

Gastrointestinal, Hepatobiliary and Pancreatic Pathology

A Role for Serotonin (5-HT) in Hepatic Stellate Cell Function and Liver Fibrosis

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Hepatic stellate cells (HSCs) are key cellular components of hepatic wound healing and fibrosis. There is emerging evidence that the fibrogenic function of HSCs may be influenced by neurochemical and neurotrophic factors. This study addresses the potential for the serotonin (5-HT) system to influence HSC biology. Rat and human HSCs express the 5-HT_{1B}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, and 5-HT₇ receptors, with expression of 5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2B} being induced on HSC activation. Induction of 5-HT_{2A} and 5-HT_{2B} was 106 ± 39- and 52 ± 8.5-fold that of quiescent cells, respectively. 5-HT_{2B} was strongly associated with fibrotic tissue in diseased rat liver. Treatment of HSCs with 5-HT₂ antagonists suppressed proliferation and elevated their rate of apoptosis; by contrast 5-HT was protective against nerve growth factor-induced apoptosis. 5-HT synergized with platelet-derived growth factor to stimulate increased HSC proliferation. HSCs were shown to express a functional serotonin transporter and to participate in both active uptake and release of 5-HT. We conclude that HSCs express key regulatory components of the 5-HT system enabling them to store and release 5-HT and to respond to the neurotransmitter in a profibrogenic manner. Antagonists that selectively target the 5-HT class of receptors may be exploited as antifibrotic drugs. (*Am J Pathol* 2006, 169:861–876; DOI: 10.2353/ajpath.2006.050767)

sis.^{1–3} In the normal healthy liver, HSCs function to regulate sinusoidal blood flow and the traffic of macromolecules across the space of Disse and also act as a store for vitamin A. In response to hepatic injury, HSCs undergo a gross morphological change in terms of both function and phenotype in a process termed “activation”, transforming to that of myofibroblast-like cell.^{1–3} The myofibroblast-like activated HSC (aHSC) is characterized by the expression of smooth muscle α -actin (α -SMA), enhanced collagen production, expression of the tissue inhibitor of metalloproteinases-1, and the loss of vitamin A stores. Additionally, on activation, the normally quiescent HSC enters the cell cycle and, in response to both auto-crine and paracrine stimulators, proliferates to produce a population of profibrogenic cells in the injured liver. Because the aHSC phenotype is relatively resistant to apoptosis due in part to the antiapoptotic effects of tissue inhibitor of metalloproteinases-1 and their high basal nuclear factor- κ B activity,^{4,5} there is a propensity in the chronically injured liver for aHSCs to persist and perpetuate. This leads to the excess deposition of cross-linked collagen resulting in both qualitative and quantitative modification of the hepatic extracellular matrix (ECM).⁶ If this process of ECM remodeling continues, then the liver becomes fibrotic, and cirrhosis eventually develops, accompanied by life threatening disturbance of normal liver physiology. There is currently much interest in improving our understanding of how HSC proliferation and apoptosis are regulated because *in vivo* experimental manipulation of these processes is known to attenuate the fibrogenic process.^{5,7–9} In particular, there is a drive to discover novel surface receptors on aHSCs that are able to attenuate proliferation and/or apoptosis in response to specific ligands.

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The hepatic stellate cell (HSC) is recognized as one of the key mediators in the progression of hepatic fibro-

Several mitogens promote the proliferation of aHSCs including platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1) and connective tissue growth factor (CTGF).^{7,10,11} Of these factors, PDGF is the most potent mitogen. Hepatic PDGF levels rise during liver injury, and HSC activation is accompanied by their acquisition of the surface expression of PDGF receptors.¹² Blockade of PDGF signaling via the PDGF receptor results in inhibition of aHSC proliferation and attenuation of experimentally induced liver fibrosis.⁷ The signaling events that mediate PDGF stimulation of aHSC proliferation have been investigated and involve various pathways including those leading to the activation of Phospholipase C γ , phosphatidylinositol 3-kinase and ERK1/2.¹³ A great deal of attention has recently focused on the process of aHSC apoptosis because stimulation of this process *in vivo* promotes accelerated rates of recovery from rat liver fibrosis.^{5,8} Surface receptors implicated in the regulation of aHSC apoptosis include the p75 low affinity nerve growth factor (NGF) receptor, transforming growth factor (TGF)- β and tumor necrosis factor- α receptors, $\alpha_v\beta_3$ integrin, N-cadherin, cannabinoid receptor 2, tumor necrosis factor-related apoptosis-inducing ligand, and Fas receptors.¹⁴⁻¹⁸ However, there still remains a need to identify novel surface receptors of aHSCs that can respond to specific pharmacological agonists/antagonists because these receptors would provide a rapid route to the development of antifibrogenic drugs.

Serotonin (5-hydroxytryptamine [5-HT]) is a biogenic amine that exerts its biological activities via seven major receptor families (5-HT₁ to 5-HT₇).¹⁹ The 5-HT₂ family of receptors comprises three members, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}, which are coupled through the G_q protein to phospholipase C and phospholipase A₂.²⁰ The 5-HT₂ receptor subtypes are targets for a vast array of drugs that are used clinically as antipsychotics, antidepressants, and antihistamines.^{21,22} 5-HT has been linked to abnormal cellular proliferation and to fibrotic diseases, although to date, there is no literature on the role of 5-HT in liver fibrosis. However, studies in renal mesangial cells, which are phenotypically and functionally similar to HSCs, have shown that serotonin is mitogenic and stimulates production of TGF- β 1 and CTGF via activation of the 5-HT_{2A} receptor and ERK1/2.^{23,24} Hence, studies on the role of the 5-HT system in the regulation of aHSC function and liver fibrosis are warranted.

In the present study, we report the expression of functional 5-HT receptors on human (*in vitro*) and rat (*in vitro* and *in situ*) aHSCs and demonstrate that 5-HT receptor antagonists attenuate the proliferative and enhance the apoptotic properties of aHSCs. We define a fibrogenic role for 5-HT in the liver by showing that it functions in synergy with PDGF to stimulate proliferation. Additionally, we present evidence that culture-activated HSCs express the serotonin transporter (SERT) and are able to release 5-HT into the culture media. We therefore describe for the first time an autocrine pathway by which 5-HT can influence the proliferation and apoptosis of aHSCs and demonstrate that antagonists selective for the 5-HT receptor family may have antifibrogenic potential.

Materials and Methods

Cell Isolation

Rat HSCs were isolated from the livers of normal male Sprague-Dawley rats (400 \pm 50 g) by sequential perfusion with pronase and collagenase as previously described.⁸ HSCs were seeded onto plastic, cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Rockville, MD) supplemented with 16% fetal calf serum (FCS; Life Technologies, Inc.), and maintained at 37 °C in an humidified atmosphere of 5% CO₂. Human HSCs were extracted from the margins of normal human liver resected for colonic metastatic disease as previously described.⁸ Human HSCs were used for experimentation after activation in primary culture or before the fourth passage. The use of human liver tissue for scientific investigation was approved by the UK South and West Local Research Ethics Committee and was subject to patient consent. COS-7 cells were maintained as previously described.²⁵

Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis

Whole-cell protein extracts were prepared by lysis of phosphate-buffered saline (PBS)-washed cultures in 150 mmol/L NaCl, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 2 mmol/L ethylenediamine tetraacetic acid, and 10 mmol/L Na₂HPO₄. Equal quantities (20 μ g) of whole-cell protein were then fractionated by electrophoresis through a 9% SDS-polyacrylamide gel. Gels were run at a constant 100V for 1.5 hours before transfer onto nitrocellulose as previously described. After blockade of nonspecific protein binding, blots were incubated for 1 hour with primary antibodies (diluted in PBS/Tween 20 [0.05%]) containing 3% Marvel. Mouse monoclonal antibodies recognizing the 5-HT_{2A} and 5-HT_{2B} receptors (BD Biosciences Pharmingen, Franklin Lakes, NJ) were used at a 1:1000 dilution. Blots were then washed four times with PBS/Tween 20 before incubation for 1 hour with rabbit anti-mouse horseradish peroxidase antibody (1:1000) and after extensive washing in PBS/Tween 20 before being processed to distilled water for detection of antigen using the ECL system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Extraction of RNA from HSCs

Subconfluent (80 to 90%) rat and human HSCs were first washed three times with ice-cold PBS and placed on ice before total RNA isolation using the RNeasy mini kit (Qiagen, Valencia, CA) per the manufacturer's instructions. Total RNA was then treated with 1 μ l of RQ1 RNase-free DNase I (1U/ μ L; Promega, Madison, WI) per 1 μ g of total RNA for 1 hour at 37°C to ensure complete removal of all DNA contamination. Enzymatic digestion was terminated by the addition of stop buffer containing 20 mmol/L EGTA, pH 8.0. Total RNA concentration was then estimated by spectrophotometry at 260 nm.

Table 1. Details of the Oligonucleotides Used in PCR Reactions

Target gene	Primer sequence	Annealing temperature (°C)	Product size (bp)
<i>Rat 5-HT_{1A}</i>	Forward 5'-GGGCAGTGAGGCAGGGTGAC-3' Reverse 5'-GGCGGGGCATAGGAGTTAGAT-3'	59.2	122
<i>Rat 5-HT_{1B}</i>	Forward 5'-CACGGTGGGGGCTTTCTATTTC-3' Reverse 5'-TGCCGTCTTGTGGGTGTC-3'	56.0	110
<i>Rat 5-HT_{1D}</i>	Forward 5'-AGCCCAGAAAGGAAAGCCACTA-3' Reverse 5'-GGGGAGGACCAAGGATACCACAA-3'	55.0	97
<i>Rat 5-HT_{1F}</i>	Forward 5'-AGTCGAGTATGCCAGGAAGAGGA-3' Reverse 5'-GAGGAGGCACGGAGATGAACAC-3'	53.6	92
<i>Rat 5-HT_{2A}</i>	Forward 5'-TACGCAGGCCGAAGGACGAT-3' Reverse 5'-GGGCACCACATTACAACAAACAGG-3'	55.5	98
<i>Rat 5-HT_{2B}</i>	Forward 5'-TTTGGGTCACTGGCTGCTTTCTTT-3' Reverse 5'-AGTCCACCGTGTAGGCGTTGAG-3'	55.3	132
<i>Rat 5-HT_{2C}</i>	Forward 5'-CACACCGAGGAGGAAGTGGCTAATA-3' Reverse 5'-TCTGGACGCTTTTCTTCTTCTTT-3'	55.1	141
<i>Rat 5-HT₃</i>	Forward 5'-GATAGCCCTCTCCACCAC-3' Reverse 5'-AACCACTCCCTGCCACCTC-3'	55.9	125
<i>Rat 5-HT₄</i>	Forward 5'-CAGGCAGCTCAGGAAAATAAAAACC-3' Reverse 5'-TCCGGACCAGGCAAAACATCTC-3'	54.9	149
<i>Rat 5-HT_{5A}</i>	Forward 5'-GGTCAGTCGCGAGCCTTCCTA-3' Reverse 5'-TCGCAGCCTTGTAATCTTCCAGTA-3'	55.9	104
<i>Rat 5-HT_{5B}</i>	Forward 5'-TGGCGGGAGCAGAAGGAGAA-3' Reverse 5'-TGAATAGCCAAGCCACAGGAATA-3'	57.7	165
<i>Rat 5-HT₆</i>	Forward 5'-GCTGGGTGGCAGCAACT-3' Reverse 5'-GCGGGCAGCCAGAAGGAT-3'	59.1	160
<i>Rat 5-HT₇</i>	Forward 5'-CGTGCAGCCGAGAGTGTG-3' Reverse 5'-CCCGCTTGAAGATGGAGATGTT-3'	55.4	124
<i>Rat SERT</i>	Forward 5'-CACAGCGGACAGGGCAGAGC-3' Reverse 5'-GGAGGCGATGTAAGGCGATGAT-3'	60.2	673
<i>Human 5-HT_{2A}</i>	Forward 5'-ACCACAGCCGCTTCAACTCCA-3' Reverse 5'-AAAGGGCACCACATCACCACAAA-3'	57.2	471
<i>Human 5-HT_{2B}</i>	Forward 5'-GGCCCCTCCCCTTGTCTAT-3' Reverse 5'-GGCGTTGAGGTGGCTTGTTT-3'	55.3	413
<i>Human 5-HT_{2C}</i>	Forward 5'-TGTGCCCGCTCTGGATTCTTTAG-3' Reverse 5'-TCGGCGTGCCTTCTGGTCTT-3'	58.1	487
<i>BTF3</i>	Forward 5'-TGGCAGCAAAACCTTCACC-3' Reverse 5'-AGCTTCAGCCAGTCTCCTCAAAC-3'	55.4	124
<i>CTGF</i>	Forward 5'-AGCGCCTGTTCTAAGACCTGTG-3' Reverse 5'-TTTGGCCCTTCTTAATGTTTTCCTC-3'	56.0	154

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Five hundred nanograms of total RNA extracted at regular time intervals from HSCs undergoing culture activation and passage 2 human HSCs (as described above) was used to generate first-strand cDNA using a random hexamer primer (oligo(dN)₆). PCR amplification of rat and human 5-HT receptors, SERT basic transcription factor 3 (BTF3), and β -actin cDNAs was performed using specific oligonucleotide primers selected within the coding regions of each particular gene. Please refer to Table 1 for exact details of primer sequences, optimal annealing temperatures, and PCR product size. Semiquantitative PCR reactions were composed of 1 μ l of cDNA, 100 ng each of sense and antisense oligonucleotide primers, and 12.5 μ l of 2 \times PCR master mix (Promega) containing 50 units/ml *Taq*DNA polymerase, 400 μ mol/L dATP, 400 μ mol/L dGTP, 400 μ mol/L dCTP, 400 μ mol/L dTTP, and 3 mmol/L MgCl₂ in a final volume of 25 μ l. After an initial 5-minute incubation at 94°C, PCR was performed using a 1-minute annealing step at temperature indicated for each primer pair, followed by a 1-minute elongation step at 72°C and a 1-minute denaturation step at 94°C. Up to a maximum of 40 PCR cycles were performed

when performing semiquantitative PCR analysis, followed by a final elongation reaction for 5 minutes at 72°C. PCR products were separated by electrophoresis at 50V for 1 hour through a 1% Tris acetate buffer agarose gel and were detected using ethidium bromide staining. Expected sizes of specific PCR products were verified by reference to a 1-kb DNA ladder (Promega). Real-time PCR analysis reactions comprising 1 μ l of cDNA, 1 μ mol/L each of sense and antisense oligonucleotide primer, and 7.5 μ l of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a final 15- μ L reaction volume. After an initial 10-minute incubation at 95°C, the following reaction conditions were used: 95°C for 20 seconds, optimal annealing temperature for oligonucleotide pair (refer to Table 1) for 20 seconds followed by 20 seconds at 72°C. A maximum of 40 cycles of PCR were performed, after which time, melt analysis of the DNA generated by the PCR was performed using the following conditions: the temperature was increased from 60 to 99°C by raising 1°C at each step with the first step being held for 20 seconds and the remaining steps being held for 4 seconds before sample fluorescence was measured. PCR was performed using an RG-3000 from Corbett Research (NSW, Sydney, Australia).

Quantification of Apoptosis

Apoptotic HSCs were visualized by staining with a 1 $\mu\text{g/ml}$ solution of acridine orange (Sigma) in 10 mmol/L HEPES buffer (pH 7.4). Apoptotic cells in five random fields were counted in duplicate wells at $\times 20$ magnification using an FITC filter, cells were counted in four independent experiments. Caspase 3 activity was determined using the caspACE 3 (DEVDase) colorimetric assay and was calculated as described by the manufacturer (Promega).

Measurement of [^3H]Serotonin Uptake

Seven-day culture-activated rat HSCs or COS-7 cells transfected with rat SERT cDNA using Lipofectamine (as previously described²⁵) were seeded in 6-well plates or 12-well plates, respectively. HSCs were left for 24 hours before being placed in low-serum growth medium (0.01%) overnight. HSCs were then washed twice with warm PBS (37°C) and incubated with 5-hydroxy[G- ^3H]tryptamine creatinine sulfate (10 to 20 Ci/mmol; Amersham) in a medium containing 120 mmol/L NaCl, 5 mmol/L KCl, 1.2 mmol/L CaCl_2 , 1.2 mmol/L MgSO_4 , 5.6 mmol/L glucose, 4 mmol/L Tris-HCl, 6.25 mmol/L HEPES, and 0.5 mmol/L ascorbic acid, pH 7.4 (uptake buffer). COS-7 cells were left for 48 hours after transfection before being incubated with 5-hydroxy[G- ^3H]tryptamine creatinine sulfate diluted in uptake buffer. Under these conditions, uptake by rat HSCs and COS-7 cells was linear for at least 10 minutes. Therefore, assays were performed for 5 minutes at 37°C (total uptake) and 4°C (nonspecific uptake). At the end of the incubation period, the medium was removed, and cells were washed three times with uptake buffer. Cells were then lysed by adding 200 μl of 0.2 N NaOH, and the radioactivity of the lysates was counted by liquid scintillation spectrometry. Uptake is expressed as femtomoles of [^3H]5-HT taken up per 10^6 cells per minute.

Determination of 5-HT Concentration in HSC Growth Medium

Day-7 culture-activated rat HSCs were washed twice with Dulbecco's modified Eagle's medium (DMEM) containing 0% FCS and incubated for 16 hours in DMEM containing 0% FCS before the commencement of experiments. At the beginning of each experiment, growth medium was again changed to DMEM containing zimelidine (10 $\mu\text{mol/L}$), which in turn was harvested at 10 and 360 minutes after cell application. 5-HT concentration was determined using enzyme-linked immunosorbent assay (ELISA; IBL Hamburg, Hamburg, Germany) according to the manufacturer's instructions.

Determination of Cellular Proliferation

Cellular proliferation was determined by one of two methods. In both assays, day-7 to -10 culture-activated rat HSCs in medium containing 16% FCS were seeded in 96-well plates at densities of 10^4 cells per well. The cells

were then left for 24 hours before the cells were washed with Hanks buffer saline solution/), and the growth medium was replaced with DMEM containing 0.01% FCS and 50 mmol/L HEPES for 16 hours. Cells were then treated as indicated in the legend to each figure for 2 hours before the addition of bromodeoxyuridine (BrdU), and cells were left for 14 hours before BrdU incorporation was determined according to the manufacturer's instructions (Oncogene Research Products, San Diego, CA). Another method used to determine cell proliferation was the CellTiter96 AQ_{ueous} One Solution Reagent (Promega) containing [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt: MTS]. Cells were treated in an identical manner as described above except that cells were treated for a total of 24 hours before the addition of 20 μl per well of the CellTiter96 AQ_{ueous} One Solution Reagent. Cells were then incubated for a further 2 hours at 37°C before the absorbance of each well being read at 490 nm.

Carbon Tetrachloride (CCl_4) Model of Liver Injury

Adult male Sprague-Dawley (200 to 225 g) rats were administered with CCl_4 (CCl_4 /olive oil, 1:1 [v/v]) 1 ml/kg body weight by intraperitoneal injection twice weekly for 4 weeks. Three days after the last CCl_4 injection, animals were killed by CO_2 asphyxiation, and livers were harvested and prepared for immunohistochemical analysis.

5-HT_{2A} Receptor and 5-HT_{2B} Receptor Immunostaining

Slides were de-waxed in xylene and dehydrated in alcohol, and antigen retrieval was achieved by microwaving in citric saline for 15 minutes. Endogenous peroxidase activity was blocked by hydrogen peroxide pretreatment for 15 minutes and then further blocked using an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA), 3 drops per section for 20 minutes, with Tris-buffered saline (TBS) washes between each stage. Slides were incubated with complete culture medium for 20 minutes followed by addition of mouse monoclonal 5-HT_{2A} receptor and 5-HT_{2B} receptor antibodies (BD Biosciences Pharmingen) diluted 1:80 and 1:160, respectively, in TBS applied to the slides and incubated overnight at 4°C. Slides were washed in TBS, and then the secondary and the anti-IgG horseradish peroxidase-conjugated tertiary antibodies were incubated for 20 minutes with TBS washes between antibody incubation (Vector Laboratories). 5-HT_{2A} receptor- and 5-HT_{2B} receptor-positive cells were visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining. Slides were counterstained with Mayer's hematoxylin for 30 seconds, dehydrated, cleared in xylene, and mounted in DPX.

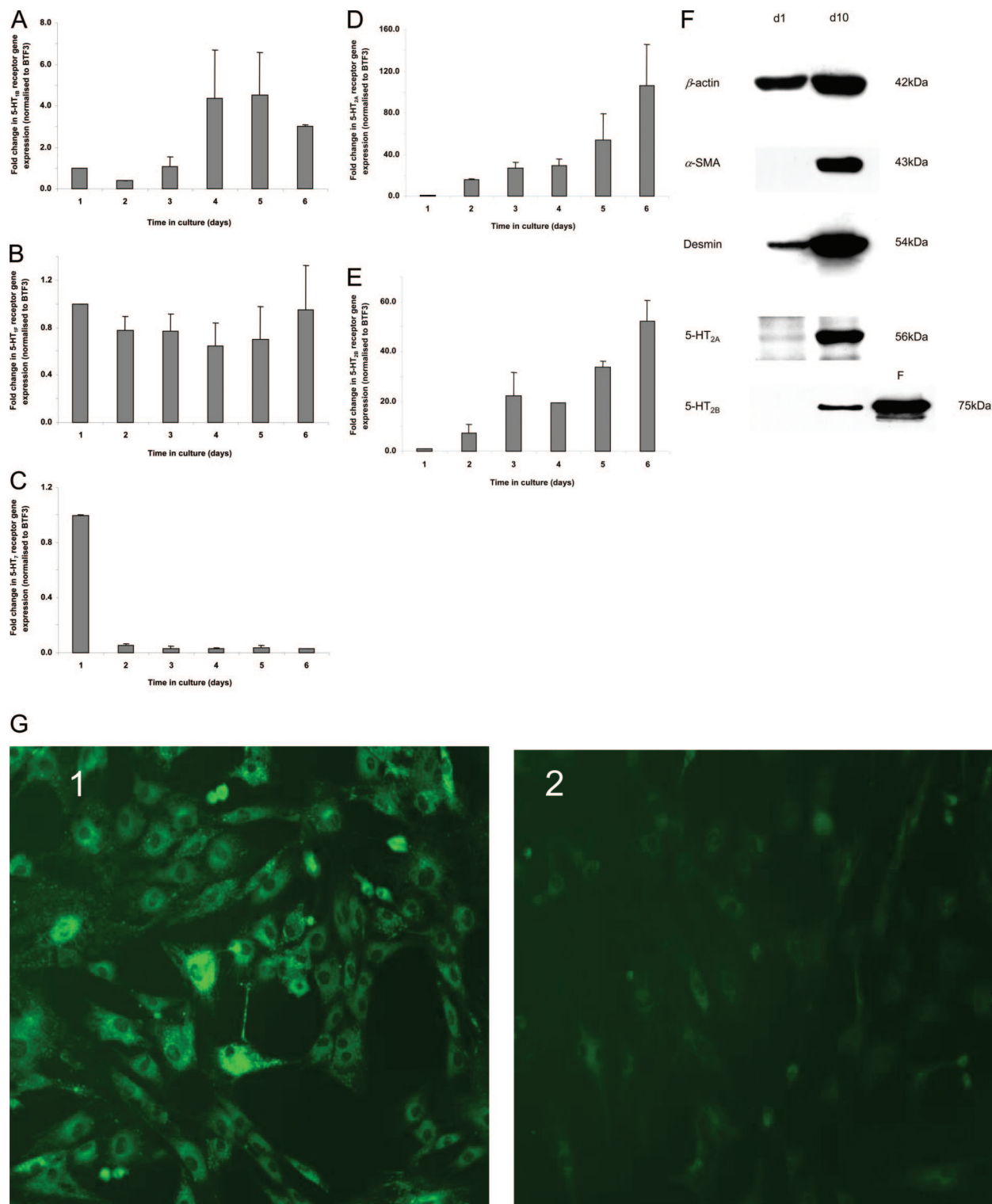


Figure 1. Detection of 5-HT receptor subtype expression in rat HSCs. **A–E:** Real-time RT-PCR analysis was performed on total mRNA isolated from rat HSCs at regular 24-hour time periods after between 1 and 6 days of culture on plastic dishes. Primers specific to the individual subtypes of the rat 5-HT receptors were used to prime PCR reactions (see Table 1 for list). 5-HT receptor expression levels were normalized to the expression levels of BTF3, which was found not to change significantly with HSC activation. Results shown are for all receptor subtypes that were within the assay detection limits; those receptors absent were not detected. All PCR reactions had efficiencies $\geq 95\%$, and melt analysis of the amplified DNA demonstrated the generation of a single DNA species in each reaction (results not shown). Western blotting analysis (**F**) was also performed on 20 μg of whole-cell protein extracted from day-1 and day-10 rat HSCs. Once transferred to nitrocellulose membranes, the proteins were probed with antibodies specific to β -actin, α -SMA, desmin, 5-HT_{2A}, and 5-HT_{2B} receptors. Approximate molecular weights of the visible bands were estimated from prestained protein markers run simultaneously with the whole-cell protein. **F:** Whole-cell protein extracted from the rat stomach fundus used as a positive control for the 5-HT_{2B} receptor. **G:** 5-HT₂ receptor/ligand interaction was visualized using a fluorescently labeled spiperone derivative (10 $\mu\text{mol/L}$ NBD-spiperone) (panel 1), and binding specificity was demonstrated when rat HSCs were incubated with both NBD-spiperone and unlabeled 10 $\mu\text{mol/L}$ spiperone (panel 2). The results shown here are representative of three independent experiments from at least three separate cell preparations.

Results

Rat and Human HSCs Express 5-HT₂ Family Receptors

To determine which 5-HT receptors are expressed by rat HSCs, we initially performed real-time RT-PCR to detect transcripts in culture-activating (up to day 10) rat HSCs (aHSCs). Rat HSCs were found to express mRNA for a number of 5-HT receptors, including the 5-HT_{1B} receptor, which was induced during HSC activation (Figure 1A), and the 5-HT_{1F} receptor, which was found to be present at consistent expression levels (Figure 1B), whereas the mRNA for the 5-HT₇ receptor underwent a considerable decrease in expression 2 days after isolation (Figure 1C). Transcripts for both 5-HT_{2A} and 5-HT_{2B} receptors were induced with HSC activation (Figure 1, D and E). The remaining rat 5-HT receptor subtypes (1A, 1D, 2C, 3, 4, 5A, 5B, and 6) were below the level of assay detection (data not shown). Results were normalized to the recognized housekeeping gene, BTF3, which in our hands demonstrated no changes in HSC expression with culture activation (results not shown). Expression of the 5-HT_{2A} and 5-HT_{2B} receptors was further confirmed by immunoblotting. First, we confirmed the phenotypic status of the freshly isolated and activated HSCs with reference to expression of classic markers of HSC activation (Figure 1F). As expected, freshly isolated rat HSCs expressed undetectable levels of α -SMA and low levels of desmin, whereas aHSCs expressed high levels of both proteins. Abundant levels of both 5-HT_{2A} and 5-HT_{2B} proteins were detected in activated rat HSCs; by contrast freshly isolated rat HSCs expressed low levels of 5-HT_{2A}, whereas expression of 5-HT_{2B} was undetectable. We also determined that human aHSCs express 5-HT_{2A} and 5-HT_{2B} transcripts and proteins (Figure 2), but as observed with rat aHSCs, human HSCs lacked detectable expression of 5-HT_{2C} (data not shown). To confirm surface expression of functional 5-HT receptors, aHSCs were incubated with 4-nitrobenzo-2-oxa-1,3-diazol (NBD)-aminohexanoylaminophenylethylspiperone (NBD-spiperone), which is a fluorescent analog of the 5-HT_{1/2} receptor selective antagonist spiperone. As shown in Figure 1G, all cells in the culture were labeled at their surface with fluorescent spiperone, and this was inhibited by pre-incubation with unlabeled spiperone (Figure 1G).

Antagonists Selective for 5-HT₂ Receptors Induce aHSC Apoptosis and Suppress Proliferation

We next investigated the potential for surface 5-HT₂ receptors to influence two key phenotypic features of the aHSCs, their apoptotic index, and their proliferation. We initially determined the ability of 5-HT₂ receptor-selective antagonists to induce apoptosis by manual counting of acridine orange-stained cells as previously described.⁴ Incubation of rat aHSCs with spiperone, LY53,857, and methiothepin induced elevated numbers of apoptotic aHSCs in a dose-dependent fashion (Figure 3, A and B).

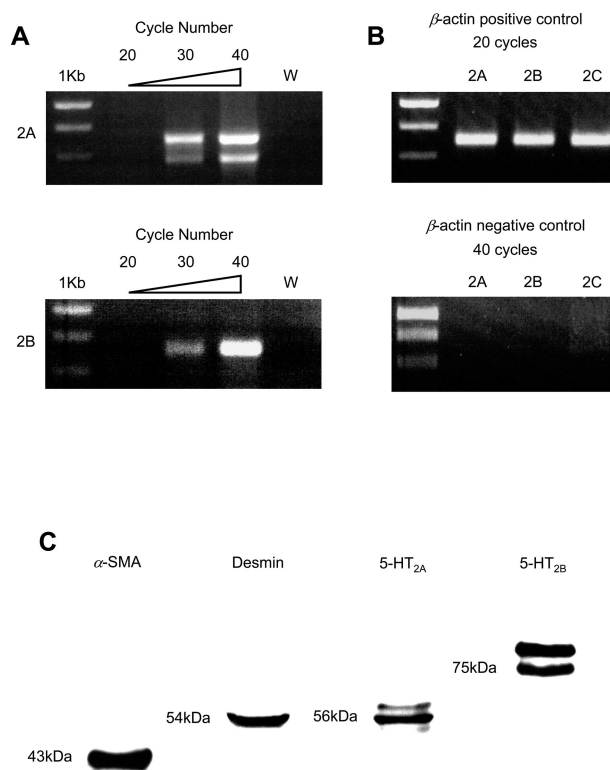


Figure 2. RT-PCR and Western blotting detection of 5-HT₂ receptor expression in human HSCs. **A:** RT-PCR analysis was performed on total mRNA isolated from passage 3 human HSCs. Primers specific to the human 5-HT_{2A} (2A) and 5-HT_{2B} (2B) receptors were used to prime the PCR reaction. PCR reactions were terminated after a predetermined number of cycles (no greater than 40). Controls performed were as follows: water replacing cDNA (lane W) was run simultaneously with test conditions, PCR was performed for 40 cycles using β -actin primers (**B**) where cDNA was replaced by mRNA (negative control), and the positive control was detection of β -actin gene expression using 20 cycles of PCR analysis. The approximate size of each amplified fragment was estimated from a 1-kb DNA ladder. Western blotting analysis (**C**) was also performed on 20 μ g of whole-cell protein extracted from passage 3 human HSCs. Once transferred to nitrocellulose membranes, the proteins were probed with antibodies specific to α -SMA, desmin, 5-HT_{2A}, and 5-HT_{2B} receptors. Approximate molecular weights of the visible bands were estimated from prestained protein markers run simultaneously with the whole-cell protein. The results shown here are representative of three independent experiments from at least three separate cell preparations.

As shown in Figure 3C, the optimal dose for each antagonist induced an elevated rate of apoptosis (from baseline levels) after just a 3-hour exposure. As a biochemical measurement of apoptosis, we used a caspase 3 activity assay. Spiperone was able to induce caspase 3 activity in aHSCs at a level similar to that measured in cells treated with the powerful proapoptotic fungal metabolite, gliotoxin (Figure 3D). Incubation of aHSCs with spiperone and 5-HT brought about a partial but significant blockade of apoptosis, indicating that spiperone induces apoptosis via a 5-HT receptor-linked mechanism. 5-HT also inhibited NGF (100 ng/ml)-induced HSC apoptosis, with all three concentrations of 5-HT tested (1, 10, and 50 μ mol/L) and significantly inhibiting NGF-induced apoptosis by 64.8, 70.5, and 76.1%, respectively (Figure 3E).

The influence of 5-HT₂ receptor signaling on rat aHSC proliferation was determined by BrdU incorporation assay on aHSC cultures treated with varying doses of antagonists. Methiothepin, spiperone, and ritanserin (Figure

Table 2. IC₅₀ Values of 5-HT₂ Antagonist's Inhibition of BrdU Incorporation in Rat Activated HSCs

Antagonist	Spiperone	Methiothepin	Ritanserin	LY53857	Ketanserin	WAY100635
IC ₅₀ (μmol/L)	1.03 ± 0.29	2.31 ± 0.28	6.31 ± 0.15	44.2 ± 0.56	—	—

Culture-activated rat HSCs (day 10 or greater) were cultured in DMEM plus 0.1% fetal calf serum and 50 mmol/L HEPES for 24 hours before incubation with various 5-HT receptor antagonists. Cells were treated for 2 hours prior to the addition of BrdU to the cell culture medium, and the extent of the BrdU incorporation was determined 16 hours later. Data are expressed as the concentration of the antagonist that was required to inhibit BrdU incorporation by 50% relative to control (growth medium alone) ± SEM for three separate experiments done in triplicate.

4, A–C) brought about powerful dose-dependent repression of aHSC proliferation with a 50% inhibition observed at doses of 2.31, 1.03, and 6.31 μmol/L, respectively. LY53,857 was less effective but still achieved a 50% inhibition of proliferation at a dose of 44.2 μmol/L (Figure 4D). For reasons we are at present unable to explain, ketanserin, which has selectivity for 5-HT_{2A} and 5-HT_{2C} receptors, was without effect (Figure 4E). But of note, WAY100635, which has specificity for the 5-HT_{1A} receptor not detected on aHSCs, was without effect on aHSC proliferation (Figure 4F). For summary of IC₅₀ values for the inhibition of BrdU incorporation associated with each individual 5-HT antagonist, see Table 2. Given that antagonists selective for the 5-HT₂ receptor family inhibit aHSC proliferation, it is likely that 5-HT stimulates proliferation. However, we repeatedly failed to observe any stimulatory effects of 5-HT on rat aHSCs (Figure 5A). We therefore explored the possibility that 5-HT may operate in synergy with other HSC growth factors such as PDGF. As shown in Figure 5B and Table 3, supplementation of media with 10 μmol/L 5-HT significantly enhanced the stimulatory effects of PDGF-BB on aHSC proliferation. Supplementation of the HSC growth media with a fixed dose of PDGF-BB (20 ng/ml) and increasing concentrations of 5-HT 100 nmol/L to 100 μmol/L was also found to increase cell proliferation (Figure 5C). By contrast 5-HT had no effect on IGF-1-stimulated proliferation (Table 3). Although 5-HT (up to 100 μmol/L) alone had no effect on proliferation rates, a significant increase in the cellular levels of CTGF (328% that of control) was observed after HSCs were incubated with 5-HT at various concentrations for 3 hours (Figure 5D).

HSCs Express the SERT and Are Able to Actively Transport Serotonin across the Plasma Membrane

Our observation that 5-HT antagonists are able to influence HSC apoptosis and proliferation suggests that in addition to aHSCs expressing 5-HT receptors, they may also be able to uptake and release 5-HT. RT-PCR and immunoblot analysis revealed that rat HSCs express SERT, which appears to be substantially induced on culture activation (Figure 6, A–C). To assess the ability of aHSCs to uptake 5-HT, we incubated culture-activated rat HSCs with [³H]serotonin at either 4°C (nonspecific transport) or 37°C (active transport) for 10 minutes before cell lysis and quantification of intracellular [³H]serotonin. Table 4 shows the degree of active 5-HT uptake by activated rat HSCs (intracellular [³H]serotonin at 37°C; intracellular [³H]serotonin at 4°C) with a V_{max} of 343.7 ±

50.3 pmol/1 × 10⁶ cells/minute and a K_m value of 302.9 ± 18.4 nmol/L. Comparison of these data with data gathered using an identical method from COS-7 cells (cells lacking a native SERT) transiently transfected with rat SERT demonstrated similar K_m values, both being between 300 and 400 nmol/L. However, the transiently transfected COS-7 did display a slightly elevated V_{max} when compared with activated rat HSCs, suggesting a higher level of SERT expression in COS-7 cells. We next determined whether aHSCs actively released 5-HT. Rat aHSCs were incubated in serum-free media containing zimelidine, which blocks the reuptake of 5-HT via SERT. Medium was then harvested at 10 and 360 minutes after exchange of cells into zimelidine-containing media, and 5-HT concentration in the media was determined by ELISA and with reference to a standard curve (not shown). An elevation of 5-HT was detected in culture media at both time points, with 11.5 and 9 ng/ml measured at 10 and 360 minutes, respectively (Figure 7). Taken together with the 5-HT uptake data, these results suggest that aHSCs are able to actively cycle 5-HT via SERT between the intracellular and extracellular compartments of the cell.

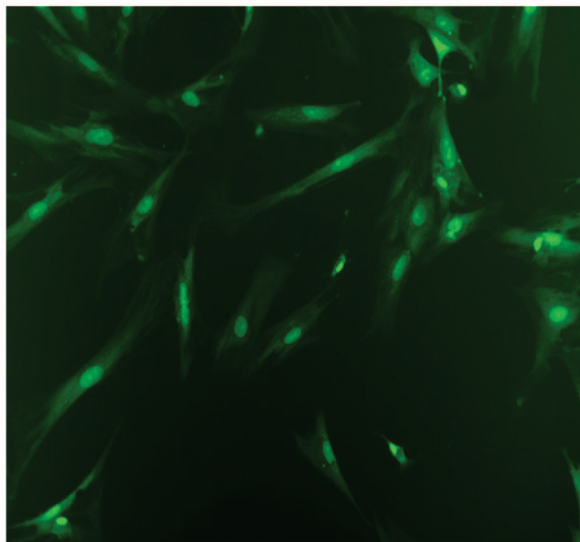
The 5-HT_{2B} Receptor Is a Selective Marker for aHSCs in Fibrotic Rat Liver

To establish whether 5-HT₂ receptors are also expressed in fibrotic liver, we performed immunohistochemical staining for 5-HT_{2A} and 5-HT_{2B} in rat livers injured for 8 weeks by twice weekly intraperitoneal administration of either CCl₄ or olive oil (control). Staining for 5-HT_{2A} in uninjured liver displayed weak diffuse expression that was increased in intensity (but again in a diffuse manner) throughout injured livers (Figure 8A). By contrast 5-HT_{2B} expression was essentially absent in control livers but was selectively induced in elongated cells associated with fibrotic bands. High-power analysis of the tissue sections also revealed weak hepatocyte staining for 5-HT_{2B} (Figure 8B). From these data, we propose that the 5-HT_{2B} receptor is a selective marker of aHSCs in fibrotic rat liver.

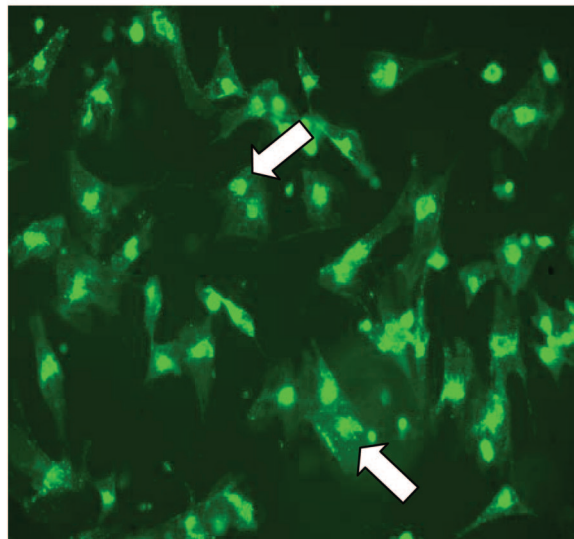
Discussion

The cellular mechanisms that underlie the persistence and proliferation of collagen-producing activated HSCs in chronic liver disease are now being revealed in detail. A key goal of these studies was the identification of molecular regulators of the phenotype and life span of aHSCs so as to

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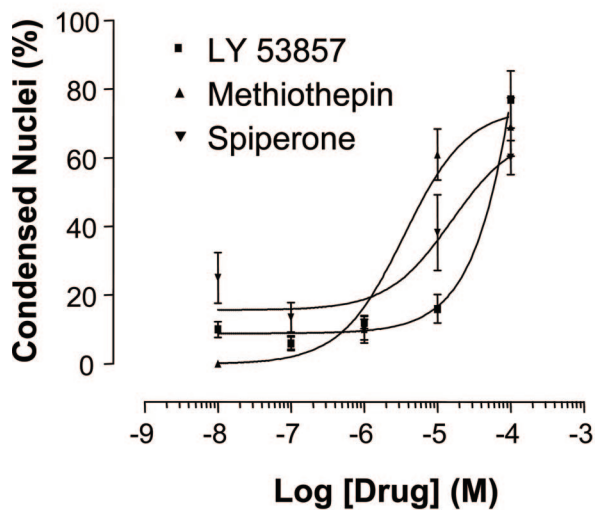


Control: vehicle only 24 hours

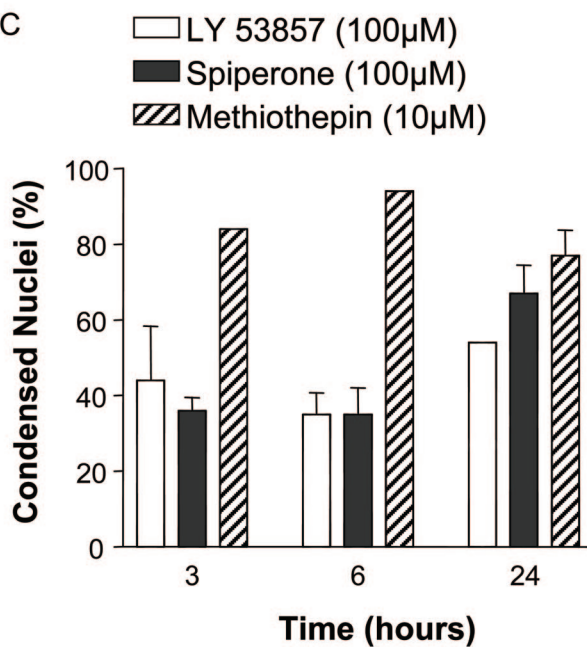


Spiperone 10µM 24 hours

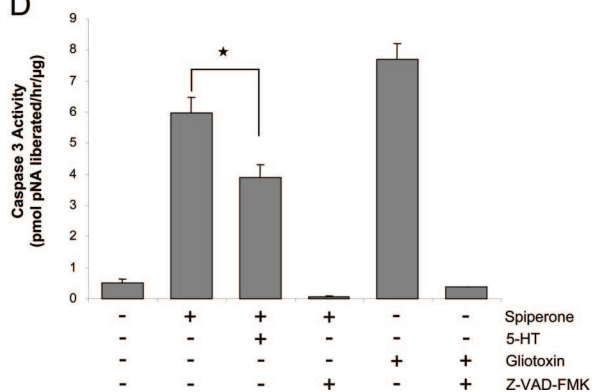
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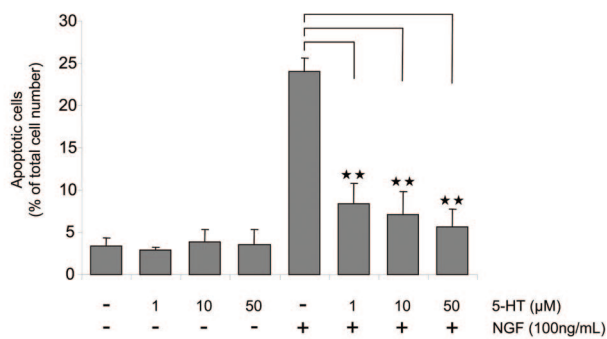
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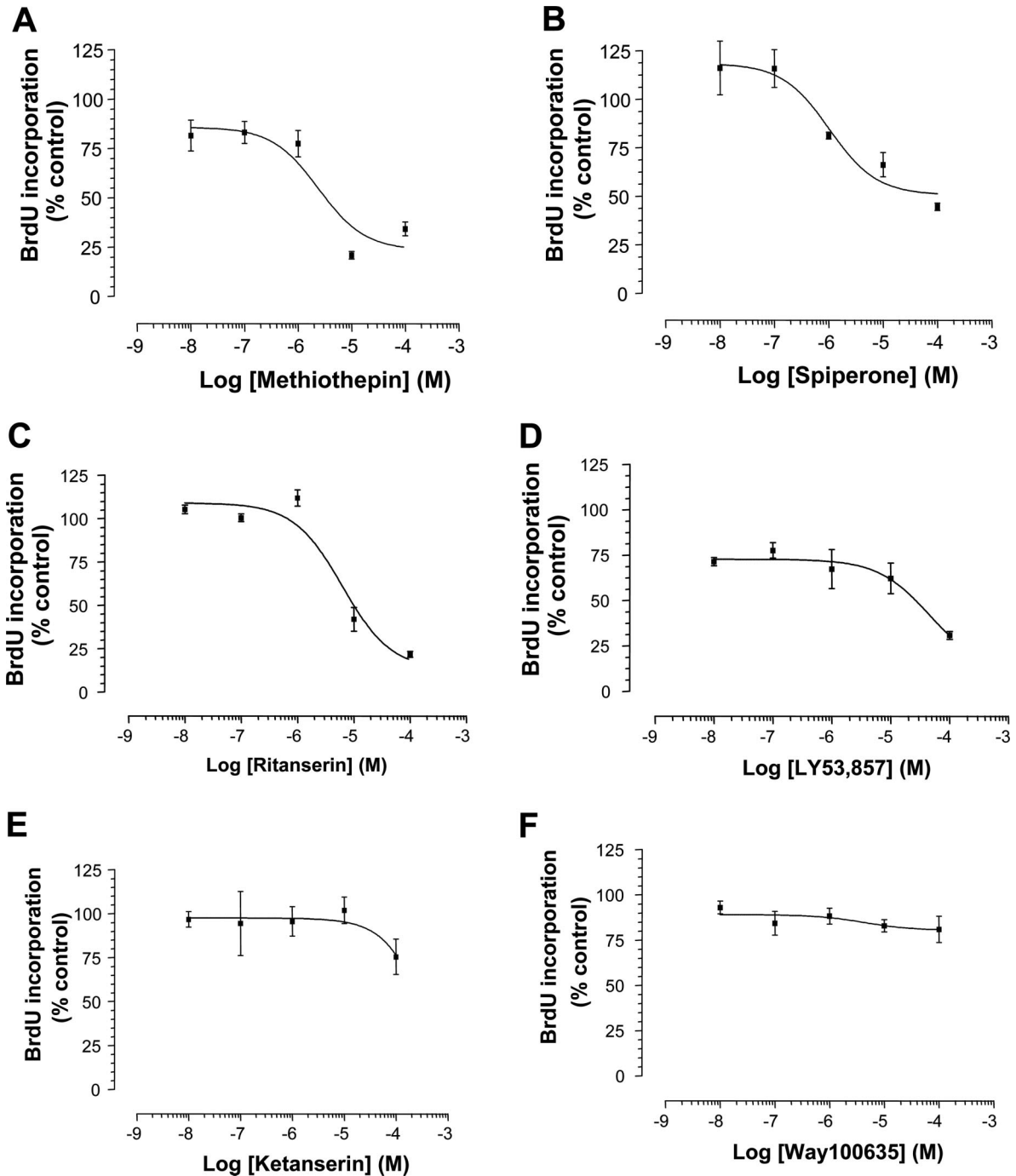


Figure 4. Effects of 5-HT₂ antagonists on activated rat HSC proliferation. Culture-activated rat HSCs (day 10 or greater) were cultured in DMEM plus 0.1% fetal calf serum and 50 mmol/L HEPES for 24 hours before incubation with various 5-HT receptor antagonists methiothepin maleate (**A**), spiperone (**B**), ritanserin (**C**), LY53,857 (**D**), ketanserin (**E**), and WAY100635 (**F**) at the indicated concentrations. Cells were treated for 2 hours before the addition of BrdU to the cell culture medium, and the extent of the BrdU incorporation was determined 16 hours later. Data are expressed relative to control values and are presented as mean values \pm SEM for three separate experiments done in triplicate.

Figure 3. Induction of rat HSC apoptosis by 5-HT₂ receptor-specific antagonists. **A:** After treatment with 10 μ mol/L spiperone for 24 hours, nuclear condensation and fragmentation was visualized with acridine orange (1 μ g/ml) (as indicated by white arrows). **B:** Cells were treated with indicated compounds (all known to bind members of the 5-HT₂ receptor family) at indicated concentrations for 24 hours. Condensed and fragmented nuclei were then counted, and results were expressed as a percentage of total number of nuclei visible. The concentration of the indicated compound that caused optimal nuclear condensation in part (**B**) was then incubated with rat HSCs for various time periods as indicated (**C**). Again, condensed and fragment nuclei were counted and expressed as a percentage of the total number nuclei visible. The specific activity of caspase-3 (**D**) was then assessed after aHSC treatment with spiperone (100 μ mol/L for 24 hours) in the absence or presence of 10 μ mol/L 5-HT, Z-VAD-FMK caspase 3 inhibitor (50 μ mol/L), and gliotoxin (1.5 μ mol/L for 3 hours) in the absence or presence of Z-VAD-FMK (50 μ mol/L). The ability of 5-HT to inhibit apoptosis in HSCs initiated by NGF was also investigated (**E**). Cells were preincubated with various 5-HT concentrations for 30 minutes before the application of 100 ng/ml NGF for 6 hours. Nuclear condensation and fragmentation (as identified by acridine orange) were then assessed and expressed as a percentage of total cell nuclei visible. The results shown here are representative of three independent experiments from three separate cell preparations, and in the case of nuclear condensation and fragmentation, data were generated from three separate fields of view per treatment per data point. */** indicates SD values ($P < 0.05/0.01$).

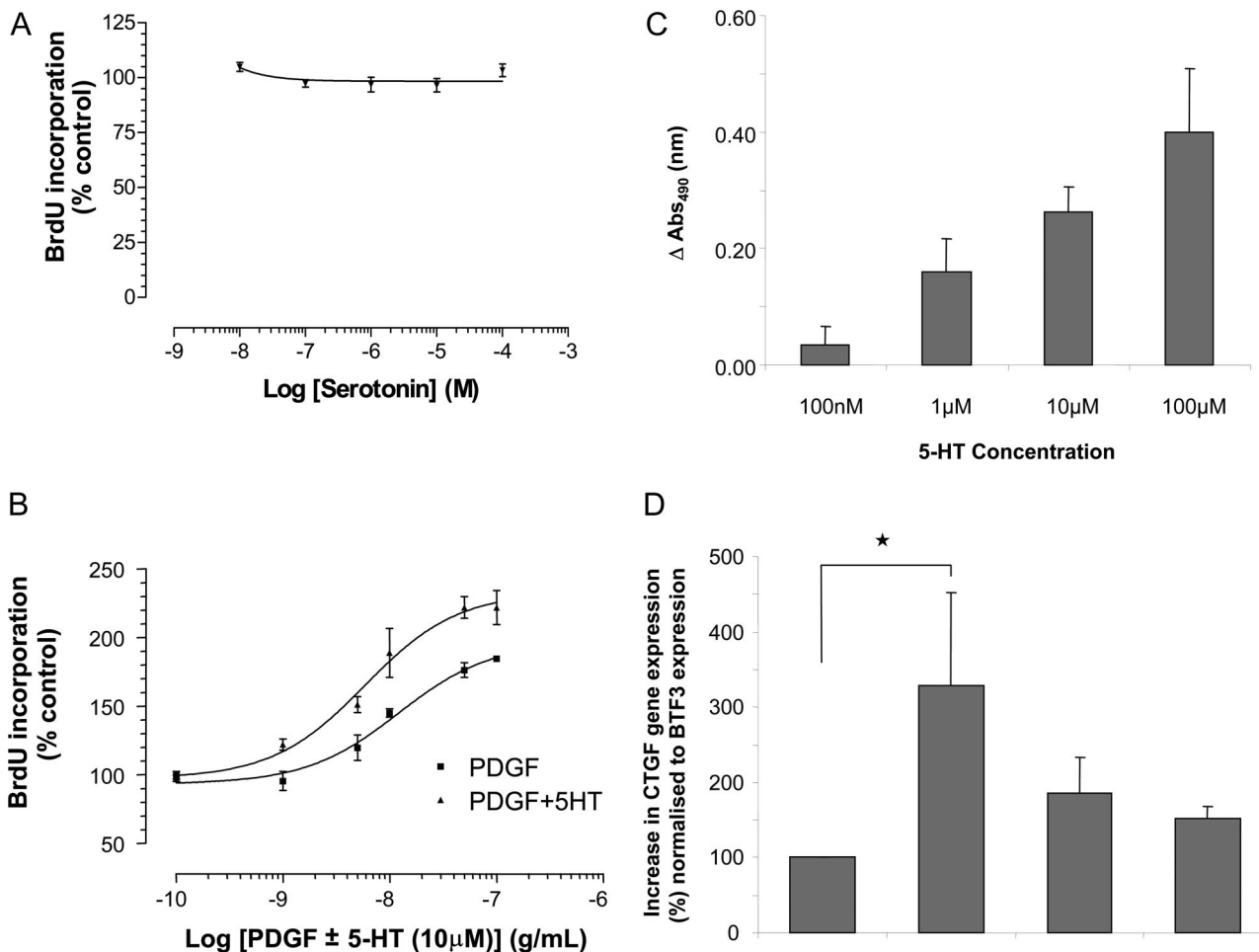


Figure 5. Effects of 5-HT proliferation and CTGF gene expression in rat-activated HSCs. Culture-activated rat HSCs (day 10 or greater) were cultured in DMEM plus 0.1% fetal calf serum and 50 mmol/L HEPES for 24 hours before incubation with 5-HT (A) or PDGF-BB (B) ($\pm 10 \mu\text{mol/L}$ 5-HT) at the indicated concentrations. Cells were treated for 2 hours before the addition of BrdU to the cell culture medium, and the extent of the BrdU incorporation was determined 16 hours later. C: Using a single submaximal concentration of PDGF-BB (20 ng/ml), the dose dependency of the effects of 5-HT on PDGF BB-stimulated proliferation was also tested. Cells were deprived of serum for 16 hours before cells were treated with 5-HT (at the indicated concentrations) for 30 minutes. PDGF-BB (10 ng/ml) was then introduced into the culture medium for a further 23.5 hours. After 24 hours, 20 μl of an MTS tetrazolium reagent were added to each well, and the absorbance was read after 2 hours. Results here were corrected for the change in absorbance at 490 nm induced by PDGF-BB alone. Serum-deprived, activated HSCs were also incubated with various concentrations of 5-HT for 3 hours before RNA extraction and first-strand cDNA synthesis. Changes in CTGF RNA expression were monitored after 5-HT incubation (D) using real-time PCR SYBR green technology. Data for all experiments are expressed relative to control values and are presented as mean values \pm SEM for three separate experiments done in triplicate. * indicates SD values ($P < 0.05$).

inform the design of therapeutics that attenuate the fibrogenic process. In the present study, we show that aHSCs can uptake, release, and respond to 5-HT due to their expression of SERT and members of the 5-HT receptor family. Moreover, we also demonstrate the enhanced proliferation of aHSCs in response to PDGF-BB and 5-HT co-stimulation, increased CTGF transcription in response to 5-HT, and the ability of specific 5-HT₂ receptor antagonists

to inhibit proliferation and stimulate HSC apoptosis. Because 5-HT₂ receptor antagonists are already undergoing clinical trial for the treatment of a range of disorders, including Raynaud's phenomenon,²⁶ various psychological disorders such as depression and dysphoric mania,²⁷ and erythrocytosis,²⁸ our study raises the possibility that this class of drug may also be used for the treatment of liver disease.

Table 3. EC₅₀ Values of PDGF-BB \pm 5-HT and IGF-1 \pm 5-HT Stimulated BrdU Incorporation in Rat Activated HSCs

Agonist	5-HT	PDGF BB	PDGF BB + 5-HT	IGF-1	IGF-1 + 5-HT
EC ₅₀ (ng/ml)	—	12.7 \pm 0.09	6.3 \pm 0.15*	106 \pm 89	102 \pm 130

Culture-activated rat HSCs (day 10 or greater) were cultured in DMEM plus 0.1% fetal calf serum and 50 mmol/L HEPES for 24 hours before incubation with 5-HT or PDGF-BB ($\pm 10 \mu\text{mol/L}$ 5-HT) at the indicated concentrations. Cells were treated for 2 hours before the addition of BrdU to the cell culture medium, and the extent of the BrdU incorporation was determined 16 hours later. Data are expressed as the concentration of PDGF-BB/IGF-1 \pm 5-HT required to stimulate BrdU incorporation by 50% relative to maximal BrdU incorporation \pm SEM for three separate experiments done in triplicate.

*SD values from cells treated with PDGF-BB ($P < 0.05$).

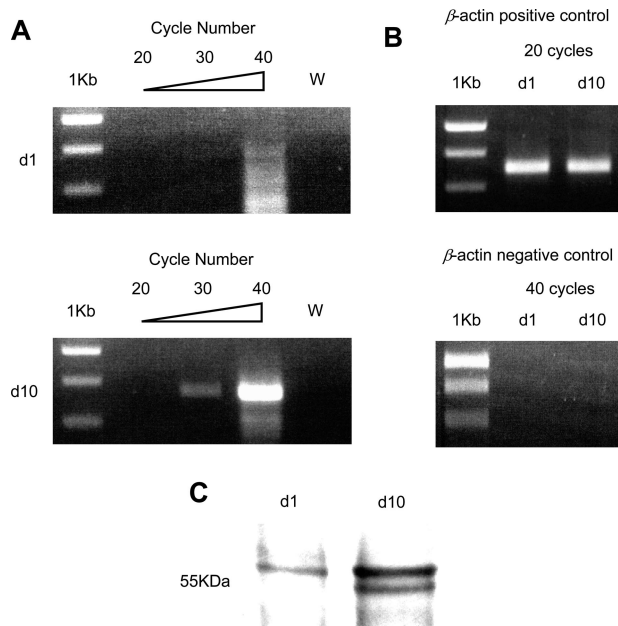


Figure 6. The expression and function of the serotonin transporter in rat HSCs. **A:** RT-PCR analysis was performed on total mRNA isolated from day-1 (d1) and day-10 (d10) culture-activated rat HSCs. Primers specific to the rat SERT were used to prime the PCR reaction. PCR reactions were terminated after a predetermined number of cycles (no greater than 40). Controls performed were as follows: water replacing cDNA (lane W) was run simultaneously with test conditions, PCR was performed for 40 cycles using β -actin primers (**B**) where cDNA was replaced by mRNA (negative control), and the positive control was detection of β -actin gene expression using 20 cycles of PCR analysis. The approximate size of each amplified was estimated from a 1-kb DNA ladder. **C:** Western blotting analysis was also performed on 20 μ g of whole-cell protein extracted from d1 and d10 rat HSCs. Once transferred to nitrocellulose membranes, the proteins were probed with rabbit polyclonal antibodies raised against the rat SERT. Approximate molecular weights of the visible bands were estimated from prestained protein markers run simultaneously with the whole-cell protein. The results shown here are representative of three independent experiments from at least three separate cell preparations.

5-HT and 5-HT₂ receptors in particular have been implicated in the etiology of several fibrotic disorders including retroperitoneal fibrosis, carcinoid heart disease, pulmonary hypertension, and aortic valve disease^{29,30}; however, to date, there have been no reports indicating a profibrogenic role for 5-HT in the diseased liver. Several studies, however, have hinted at functions for 5-HT in the normal and fibrotic liver. In normal liver, several members of the 5-HT receptor family are expressed in a variety of cells including hepatocytes (5-HT₂), cholangiocytes (5-

Table 4. Uptake of [³H]5-HT by Rat aHSC and COS-7 Cells Transiently Expressing the Rat SERT

Cell	V_{max} (pmol/1 × 10 ⁶ cells/minute)	K_m (nmol/L)
Activated rat HSCs	343.7 ± 50.3	302.9 ± 18.4
COS-7	604.6 ± 35.09	373.1 ± 32.17

Day-10 rat HSC and COS-7 cells transiently expressing the rat SERT were incubated with [³H]serotonin at 4°C (nonspecific transport) or 37°C (active transport) for 5 minutes. Cells were then washed three times with ice-cold PBS before cell lysis, and the amount of [³H]serotonin taken up was quantified. Nonspecific transport was subtracted from total active transport giving the specific [³H]serotonin transport V_{max} (pmol/1 [times] 10⁶ cells/minute) and K_m (nmol/L) The results shown here are representative of at least three independent experiments from at least three separate cell preparations.

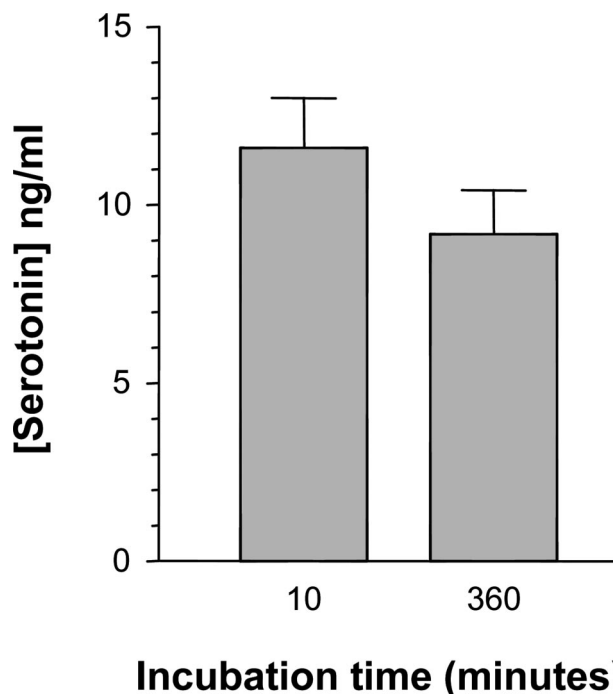


Figure 7. Quantitative determination of 5-HT in rat HSC growth medium by ELISA. Culture-activated rat HSCs were washed twice with DMEM containing 0% FCS and incubated overnight in DMEM containing 0% FCS. Growth medium was again changed, this time to DMEM containing zimelidine (10 μ mol/L), which was used to block the uptake of serotonin by rat HSCs. Medium was then harvested at indicated times where 5-HT concentration was determined by ELISA. Data are representative of two experiments done in duplicate.

HT_{1A} and 5-HT_{1B}), and sinusoidal endothelial cells (5-HT₂). In these various cell types, 5-HT receptors are known to regulate cell proliferation, growth of the biliary tree, and local blood flow.³¹⁻³³ Levels of unconjugated (active) plasma 5-HT are significantly higher in patients with cirrhosis, and conversely, levels of the 5-HT metabolite 5-hydroxyindole acetic acid are significantly decreased in patients with cirrhosis.³⁴ Data presented by Marasini et al³⁵ and Laffi et al³⁶ also found that serotonin levels were influenced by hepatic injury; more specifically, a decrease in the intraplatelet serotonin concentration was observed, which was postulated to be responsible for the bleeding tendency of cirrhotic patients.^{35,36} CCl₄-induced cirrhosis of spontaneously hypertensive rats and Wistar-Kyoto rats is associated with diffuse serotonin particles, and numerous mast cells (a source of 5-HT) were found in the fibrotic matrix, indicating that serotonin particles and mast cells participate at some stage in the response to liver injury.^{37,38} These studies suggest that 5-HT does have an important role to play in the progression of hepatic fibrosis. However, it is unclear whether altered 5-HT homeostasis is a driving force in the progression of hepatic fibrosis or is instead a consequence of fibrosis. Previous studies in cell types other than HSCs have demonstrated the ability of the 5-HT₂ receptor family to modulate the expression of key regulators of the extracellular matrix and fibrogenesis. For example, 5-HT₂ receptors positively regulate the expression of IGF-1, collagen type IV, TGF- β 1, and MMP1³⁹⁻⁴³

and negatively regulate the expression of type I collagen, laminin $\beta 1 + \beta 2$, fibronectin, and type III collagen.⁴¹ These observations coupled with our demonstration of expression of 5-HT₂ receptors on the surface of HSCs led us to investigate the potential for this class of 5-HT receptor to function as regulators of the HSC phenotype.

Culture activation of both rat and human HSCs led to a stable increase in the expression of 5-HT_{2A} and 5-HT_{2B} receptors. These receptors were functional in terms of their ability to elicit the effects of 5-HT including enhancing cell proliferation, protecting against NGF-induced apoptosis, and enhancing CTGF gene expression. aHSCs incubated with 5-HT alone did not demonstrate an elevated level of BrdU incorporation. However, aHSCs incubated with PDGF-BB and 5-HT simultaneously displayed an elevated level of proliferation when compared with cells incubated with PDGF-BB alone. A similar observation was made by Eto et al,⁴⁴ who demonstrated the cumulative effects of 5-HT and PDGF-BB incubation on 5-HT_{2A} receptor ex-

pressing mesangial cells when compared with the effects of 5-HT or PDGF-BB alone. 5-HT_{2B} receptors have also been shown to interact with the signaling cascade associated with the PDGF receptor resulting in cell cycle progression via ERK1/2.⁴⁵ In the experiments presented here, the reason why 5-HT was unable to enhance HSC proliferation in the absence of PDGF-BB remains unclear. One possible explanation is that the relatively high rate of proliferation of aHSCs and the high endogenous levels of mitogen-activated protein kinase activity (R.G. Ruddell, unpublished observation) may mask the mitogenic effects elicited by 5-HT that are observed in less rapidly dividing cells. In contrast to its ability to augment PDGF-BB-induced proliferation, 5-HT was unable to alter HSC responsiveness to the mitogen IGF-1. IGF-1 is known to signal via the IGF-1 receptor, enhancing HSC proliferation through the activation of phosphatidylinositol 3-kinase activation, which contributes to the activation of ERK1/2 and Ras.⁴⁶ Because 5-HT₂ receptors are able

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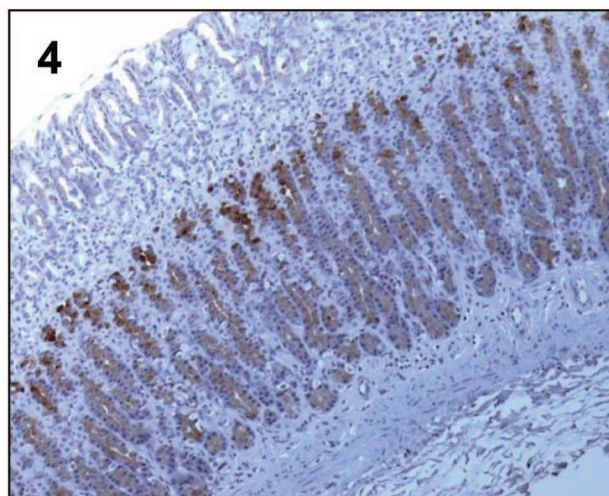
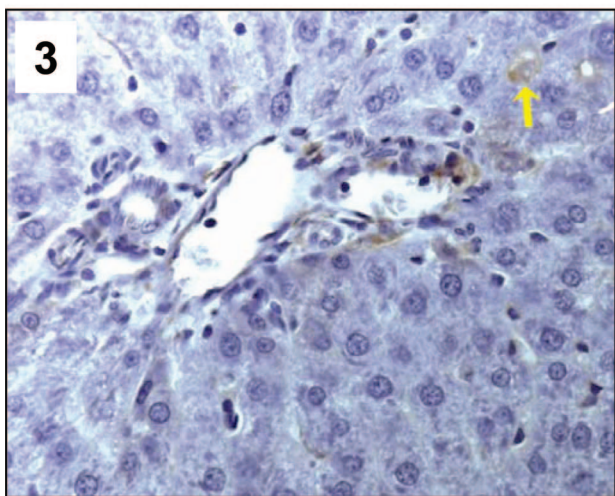
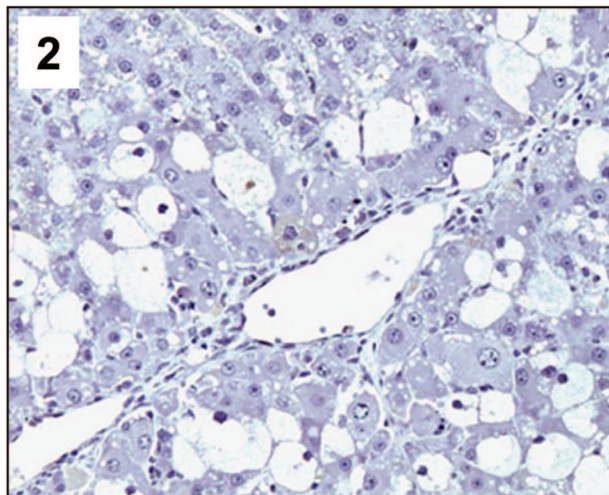
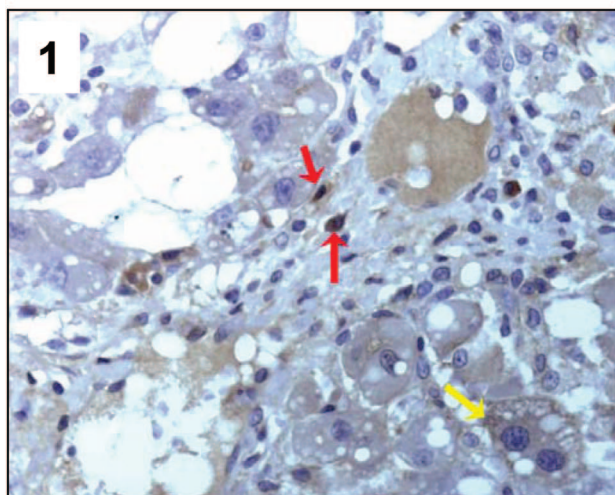


Figure 8.

to interact with signaling pathways associated with other receptor tyrosine kinases such as the PDGF receptor, it would in theory be possible for 5-HT to up-

regulate HSC responsiveness to IGF-1. However, our data suggest that at least for aHSCs, there is selectivity concerning the cross-talk between 5-HT and receptor

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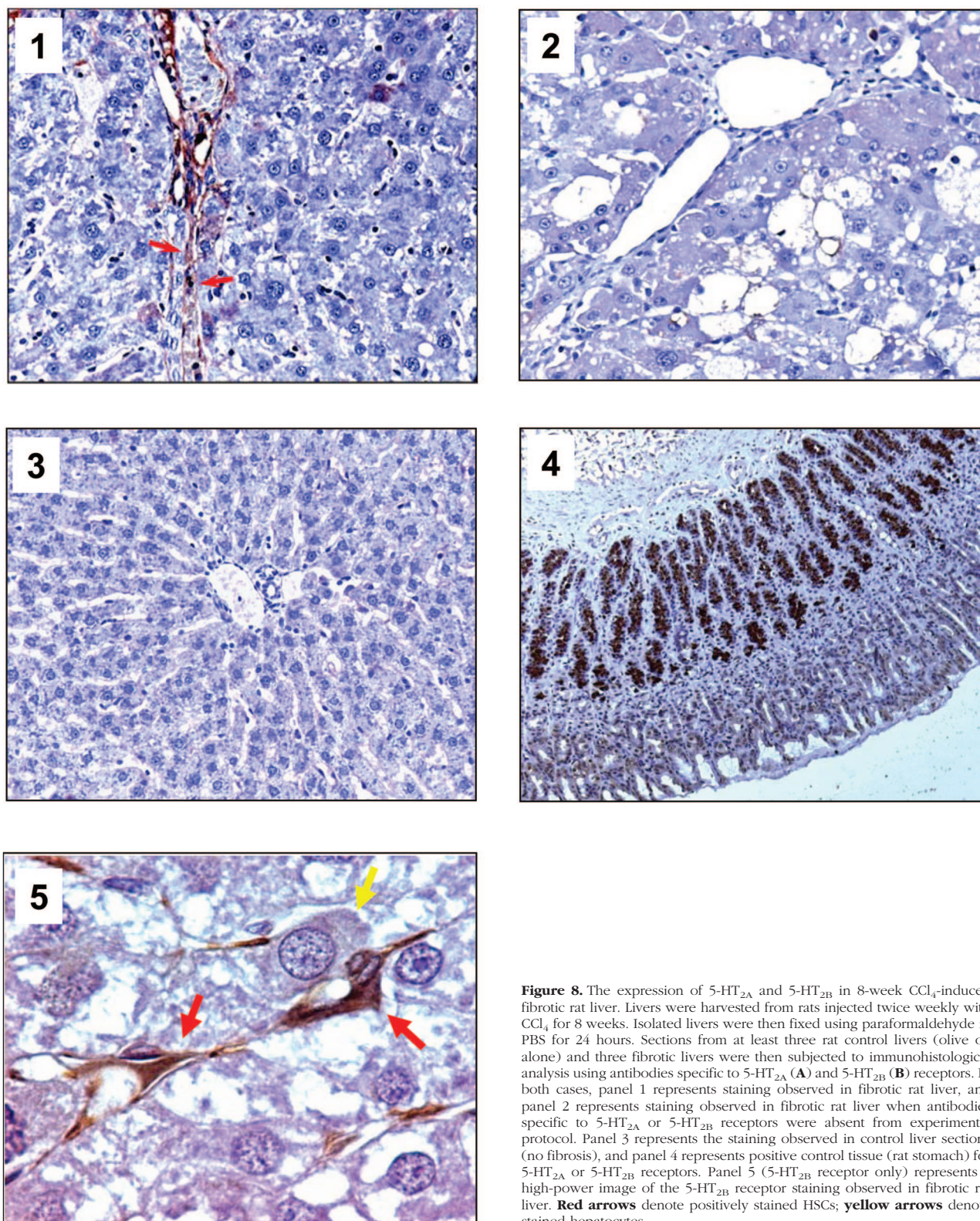


Figure 8. The expression of 5-HT_{2A} and 5-HT_{2B} in 8-week CCl₄-induced fibrotic rat liver. Livers were harvested from rats injected twice weekly with CCl₄ for 8 weeks. Isolated livers were then fixed using paraformaldehyde in PBS for 24 hours. Sections from at least three rat control livers (olive oil alone) and three fibrotic livers were then subjected to immunohistological analysis using antibodies specific to 5-HT_{2A} (**A**) and 5-HT_{2B} (**B**) receptors. In both cases, panel 1 represents staining observed in fibrotic rat liver, and panel 2 represents staining observed in fibrotic rat liver when antibodies specific to 5-HT_{2A} or 5-HT_{2B} receptors were absent from experimental protocol. Panel 3 represents the staining observed in control liver sections (no fibrosis), and panel 4 represents positive control tissue (rat stomach) for 5-HT_{2A} or 5-HT_{2B} receptors. Panel 5 (5-HT_{2B} receptor only) represents a high-power image of the 5-HT_{2B} receptor staining observed in fibrotic rat liver. **Red arrows** denote positively stained HSCs; **yellow arrows** denote stained hepatocytes.

tyrosine kinase pathways such that 5-HT can synergize with PDGF-BB but not IGF-1. In its own right, 5-HT was also able to alter the mRNA levels of CTGF in a dose-dependent fashion. This event has previously been demonstrated in mesangial cells by Hahn et al²⁴ and was shown to be mediated by the 5-HT_{2A} receptor and pertussis toxin-insensitive G proteins. Whether this is also the case in the activated HSCs remains to be elucidated.

The extracellular levels of 5-HT and therefore its physiological functionality are regulated by the serotonin transporter. After the Na⁺/Cl⁻-dependent uptake process is complete, the internalized serotonin is either degraded by monoamine oxidases or repackaged into vesicles, where it remains until released back into the extracellular environment. Normal human and mouse liver do not express SERT mRNA or protein.^{47,48} Here, we show that culture-activated HSCs do express a functional SERT, and our data are in agreement with previous studies regarding the relative affinity of the SERT for 5-HT, with previously cloned rat SERT having an almost identical K_m value of 320 nmol/L⁴⁹ to that reported in this study (302.9 ± 18.4 nmol/L). The reasons for expression and function of the SERT on the activated HSCs are open to speculation, but our data provide further evidence implicating the HSCs as a site of action for 5-HT. Expression of the SERT appears to be regulated, at least in part, by various stress-related factors including hypoxia and the release of growth factors, namely basic fibroblast growth factor.^{50,51} On mesangial cells, the SERT is proposed to play a protective role against the deleterious effects of 5-HT on glomeruli, the proliferative and fibrogenic effects of 5-HT being countered by the removal and subsequent degradation of 5-HT.⁵² In contrast, SERT expression in hypoxic pulmonary artery smooth muscle cells is thought to sensitize pulmonary artery smooth muscle cells to the mitogenic effects of 5-HT.⁵⁰ Much further investigation is warranted to determine the role of the SERT in liver fibrosis; however, it is plausible that the SERT may be taking on an antiproliferative role (similar to that in mesangial cells) and could in part explain the limited effects of 5-HT on HSC proliferation. Analysis of 5-HT concentration in HSC culture media demonstrated that in the absence of external sources of 5-HT, HSC culture medium still contained significant amounts of extracellular 5-HT even after complete serum deprivation. Whether the source of this 5-HT was the HSCs or whether the HSCs had internalized and stored the 5-HT from earlier culture growth medium remains to be determined. However, the consequences of the ability of HSCs to store and release 5-HT would be considerable as in effect the cells would have a self-perpetuated serotonergic/mitogenic stimulus. This would also explain why 5-HT₂ receptor antagonists were able to affect HSC proliferation and apoptosis. Future studies that are beyond the scope of the present study will need to address the important question of whether, in addition to being able to transport and respond to 5-HT, HSCs can also synthesize or concentrate 5-HT.

In terms of the therapeutic potential of our discovery of the acquisition of 5-HT₂ receptor expression on aH-

SCs, the most relevant finding was that 5-HT₂-specific antagonists will promote aHSC apoptosis. The effects of ritanserin, spiperone, LY53,857, and methiothepin on HSC proliferation were found to be dose dependent, with antiproliferative IC₅₀ values being in the low micromolar range. Moreover, the degree of apoptosis induced by spiperone was found to be equivalent to that induced by gliotoxin, which has been used to promote HSC apoptosis *in vivo* and enhance recovery from fibrosis in rats.⁸ Whereas the ability of various 5-HT receptors to regulate proliferation is well documented,^{31,32,44} their function as regulators of apoptosis, by contrast, is less well established. Studies by Choi et al⁵³ demonstrate a role for the 5-HT_{2B} receptor in mouse embryogenesis, with antagonism of the receptor at key stages of development causing apoptosis and abnormal sarcomeric organization in the cephalic region, heart, and neural tube. Further studies by the same group demonstrated that serotonin was able to protect cardiomyocytes from serum deprivation-triggered apoptosis via co-activation of Akt and ERK 1/2 signaling pathways.⁵⁴ We are at present unable to determine whether the 5-HT₂-specific antagonists are inducing apoptosis via the 5-HT_{2A} the 5-HT_{2B} receptor or, for that matter, via any other subtype shown here to be expressed by aHSCs. In addition, we have no information concerning the signaling pathways through which these receptors might transduce the apoptotic signal or the downstream targets of these pathways. Nevertheless, our findings raise the possibility that 5-HT receptor antagonists may have potential as future therapeutic agents in the treatment of liver disease. Recent studies have indicated that recovery from hepatic fibrosis is associated with the apoptosis of aHSCs and have shown that agents that can selectively induce HSC apoptosis may have potential as therapeutic agents.^{5,8} Unfortunately, currently available 5-HT₂ receptor antagonists are relatively nonspecific in terms of the ability of the ligand to bind to all of the 5-HT₂ receptor family members. As such, we cannot formally exclude the possibility that the antagonists used in this study may at least in part mediate their effects on HSCs function via multiple 5-HT and non-5-HT receptors (eg, D2 receptors). Evolution of more specific 5-HT_{2A} and 5-HT_{2B} receptor ligands may allow the role of each individual receptor in liver fibrosis to be determined; however, current pharmacological tools available do not allow this. Of note, our immunohistochemical analysis of the *in situ* expression of the 5-HT_{2A} and 5-HT_{2B} receptors indicated that the latter receptor is likely to be the most promising target. Although 5-HT_{2A} staining was of a diffuse nature in both normal and diseased liver, by contrast, expression of 5-HT_{2B} was absent in healthy liver and was selectively associated with aHSCs in fibrotic liver. 5-HT_{2B} receptors are therefore a new marker for aHSCs and a potential therapeutic target.

Our data provide evidence that aHSCs *in vivo* and *in vitro*, from both human and rat sources, express several 5-HT receptor types but especially the 5-HT_{2A} and 5-HT_{2B} receptors, which in turn are able to mediate

changes in HSC proliferation, transcription, and apoptosis. The expression of several 5-HT receptor types may also be of physiological relevance because 5-HT is able to bind to all and signal through all, leading to a complex interaction of signaling intermediates triggered by 5-HT. The resulting effects of 5-HT on HSC function demonstrated in this study are possibly due to multiple receptor types. HSCs also express a functional SERT and are able to release 5-HT back into the culture medium after serum deprivation. These findings all lend weight to the conclusion that 5-HT may have a critical role to play in modulating the characteristic phenotypic changes of the HSCs in response liver injury. With the continual development of ever more specific 5-HT_{2A} and 5-HT_{2B} ligands, the roles of each individual receptor in the development of hepatic fibrosis may be answered, eventually leading to the development of novel therapeutic agents in the treatment of liver fibrosis.

Acknowledgment

We thank Hao Ngoc Nguyen for her technical assistance with the COS-7 serotonin uptake study.

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