig. S2A,B). When *Mdr2*$^{-/-}$ animals were treated with 5HTR2A/2B/2C antagonists, there were reduced serotonin levels in serum compared to *Mdr2*$^{-/-}$ mice (Supporting Fig. S2A). By IHC, we demonstrated enhanced immunoreactivity for 5HTR2A/2B/2C/TPH1 in human PSC samples compared to their healthy controls (Supporting Fig. S2C).

IN VITRO STUDIES IN IMCLs, H69, AND HHSTeCs CELL LINES

By IF, IMCLs, H69, and HHSTeCs expressed 5HTR2A/2B/2C, TPH1, and MAO-A (Supporting Figs. S3A and S4A). Treatment of: (1) ICMLs with 5HTR2A/2B/2C antagonists or p-CPA (10^{-3} M) for 24 hours and (2) HHSTeCs with p-CPA (10^{-3} M) for 24 hours[25] decreased mRNA expression of fibrosis and proinflammatory genes compared to control cells (Supporting Figs. S3B and S4B). By the mouse cytokine ELISA Plate Array I, we measured SASP levels in the supernatant of IMCLs after LPS and with or without 5HTR2A/2B/2C antagonists. Levels of TNFα, IL-1α, and IL-1β were significantly increased in the supernatant of IMCLs after LPS treatment compared to control IMCLs. Furthermore, 5HTR2A/2B/2C antagonists decreased levels of the aforementioned cytokines in the supernatant of IMCLs treated with LPS compared to IMCLs treated only with LPS (Supporting Fig. S3C). By the ELISA 5HT kits, 5HT levels decreased in the supernatant of IMCLs and HHSTeCs treated with 5HTR2A/2B/2C antagonists compared to vehicle-treated ICMLs and HHSTeCs, respectively (Supporting Figs. S3D and S4C).

Discussion

In the present study, we demonstrated that: (1) 5HTR/2A/2B/2C receptors are expressed by both cholangiocytes and HSCs and (2) expression of these receptor subtypes increases in BDL rats, *Mdr2*$^{-/-}$ mice, and human PSC samples. We demonstrated that administration of agonists for 5HTR/2A/2B/2C receptors increases IBDM and LF in normal and BDL rats as well WT and *Mdr2*$^{-/-}$ mice, whereas the administration of 5HTR/2A/2B/2C antagonists decreases IBDM and LF in both BDL rats and *Mdr2*$^{-/-}$ mice (Fig. 8). SASP levels (that were increased in BDL compared to normal rats) decreased in BDL rats treated with 5HTR2A/2B/2C antagonists compared to vehicle-treated BDL controls. We have shown that: (1) cholangiocytes, HSCs, as well as hepatocytes secrete 5HT, evidenced by expression of MAO-A and TPH1 and (2) cholangiocytes from BDL rats, *Mdr2*$^{-/-}$ mice, and human PSC samples express higher levels of TPH1 (but decreased MAO-A expression), leading to enhanced 5HT serum levels in *Mdr2*$^{-/-}$ mice and human early- and late-stage PSC. Treatment of IMCL cells with LPS increased TNFα/IL-1α/IL-1β levels compared to control IMCLs. Treatment of IMCLs with LPS and 5HTR2A/2B/2C antagonists decreased levels of TNFα, IL-1α, and IL-1β cytokines in the supernatant of IMCLs compared to only LPS-treated IMCLs. *In vitro*, we demonstrated that: (1) IMCLs and HHSTeCs express 5HTR2A/2B/2C, TPH1, and MAO-A; and (2) treatment of these cell lines with 5HTR2A/2B/2C antagonists and/or p-CPA decreased mRNA expression of fibrosis/proinflammatory genes compared to control cells.

Although there is growing information on the autocrine/paracrine role of serotonin signaling in the modulation of liver diseases, including cholangiopathies, limited information exists on the role of the 5HTR2A/2B/2C in the regulation of biliary damage and LF in models of models of extrahepatic cholestasis and PSC.[26,27] For example, a study has shown that 5HT inhibits biliary hyperplasia of BDL rats by autocrine/paracrine pathways associated with inhibition of IP_3/Ca^{2+}/protein kinase C (PKC)-dependent cAMP/protein kinase A (PKA)/Src/extracellular signal-regulated kinase 1/2 (ERK 1/2).[7] Also, inhibition of 5HT synthesis (up-regulated in CCA) inhibited CCA growth both *in vitro* and *in vivo*.[13]

Overexpression of MAO-A inhibits CCA growth by coordinated epigenetic and IL-6–driven pathways.[28] Furthermore, Omenetti et al. have shown that paracrine modulation of biliary 5HT synthesis regulates biliary remodeling.[29] 5HT has been shown to play an important role in liver regeneration after partial hepatectomy.[30] The differential transduction pathways may likely explain why activation of 5HTR1A/1B inhibits biliary hyperplasia in BDL rats,[7] whereas administration of 5HTR2A/2B/2C agonists triggers biliary proliferation and LF in both BDL rats and *Mdr2*$^{-/-}$ mice. In support of this possibility, we have performed Ingenuity Pathway Analysis and demonstrated that activation of biliary mass and LF by 5HTR2 is attributed likely to: (1) activation of mitogen-activated protein kinase and signal transducer and activator of transcription 3 mediated by 5HTR2A; (2) enhanced IP3 levels and ERK1/2 phosphorylation by 5HTR2B; and (3) increased cAMP-dependent PKA by 5HTR2C. On the contrary, interaction with 5HTR1A/1B, which we have shown reduces biliary hyperplasia in cholestatic BDL rats, is mediated by enhanced IP_3/Ca^{2+}/PKC signaling and subsequent inhibition of the cAMP/PKA/Src/ERK1/2 pathway (Supporting Fig. S5).

Our findings are consistent with previous studies showing that antagonists of 5HTR2A inhibit fibrosis mRNA expression *in vitro* in the human hepatic stellate cell line, LX2, and *in vivo* in rats with LF induced by thioacetamide.[26] Also, HSC secretion of 5HT and expression of 5HTR2B contributes to activation of HSCs and enhanced LF in rats.[11] The 5HTR2A antagonist, ketanserin, reduces the extent of ischemia-related biliary fibrosis in rats with donation after cardiac death liver transplantation.[31] The antifibrotic activity of 5HTR2A/2B/2C antagonists has also been demonstrated in other organs. For example, antagonists for 5HTR2A/2B inhibit cardiac fibrosis in mice after pulmonary artery banding to protect these animals from heart failure.[32] Also, 5HTR2A/2B antagonists decrease bleomycin-induced lung fibrosis in mice through reduced levels of TGF-β1.[33] Considering that there are 16 subtypes of 5HTR, further studies are necessary to determine the role of 5HTR2A/2B/2C agonists/antagonists on the compensatory expression and role of other 5HTR subtypes on biliary damage and LF. Consistent with this notion, 5HTR3 antagonists have been shown to ameliorate pruritus during cholestatic liver injury.[34] Antagonists for 5HTR3 improve obesity-associated fatty liver diseases in mice.[35] Moreover, antagonists for 5-HTR7 decreased liver regeneration after 60%-70% partial hepatectomy.[36] Although the role of 5HT in liver inflammation is more defined, the role of the 5HTR2A/2B/2C agonist/antagonists in liver inflammation is still unclear. Indeed, 5HT deficiency has been shown to worsen acetaminophen-induced liver toxicity in mice.[37] 5HTR7 agonists reduce CCl_4-induced oxidative stress, liver inflammation, and fibrosis.[38] Furthermore, another study has shown that 5HT modifies human macrophage polarization through interaction with HTR2B and HTR7.[39] Consistent with these findings, we have shown that 5HTR2A/2B/2C antagonists decreased mRNA expression and levels of proinflammatory cytokine in cholangiocytes from BDL rats and *Mdr2*$^{-/-}$ mice compared to control animals. Supporting our findings, AM1030 (an antagonist of 5HTR2B) displays anti-inflammatory properties against LPS-induced atopic dermatitis[40] Consistent with previous findings showing that damaged/proliferating cholangiocytes acquire neuroendocrine phenotypes and secrete a number of neuroendocrine factors (e.g., melatonin, 5HT, sex hormones, secretin, VEGF, and nerve growth factor)[4,7,17,18,20,29] that regulate biliary homeostasis during cholestasis, we

demonstrate that cholangiocytes (in addition to HSCs) express TPH1 and secrete 5HT, parameters that were increased in both BDL rats and *Mdr2*$^{-/-}$ mice and human PSC serum samples (5HT levels). Parallel to our findings in cholangiocytes and HSCs, several studies support the existence of paracrine/autocrine serotonergic networks in both the brain and peripheral organs,[41,42] given that MAO-A and TPH1 are present in the brain as well as lungs, kidneys, thyroid, heart, and liver.[7,13,43,44] Similar to what we observed in our study, the TPH1 inhibitor (LX-1031) has been shown to ameliorate irritable bowel syndrome and chronic diarrhea through reduced 5-HT levels.[45] Moreover, a study has shown that p-CPA decreases liver FGF21 expression/levels of fibroblast growth factor 21 in a mouse model of high-fat diet, suggesting the potential role of 5HT in modulating LF in nonalcoholic fatty liver disease/nonalcoholic steatohepatitis patients.[46]

We next performed *in vitro* experiments aimed to demonstrate that the autocrine/paracrine action of serotonin on biliary proliferation/liver fibrosis is not attributed to secondary *in vivo* effects, but rather to a direct interaction with both cholangiocytes and HSCs. In these studies, we demonstrated that treatment of IMCLs and HHSteCs with 5HTR2A/2B/2C antagonists and/or TPH1 blocker reduces the expression of inflammation and fibrosis genes. A shortcoming of our studies (part of another ongoing project) is that we did not pinpoint the cellular targets/trigger (cholangiocytes or HSCs) of the TPH1/MAO-A/5HT/5HTR2A/2B/2C axis and the potential role of cholangiocytes activating HSCs and stimulating LF by a paracrine mechanism releasing potential SASPs such as TGF-β1.[17]

In summary, we have demonstrated the importance of the autocrine/paracrine role of the TPH1/MAO-A/5HT/5HTR2A/2B/2C axis in the modulation of biliary damage/homeostasis in rodent models of extrahepatic obstruction and PSC. Modulation of this axis may provide new therapeutic approaches for the management of cholangiopathies including PSC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by the Dr. Nicholas C. Hightower Centennial Chair of Gastroenterology from Baylor Scott & White, a VA Research Senior Career Scientist Award, and a VA Merit award to Dr. Alpini (5I01BX000574); a VA Merit Award (1I01BX001724) to Dr. Meng; a VA Merit Award (1I01BX003031) to Dr. Francis; and the NIH grants DK058411, DK07698, DK107310, DK110035, DK062975, AA025997, and AA025157 to Drs. Alpini, Meng, and Glaser and the NIH grant DK108959 to Dr. Francis and NIH grants R01DK107682 and R01AA025208 and VA Merit Award I01CX000361 to Dr. Liangpunsakul. Portions of this study were supported by PSC Partners Seeking a Cure Award to Dr. Alpini. This material is the result of work supported by resources at both Central Texas Veterans Health Care System (Temple, TX) and Richard L. Roudebush VA Medical Center (Indianapolis, IN). The content is the responsibility of the author(s) alone and does not necessarily reflect the views or policies of the Department of Veterans Affairs or the United States government.

Abbreviations:

5HT	5-hydroxytryptamine
5HTR	5-hydroxytryptamine receptor
α-SMA	α-smooth muscle actin

BDL	bile-duct ligated
BW	body weight
CCA	cholangiocarcinoma
CK-19	cytokeratin-19
CNS	central nervous system
Col1a1	collagen, type I, alpha 1
DAPI	4′,6-diamidino-2-phenylindole
ELISA	enzyme-linked immunosorbent assay
ERK1/2	extracellular signal-regulated kinase
FGFβ	fibroblast growth factor beta
Fn-1	fibronectin-1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
HHSteCs	human hepatic stellate cells
HSCs	hepatic stellate cells
hPSCL	human PSC cell lines
IBDM	intrahepatic bile duct mass
IF	immunofluorescence
IHC	immunohistochemistry
IL	interleukin
IMCLs	immortalized murine cholangiocyte lines
INFγ	interferon gamma
IP_3	inositol 1,4,5-trisphosphate
LF	liver fibrosis
LPS	lipopolysaccharide
MAO-A	monoamine oxidase A
MCP-1	monocyte chemoattractant protein-1
NR	normal rat
p-CPA	p-chlorophenylalanine

PKA	protein kinase A
PSC	primary sclerosing cholangitis
Rantes	regulated on activation normal T-cell expressed and secreted
SASPs	senescence-associated secretory phenotypes
SCF	stem cell factor
TIMP-1	metallopeptidase inhibitor 1
TPH1	tryptophan hydroxylase
TGF-β1	transforming growth factor-fit-β1
TNFα	tumor necrosis factor
VEGF	vascular endothelial growth factor
WT	wild type

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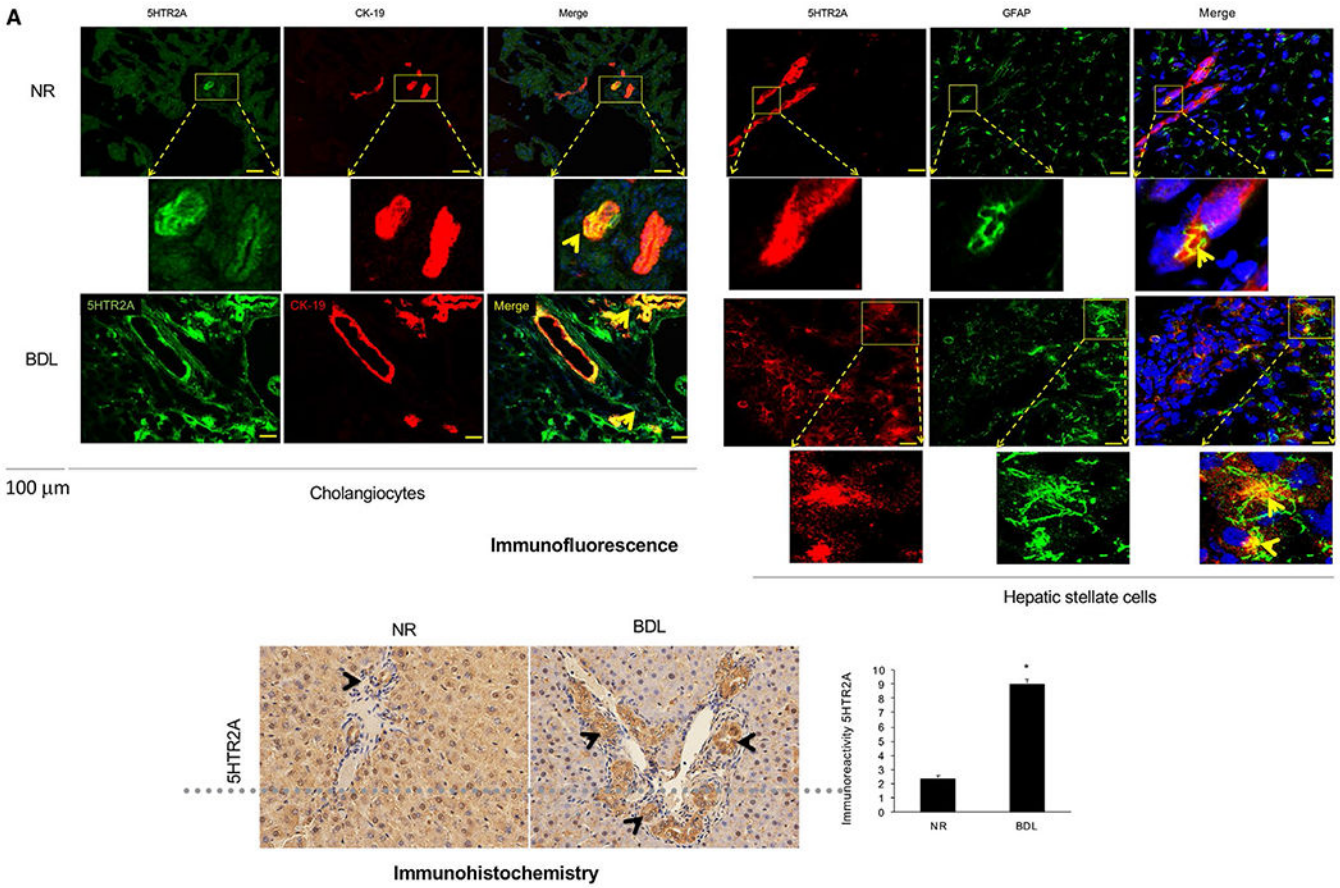


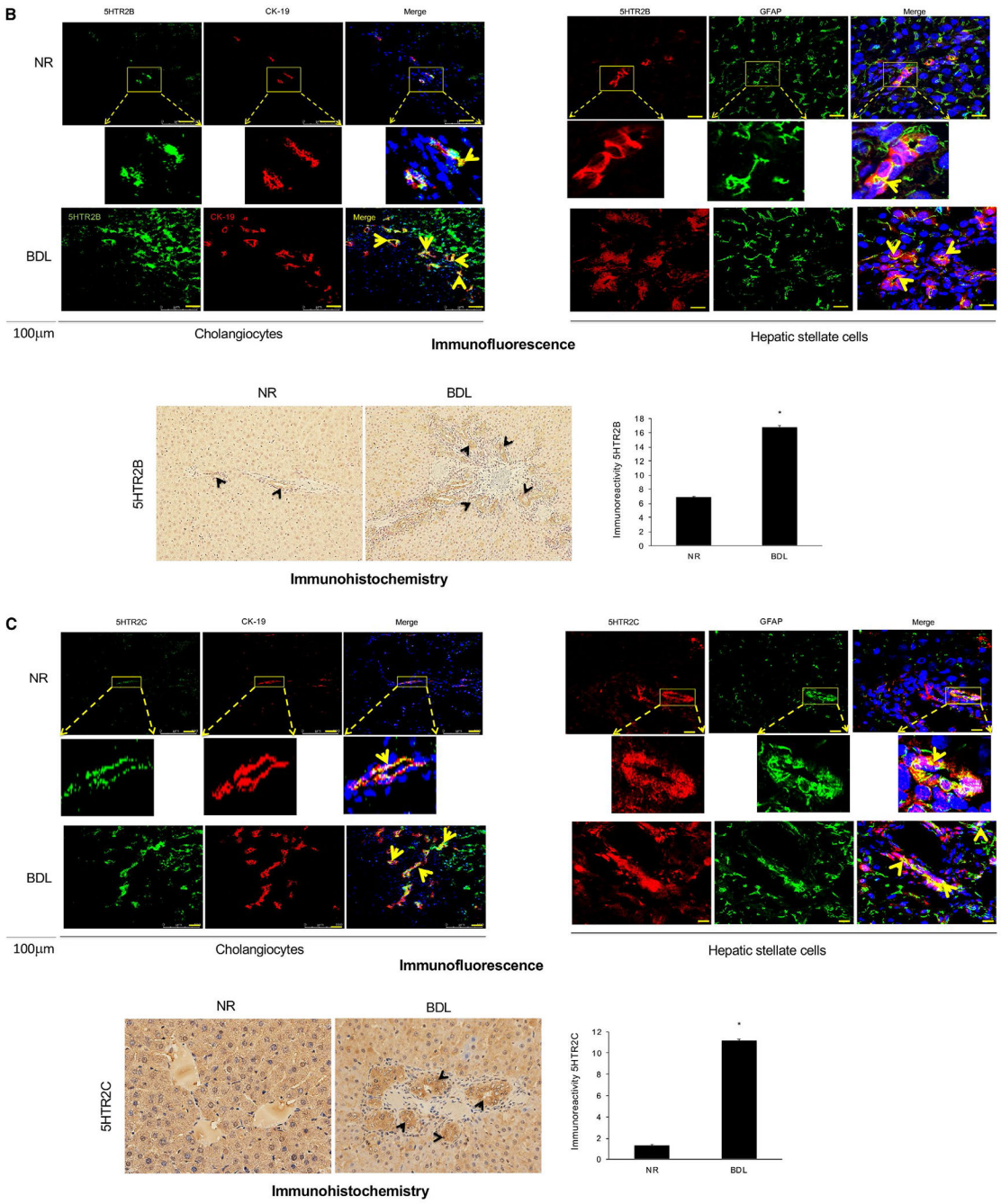


FIG. 1.

(A-C) We demonstrated immunoreactivity for 5HTR2A/2B/2C in cholangiocytes and HSCs from normal and BDL rats; nuclei were stained with DAPI (blue) in the immunofluorescent images; scale bar = 100 μm. Yellow arrows show the colocalization of 5HTR2A/2B/2C with cholangiocytes (costained with CK-19) and HSCs (costained with GFAP). There was enhanced immunoreactivity (by IHC in liver sections) for 5HT2A/2B/2C in total liver from BDL compared to normal rats. IHC quantification data are expressed as mean ± SD of four

cumulative evaluations from each group. Black arrows show the 5HTR2A/2B/2C-positive bile ducts. Original magnification, ×20 (A,C), ×10 (B). *$P < 0.05$ versus NR.

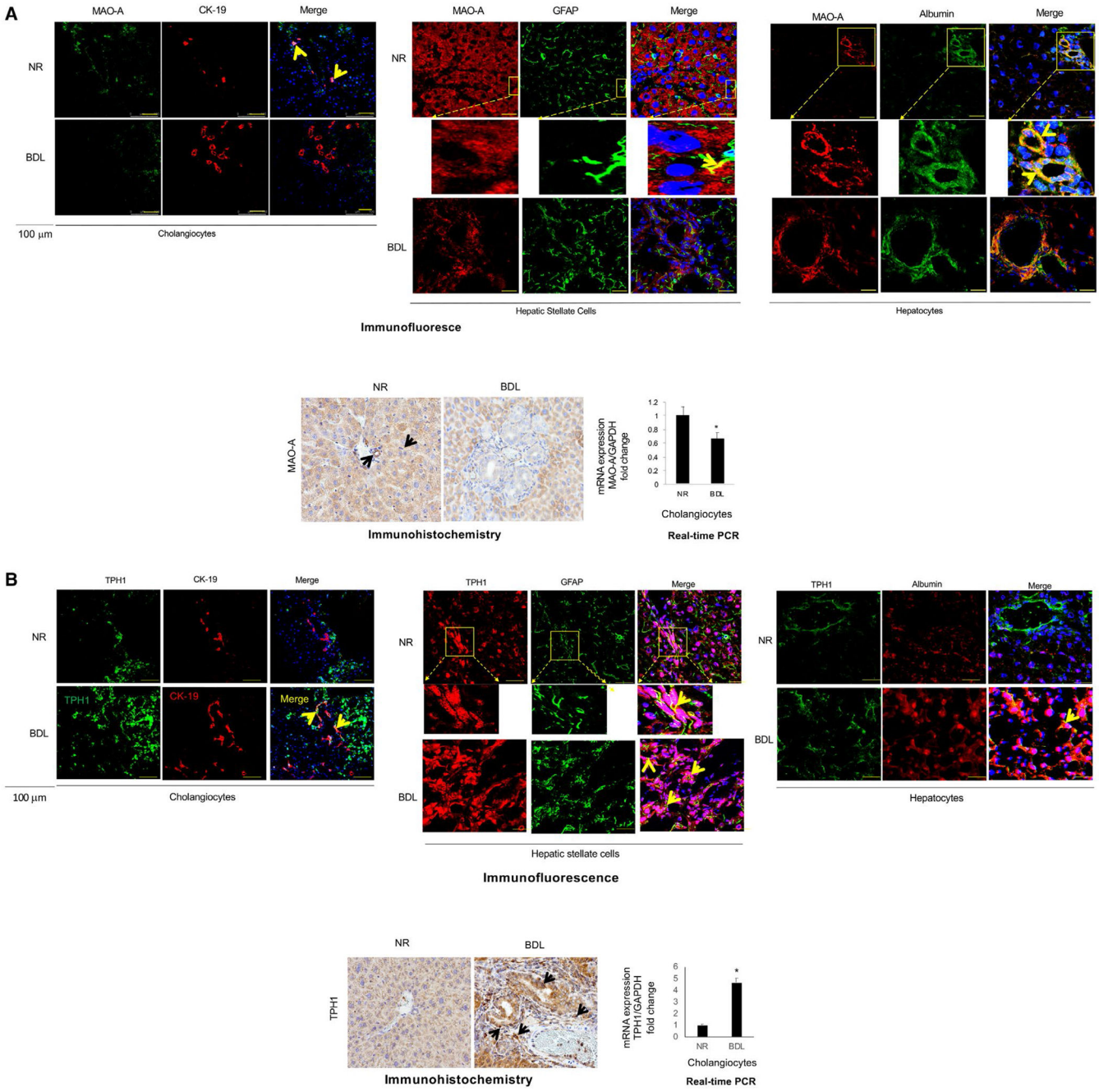


FIG. 2.

(A,B) There was decreased immunoreactivity for MAO-A, but enhanced immunoreactivity for TPH1 (both expressed by cholangiocytes, costained with CK-19, and HSCs, costained with GFAP) in liver sections from BDL compared to normal rats. By IF, we demonstrated immunoreactivity for MAO-A/TPH1 in hepatocytes of NR and BDL rats; nuclei were stained with DAPI (blue); scale bar = 100 μm. Yellow arrows show the colocalization of MAO-A/TPH1 with cholangiocytes (costained with CK-19), HSCs (costained with GFAP) and hepatocytes (costained with albumin). Black arrows show the MAO-A- and TPH1-positive bile ducts. Original magnification, ×40. By qPCR, there was decreased mRNA

expression of MAO-A, but enhanced expression of TPH1, in cholangiocytes from BDL compared to normal rats. Data are expressed as mean ± SD. Data are from four qPCR reactions from three cumulative preparations of cholangiocytes from 3 rats. *$P < 0.05$ versus NR.

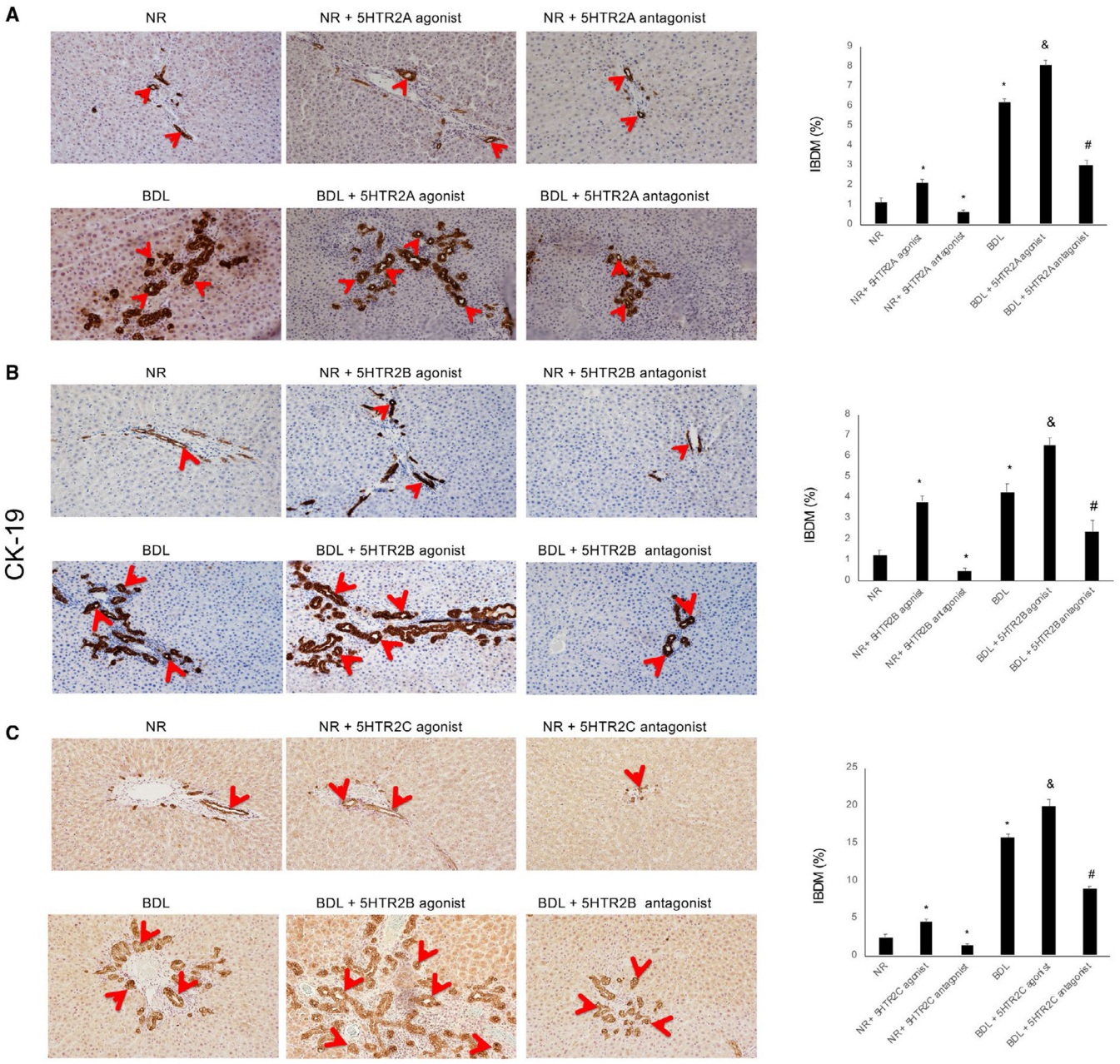
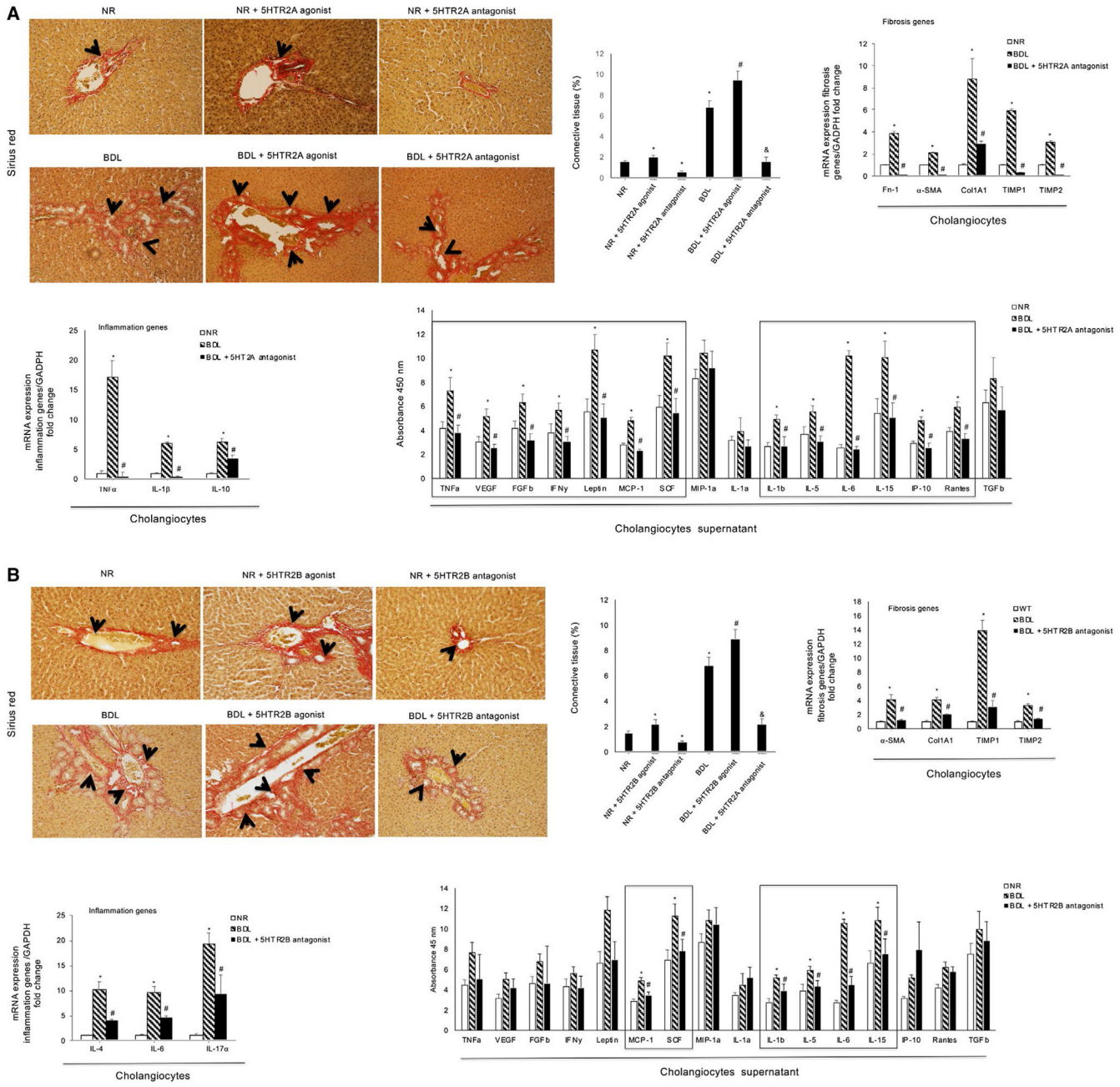


FIG. 3.

(A-C) IBDM increased in both normal and BDL rats treated with 5HTR2A/2B/2C agonists, but decreased in normal and BDL rats treated with 5HTR2A/2B/2C antagonists compared to their respective control rats. Data are expressed as mean ± SD from five cumulative values of each group. $^{*}P < 0.05$ versus NR; $^{\&\#}P < 0.05$ versus BDL rats. Red arrows show CK-19–positive bile ducts. Original magnification, ×20.



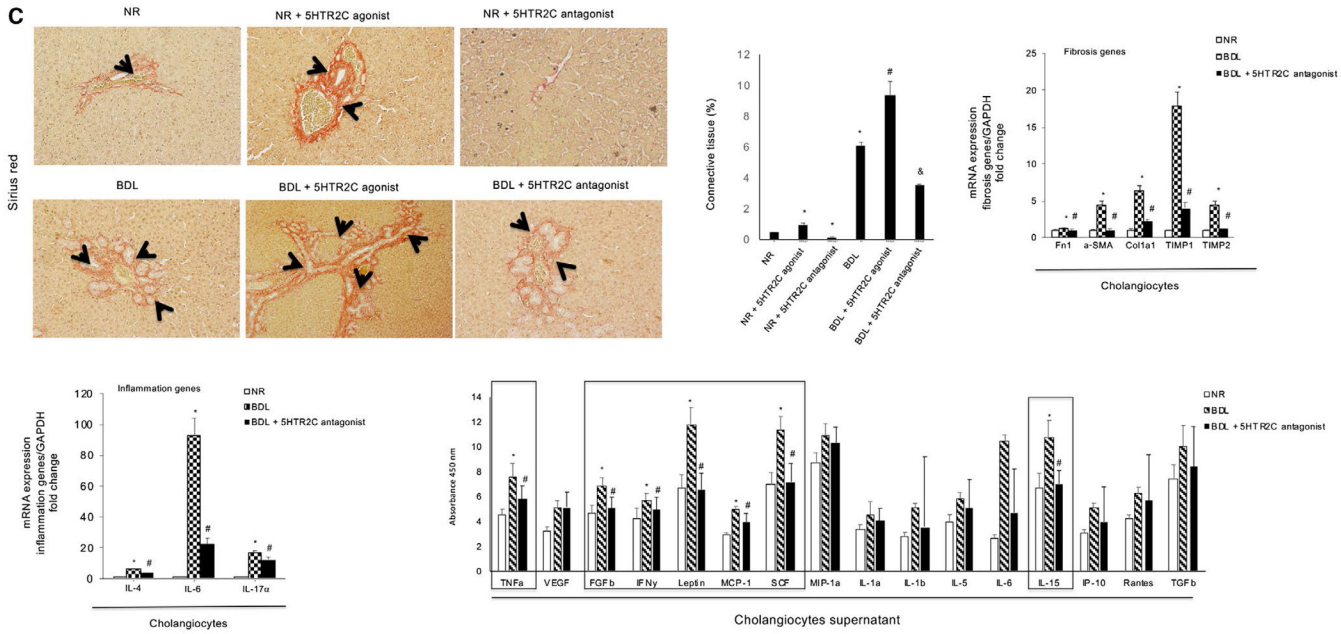


FIG. 4.

(A-C) Collagen deposition increases in BDL compared to normal rats and in normal and BDL rats treated with 5HTR2A/2B/2C agonists, but decreased in BDL and normal rats treated with 5HT2RA/2B/2C antagonists compared to their respective control rats. Original magnification, ×40. Black arrows show collagen deposition around bile ducts. By ELISA the levels of SASPs increased in cholangiocyte supernatant from BDL rats compared to normal rats, and the levels of following SASPs; TNFα, VEGF, FGFβ, INFγ, leptin, MCP-1, SCF, IL-1β, IL-5, IL-6, IL-15, and Rantes or CCL-5 decreased in supernatant from cholangiocytes from BDL rats treated with 5HTR2A antagonist; MCP-1, SCF, IL-1β, IL-5, IL-6, and IL-15 decreased in supernatant of cholangiocytes from BDL rats treated with 5HTR2B antagonist; and TNFα, FGFβ, INFγ, leptin, MCP-1, SCF, and IL-15 decreased in supernatant of cholangiocytes from BDL rats treated with 5HTR2C antagonists compared to BDL control rats. mRNA expression of fibrosis and proinflammation genes increased in cholangiocytes from BDL rats compared to normal and decreased in BDL rats treated with 5HTR2A/2B/2C antagonists compared to saline-treated BDL rats. Data are expressed as mean ± SD. Data are from cholangiocyte supernatant of 3 different rats of each group and four qPCR reactions from three cumulative preparations of cholangiocytes from 3 rats. $*P < 0.05$ versus NRs; $^{\&\#}P < 0.05$ versus BDL rats.

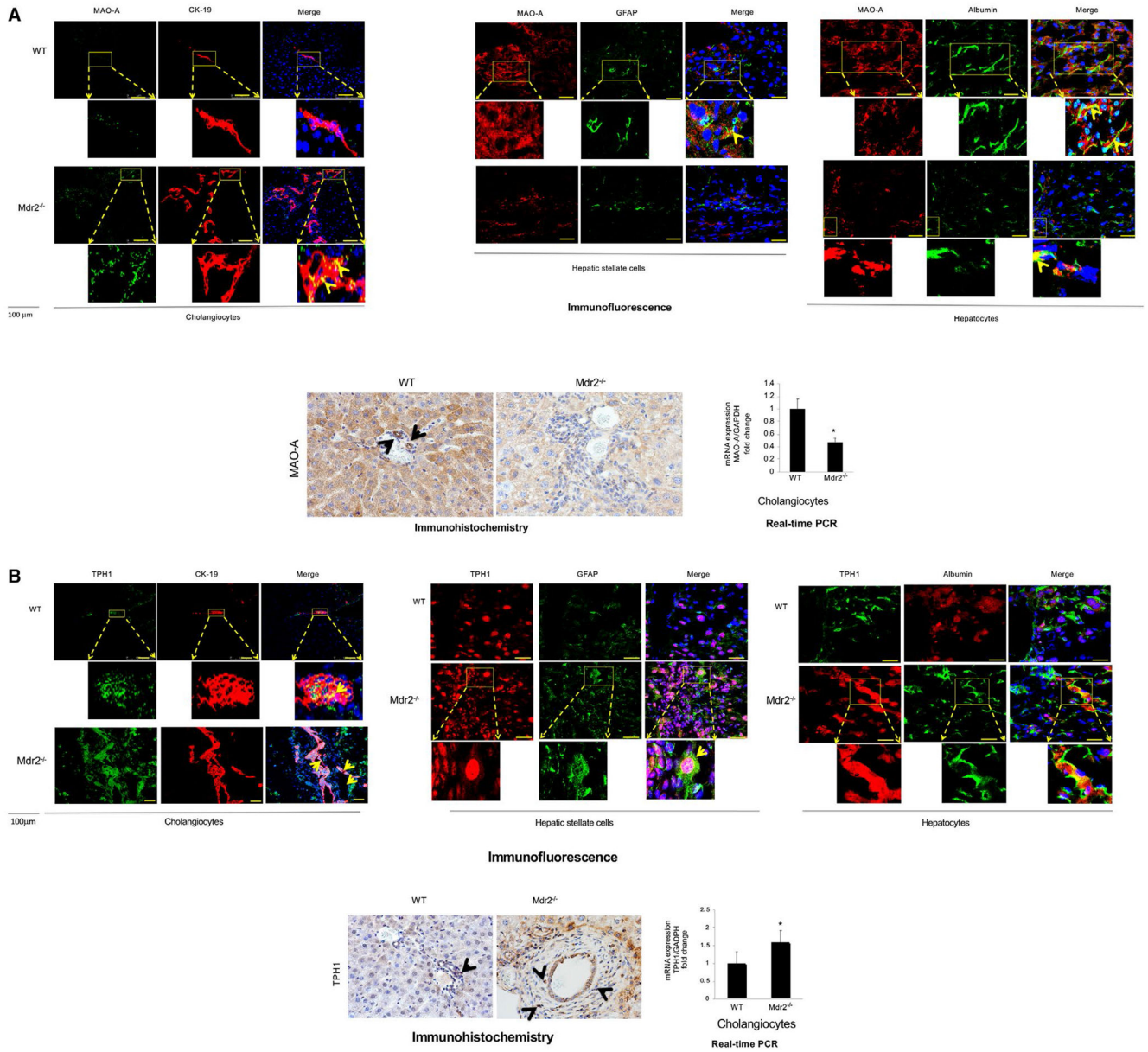


FIG. 5.

(A,B) There was decreased MAO-A immunoreactivity, but enhanced immunoreactivity of TPH1, in cholangiocytes (costained with CK-19) and HSCs (costained with GFAP) in *Mdr2*$^{-/-}$ mice compared to WT mice; hepatocytes displayed immunoreactivity for MAO-A/TPH1 (costaining with albumin); yellow arrows show the colocalization of TPH1 and MAO-A with cholangiocytes and GFAP. Nuclei were stained with DAPI (blue). Black arrows show MAO-A– and TPHl-positive bile ducts. Scale bar = 100 μm. Original magnification, ×40. By qPCR, there was decreased expression of MAO-A, but enhanced mRNA expression of TPH1, in cholangiocytes from *Mdr2*$^{-/-}$ compared to WT mice. Data are expressed as mean ± SD. Data are from four qPCR reactions from three cumulative preparations of cholangiocytes from 3 mice. *$P < 0.05$ versus WT mice.

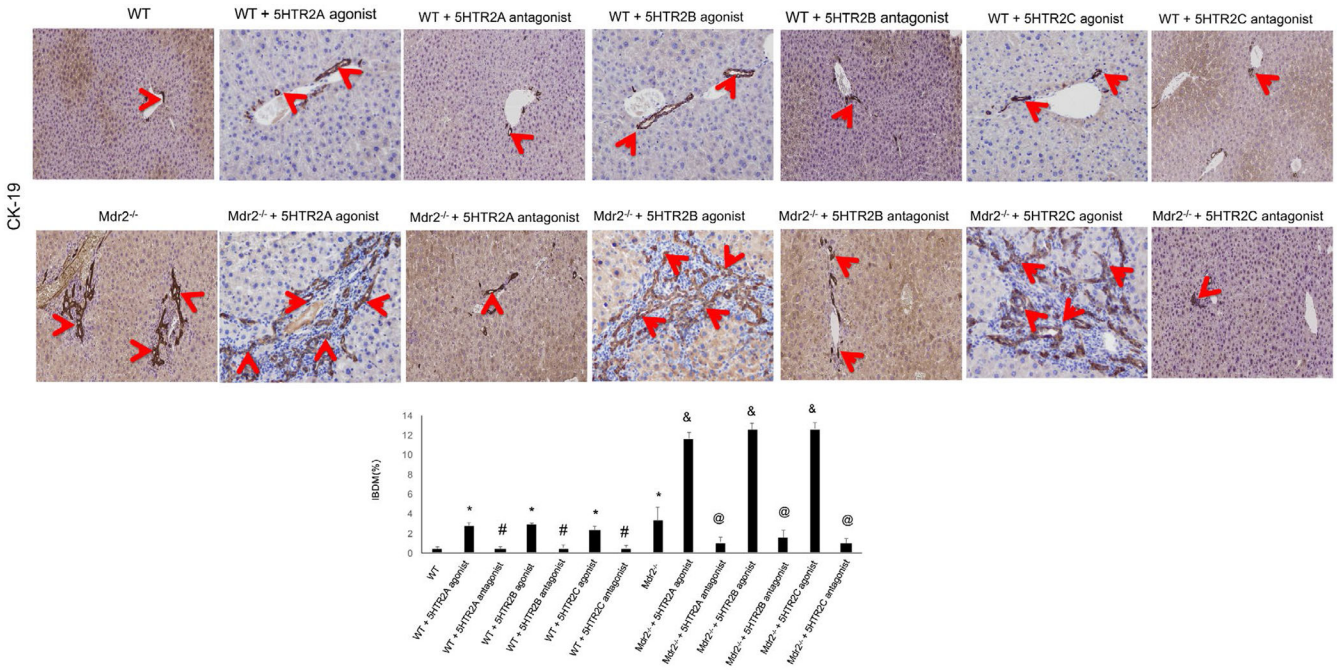


FIG. 6.

IBDM increased in both WT and *Mdr2*$^{-/-}$ mice treated with 5HTR2A/2B/2C agonists, but decreased in WT and *Mdr2*$^{-/-}$ mice treated with 5HTR2A/2B/2C antagonists compared to their respective control mice. Data are expressed as mean ± SD from five cumulative values of each group. *$P < 0.05$ versus WT; $^{\#}P < 0.05$ versus WT plus 5HTR2A/2B/2C agonists; $^{\&@}P < 0.05$ vs. *Mdr2*$^{-/-}$ mice and *Mdr2*$^{-/-}$ plus 5HTR2A/2B/2C agonists mice. Red arrows show CK-19-positive bile ducts. Original magnification, ×20.

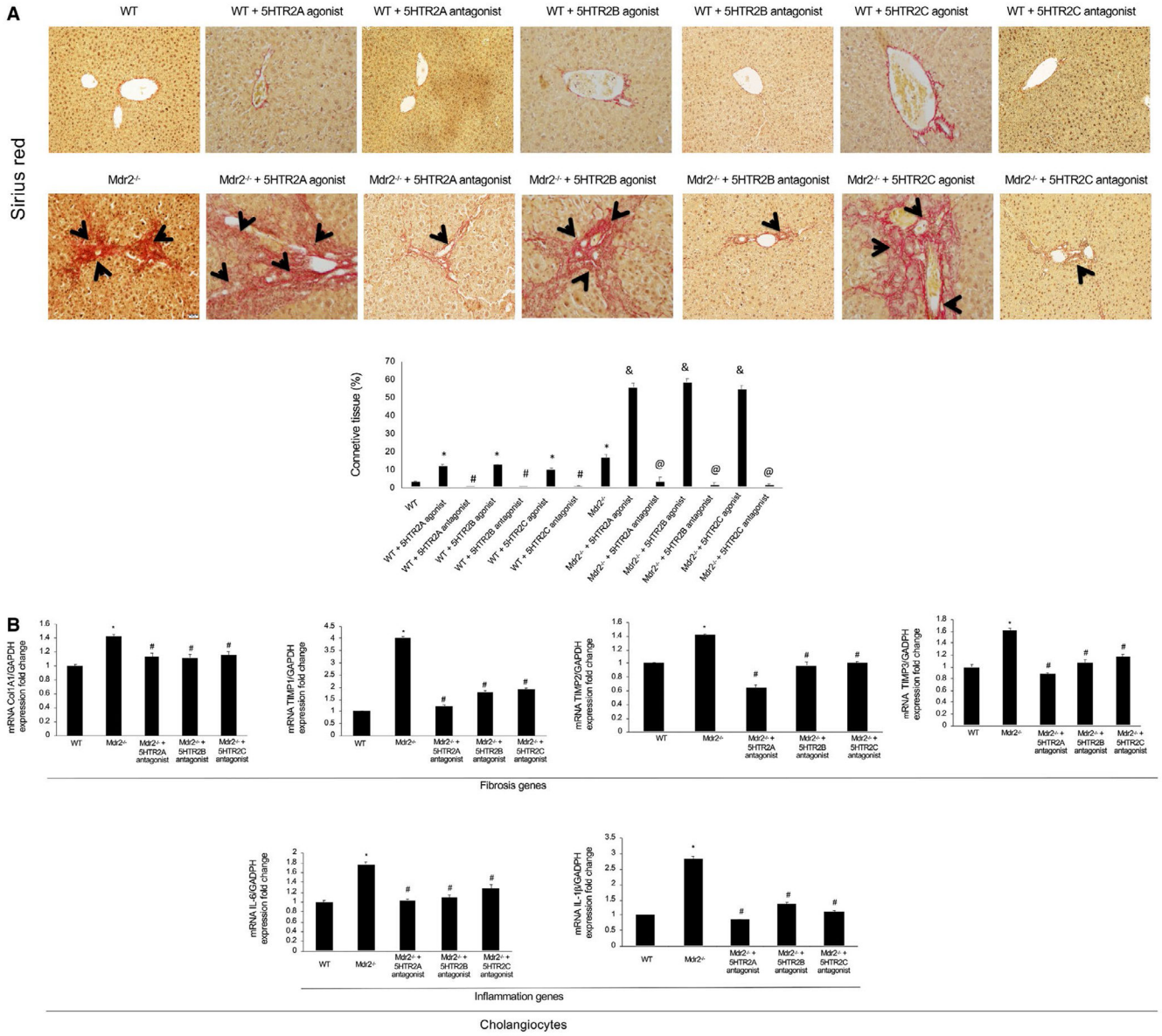


FIG. 7.

(A,B) Treatment of WT and *Mdr2*$^{-/-}$ mice with 5HTR2A/2B/2C agonists increased LF in both WT and *Mdr2*$^{-/-}$ mice, whereas treatment with 5HTR2A/2B/2C antagonists decreases LF compared to their respective control mice. Black arrows show the collagen deposition around the bile ducts. Original magnification, x40. *$P < 0.05$ versus WT; $^{\#}P < 0.05$ versus WT plus 5HTR2A/2B/2C agonists; $^{\&@}P < 0.05$ versus *Mdr2*$^{-/-}$ and *Mdr2*$^{-/-}$ plus 5HTR2A/2B/2C agonists mice. mRNA expression of fibrosis and proinflammation markers increased in cholangiocytes from *Mdr2*$^{-/-}$ compared to WT mice, but decreased in *Mdr2*$^{-/-}$ mice treated with 5HTR2A/2B/2C antagonists compared to *Mdr2*$^{-/-}$ untreated controls. Data are expressed as mean ± SD. Data are from four cholangiocytes from supernatant of 3 different mice of each group and four qPCR reactions from three cumulative preparations of cholangiocytes from 3 mice. *$P < 0.05$ versus WT mice; $^{\#}P < 0.05$ versus *Mdr2*$^{-/-}$ mice.

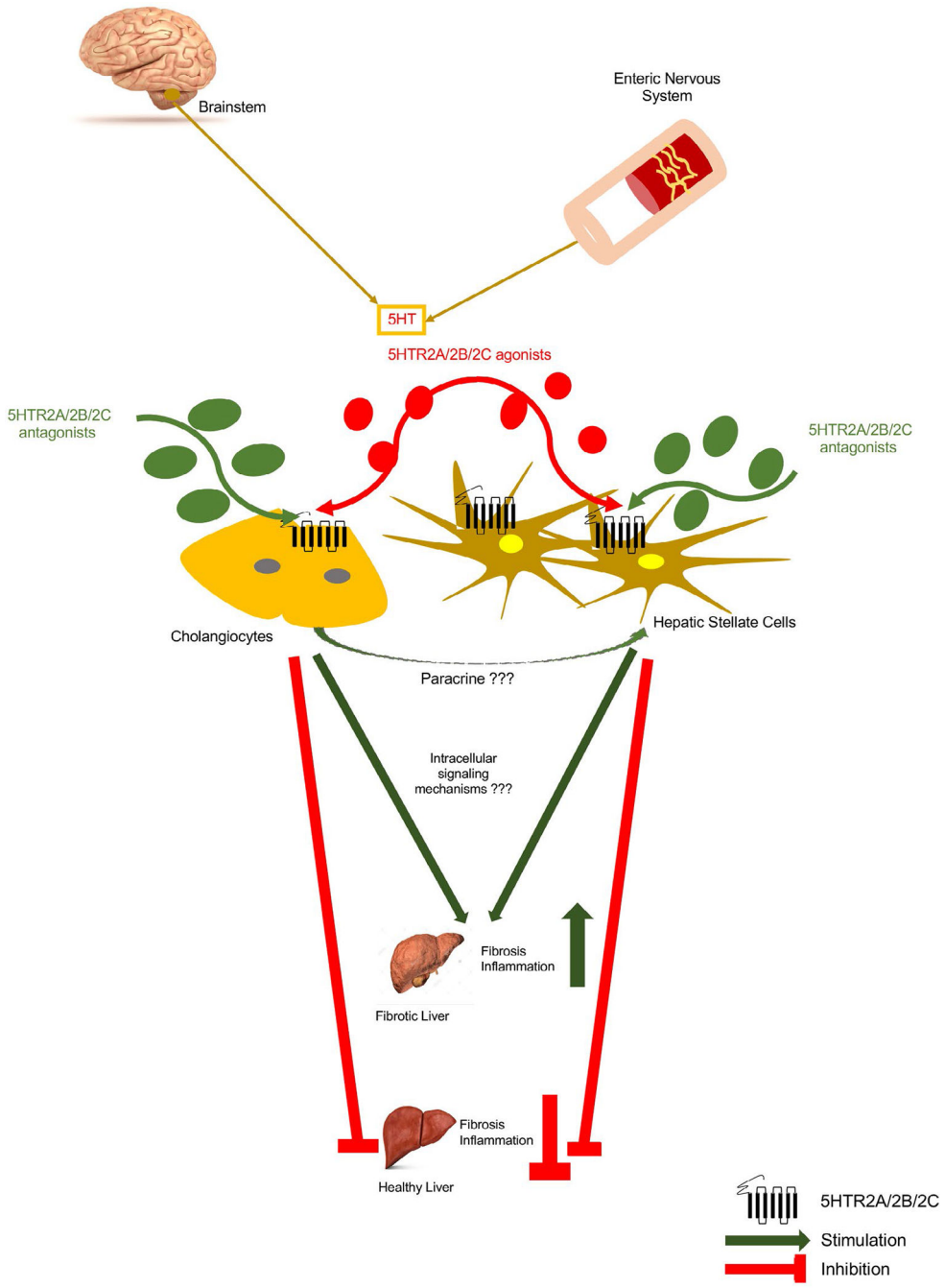


FIG. 8.

Working model related to the role of 5HTR2A/2B/2C agonists/antagonists on LF and inflammation. Agonists for the 5HTR2A/2B/2C (expressed in cholangiocytes and hepatic stellate cells) increased IBDM, LF, and inflammation of both normal and BDL rats. Administration of 5HTR2A/2B/2C antagonists to BDL rats or *Mdr2*$^{-/-}$ mice decreased IBDM, LF, and inflammation.