Neuropeptide Y inhibits biliary hyperplasia of cholestatic rats by paracrine and autocrine mechanisms

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1Research, Central Texas Veterans Health Care System, Temple, Texas; 2Scott & White Digestive Disease Research Center, Scott & White, Temple, Texas; 3Division of Research and Education, Scott & White, Temple, Texas; 4Department of Medicine, Division Gastroenterology, Texas A&M Health Science Center, College of Medicine, Temple, Texas; 5Department of Anatomical, Histological, Forensic Medicine and Orthopedics Sciences, “La Sapienza,” Rome, Italy; and 6Eleonora Lorillard Spencer-Cenci Foundation, Rome, Italy

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DeMorrow S, Meng F, Venter J, Leyva-Illades D, Francis H, Frampton G, Pae HY, Quinn M, Onori P, Glaser S, McDaniel K, Mancinelli R, Gaudio E, Alpini G, Franchitto A. Neuropeptide Y inhibits biliary hyperplasia of cholestatic rats by autocrine and paracrine mechanisms. Am J Physiol Gastrointest Liver Physiol 305: G250–G257, 2013. First published May 23, 2013; doi:10.1152/ajpgi.00140.2013.—Neuropeptide Y (NPY) exerts its functions through six subtypes of receptors (Y1–Y6). Biliary homeostasis is regulated by several factors through autocrine/paracrine signaling. NPY inhibits cholangiocarcinoma growth; however, no information exists regarding the role of NPY in the regulation of biliary hyperplasia during cholestasis. To determine 1) the expression of NPY and Y1–Y6 in cholangiocytes and 2) the paracrine/autocrine effects of NPY on cholangiocyte proliferation. Normal or bile duct ligation (BDL) rats were treated with NPY, neutralizing anti-NPY antibody, or vehicle for 7 days. NPY and NPY receptor (NPR) expression was assessed in liver sections and isolated cholangiocytes. NPY secretion was assessed in serum and bile from normal and BDL rats, as well as supernatants from normal and BDL cholangiocytes and normal rat cholangiocyte cell line [intrahepatic normal cholangiocyte culture (NRICC)]. We evaluated intrahepatic bile ductal mass (IBDM) in liver sections and proliferation in cholangiocytes. With the use of NRICC, the effects of NPY or anti-NPY antibody on cholangiocyte proliferation were determined. The expression of NPY and all NPR were increased after BDL. NPY levels were lower in serum and cholangiocyte supernatant from BDL compared with normal rats. NPY secretion from NRICC was detected at both the basolateral and apical domains. Chronic NPY treatment decreased proliferating cellular nuclear antigen (PCNA) expression and IBDM in BDL rats. Administration of anti-NPY antibody to BDL rats increased cholangiocyte proliferation and IBDM. NPY treatment of NRICC decreased PCNA expression and increased the cell cycle arrest, whereas treatment with anti-NPY antibody increased proliferation. Therapies targeting NPY-mediated signaling may prove beneficial for the treatment of cholangiopathies.

biliary epithelium; neurotransmitters; proliferation; cell cycle

CHOLANGIOCYTES ARE THE TARGET cells in cholangiopathies such as primary biliary cirrhosis and primary sclerosing cholangitis (4).

During the course of these diseases and in many other forms of liver injury, a balance between cholangiocyte proliferation and loss is critical for the maintenance of the homeostasis of biliary mass (4). Effort has gone into identifying the factors regulating biliary loss/proliferation to identify potential therapeutic targets for the maintenance of biliary mass during liver diseases.

Biliary mass is coordinately regulated by a number of growth factors and hormones during normal and cholestatic states by both autocrine and paracrine signaling (5, 12, 13, 23, 28). After bile duct ligation (BDL), cholangiocytes secrete increased amounts of growth factors such as vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) stimulating biliary proliferation (12, 13). Conversely, proliferating cholangiocytes secrete increased amounts of serotonin (23) and melatonin (28) that inhibit cholangiocyte proliferation. Serotonin and melatonin may be secreted by cholangiocytes to prevent overt biliary proliferation in response to cholestasis.

Neuropeptide Y (NPY) is a neurotransmitter mainly found in the brain, in neurons surrounding hepatic vessels throughout the gastrointestinal tract and in high concentrations in the biliary tree (15, 20). Immunoreactivity for NPY is also present in cholangiocytes (9). NPY exerts its many functions through six main receptor subtypes (Y1 through Y6) (4, 37). While little information exists regarding the role of NPY in the regulation of biliary function (8, 31), we have previously shown that NPY regulates cholangiocarcinoma proliferation (6). NPY is more highly expressed in the center of these tumors, where it may exert a local antiproliferative and antimigratory effect on cholangiocarcinoma cells, allowing for the recruitment of adequate stromal support. The effects of NPY on hyperplastic cholangiocyte proliferation are unknown. The aims of the current study were to: 1) assess the expression and secretion of NPY from cholangiocytes after BDL; 2) identify a role for NPY in biliary homeostasis; and 3) evaluate the effects of NPY depletion on biliary proliferation.

MATERIALS AND METHODS

Materials. Reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Antibodies for NPY (E-17, sc-14727) and all Y receptors except Y3 and cytokeratin-19 (CK-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). NPY (E-17, sc-14727) is an affinity-purified goat polyclonal antibody raised against a peptide mapping within an internal region of NPY of human origin and detects NPY of mouse, rat, and human origin. Y1

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(A-17, sc-21990) is an affinity-purified goat polyclonal antibody raised against a peptide mapping near the NH2-terminus of Y1, of human origin and is recommended for detection of Y1 of mouse, rat, and human origin. Y2 (L-17, sc-14736) is an affinity-purified goat polyclonal antibody raised against a peptide mapping near the COOH-terminus of Y2 of human origin and is recommended for detection of Y2 of mouse, rat, and human origin. Y4 (C-20, sc-23842) is an affinity-purified goat polyclonal antibody raised against a peptide mapping near the COOH-terminus of Y4 of human origin and detects NPY receptor 4 of human, mouse, and rat origin. Y5 (N-20, sc-23843) is an affinity-purified goat polyclonal antibody raised against a peptide mapping within an extracellular domain of Y5 of human origin and detects Y5 of human, mouse, and rat origin. Y3 (ab2074, also known as CXCR4) is a rabbit polyclonal antibody that was purchased from Abcam (Cambridge, MA). This antibody was developed against a synthetic peptide, corresponding to NH2-terminal amino acids 1–14 of human CXCR4 and reacts with mouse, rat, and human. Rat primers for NPY and all Y receptors were obtained from Qiagen SABiosciences (Frederick, MD) and were designed using sequences with the following NCBI GenBank Accession numbers: NM_012869 (NPY); NM_017008 [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)]. Antibody to Y1 (also known as CXCR4) was purchased from Abcam. We did not evaluate the expression of Y5 in cholangiocytes and normal intrahepatic cholangiocyte lines [intrahepatic normal cholangiocyte culture (NRICC)] since: 1) other studies have shown that Y5 is not expressed in the rat (4) and 2) Y5 is not expressed by bile ducts in liver sections (see RESULTS).

Animal models. Male Fischer 344 rats (150–175 g) were purchased from Charles River Laboratory (Wilmington, MA). Animals were kept in 12:12-h light-dark cycles, kept in a temperature-controlled environment (20–22°C), and fed ad libitum with free access to drinking water. We used normal and BDL rats that immediately after surgery (2) were treated with vehicle (0.9% saline) or porcine NPY (3 nmol·kg body wt·h−1·day−1) for 24, 48, or 72 h or 7 days before evaluating cell proliferation by PCNA immunoblot. NPY’s effects on cell cycle progression were also determined. NRICC were seeded in 10-cm tissue culture plates and treated with 1 μM NPY (18) for 24, 48, or 72 h or 7 days. Cells were collected by detaching with TrypLE (Invitrogen, Carlsbad, CA) and resuspending in culture medium. Cells were fixed and stained according to the Muse Cell Cycle Kit manufacturer’s instructions (EMD Millipore, Billerica, MA) followed by analysis on the Muse cell analyzer (EMD Millipore). To determine how NPY regulates biliary proliferation by autocrine mechanisms, we treated NRICC with 0.2% BSA or anti-NPY antibody for 24–72 h before evaluating NRICC proliferation by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium proliferation assay (11).

Statistical analysis. Data are expressed as means ± SE. Differences between groups were analyzed by the Student’s unpaired t-test when two groups were analyzed, and by ANOVA when more than two groups were analyzed, followed by an appropriate post hoc test.

RESULTS

Cholangiocytes express Y receptors. The expression of Y1–Y5 receptors was virtually absent in bile ducts from normal rats (Fig. 1A). The expression of Y1–Y5 receptors was higher in intrahepatic bile ducts from BDL rats (Fig. 1B). There was no immunoreactivity for Y6 in either normal or BDL rats (Fig. 1, A and B). Furthermore, purified cholangiocytes from normal and BDL rats and NRICC express the mRNA and protein for Y1–Y5 receptors (Fig. 2, A–C). By immunofluorescence, NRICC express Y1–Y5 receptors (Fig. 2D). Cholangiocytes from normal and BDL rats and NRICC express the mRNA for NPY (Fig. 2, A and B). NPY mRNA expression was increased in cholangiocytes from using normal cholangiocytes as controls. Data are expressed as relative fold-change of relative mRNA levels (GAPDH ± SE).

Immunoblotting were performed on protein (10 μg) from whole cell lysate and normalized by β-actin (28). Band intensity was determined by scanning video densitometry using the phosphoimager, Storm 860 (GE Healthcare, Piscataway, NJ), and the ImageQuant TL software version 2003.02 (GE Healthcare). In cytoplasmic culture, the expression of Y receptors and NPY was evaluated by immunofluorescence (11).

We measured NPY levels in: 1) serum and bile from normal and BDL rats and 2) supernatant from isolated cholangiocyte primary cultures (6 h) from normal and BDL rats using commercially available EIA kits (Neuropeptide Y S-1220; Bachem, Torrance, CA). To determine NPY secretion levels, cholangiocytes from normal and BDL rats and NRICC were incubated at 37°C for 0 and 6 h; NPY levels were measured in the supernatants. We measured NPY secretion in the basolateral and apical domains of NRICC by plating the cell line for 48 h on collagen-coated tissue culture inserts to produce a confluent monolayer (14); thereafter, cells were incubated for 6 h, and supernatant was collected and analyzed for NPY levels.

Evaluation of serum chemistry, cholangiocyte proliferation, and intrahepatic bile ductal mass. The serum levels of bilirubin and the transaminases glutamate pyruvate transaminases and glutamic oxaloacetic transaminase were determined by a Dimension RxL Max Integrated Chemistry system (Dade Behring, Deerfield, IL) by the Chemistry Department, Scott & White. Intrahepatic bile ductal mass (IBDM) was evaluated as the area occupied by CK-19-positive bile duct total area × 100. Ten randomly selected portal areas were evaluated (in a coded fashion) in three liver sections obtained from two animals. We evaluated cell proliferation [by immunoblots for proliferating cellular nuclear antigen (PCNA)] (28) of cholangiocytes from BDL rats treated with vehicle, NPY, or anti-NPY antibody.

Paracrine and autocrine effects of NPY on NRIC proliferation. NRICC were treated with 0.2% BSA (basal) or NPY (1.0 μmol/l, a dose used in another study) (18) for 24, 48, or 72 h or 7 days before evaluating cell proliferation by PCNA immunoblot. NPY’s effects on cell cycle progression were also determined. NRICC were seeded in 10-cm tissue culture plates and treated with 1 μM NPY (18) for 24, 48, or 72 h or 7 days. Cells were collected by detaching with TrypLE (Invitrogen, Carlsbad, CA) and resuspending in culture medium. Cells were fixed and stained according to the Muse Cell Cycle Kit manufacturer’s instructions (EMD Millipore, Billerica, MA) followed by analysis on the Muse cell analyzer (EMD Millipore). To determine how NPY regulates biliary proliferation by autocrine mechanisms, we treated NRICC with 0.2% BSA or anti-NPY antibody for 24–72 h before evaluating NRICC proliferation by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium proliferation assay (11).
BDL compared with normal rats (Fig. 2A). Immunohistochemical analysis revealed that bile ducts from normal and BDL rats express NPY (Fig. 2E). Immunofluorescence staining showed that NRICC express NPY protein (Fig. 2F). NPY serum levels were significantly lower in BDL (8.2 ± 1.15 ng/ml, n = 30) compared with normal (15.84 ± 4.9 ng/ml, n = 30) rats. NPY levels significantly decreased in cholangiocyte supernatant of BDL (0.5 ± 0.1 ng/ml, n = 24) compared with normal (2.3 ± 0.5 ng/ml, n = 24) rats. Consistent with the presence of NPY in bile and serum, NRICC secrete NPY at the basolateral (2.3 ± 0.5 ng/ml, n = 6) and apical (2.3 ± 0.5 ng/ml, n = 6) domains. NPY levels significantly increased in bile from BDL (1.12 ± 0.08 ng/ml) compared with normal (0.56 ± 0.11 ng/ml) rats. The increase of NPY observed in bile from BDL rats may be due to increased secretion of NPY from hepatocytes.

Effect of NPY on serum chemistry, cholangiocyte proliferation, and IBDM. No differences in body weight were observed among normal rats treated with saline or NPY (Table 1). There was: 1) decreased body weight of BDL compared with normal rats (2) and 2) increased liver-to-body weight ratio in BDL compared with normal rats (Table 1). Liver-to-body weight ratio was reduced in NPY-treated BDL rats compared with control-treated BDL rats (Table 1).

The serum levels of transaminases were slightly different between normal rats treated with saline or NPY (Table 1). Following BDL, the serum levels of transaminases significantly increased (compared with normal rats) (2) and decreased in NPY-treated BDL rats compared with saline-treated controls (Table 1).

In rats with BDL, there was enhanced IBDM compared with normal rats (Fig. 3A). Administration of NPY to BDL rats decreased IBDM compared with BDL rats treated with vehicle (Fig. 3A). There was reduced PCNA expression in cholangiocytes from NPY-treated BDL compared with vehicle-treated BDL rats (Fig. 3B). Administration of anti-NPY antibody to BDL rats increased IBDM in liver sections (Fig. 3A) and
PCNA protein expression in cholangiocytes (Fig. 3C) compared with the BDL rats treated with vehicle.

Paracrine/autocrine effects of NPY on NRICC proliferation. We demonstrated that NPY (1.0 μmol/l) (18) decreases the proliferation of NRICC after incubation for 72 h and 7 days (Fig. 4A). Analysis of the cell cycle progression demonstrated that there was a concomitant increase in the percentage of cells in the G0/G1 phase and a reduction in the percentage of cells in the G2/M phase after 7 days of incubation with 1.0 μmol/l NPY (Fig. 4B). In vitro treatment of NRICC with anti-NPY antibody increased cell proliferation compared with NRICC treated with BSA (Fig. 4C).

DISCUSSION

This study relates to the autocrine/paracrine role of NPY in the modulation of the homeostasis of the biliary epithelium. Bile ducts, purified cholangiocytes, and NRICC express Y1–Y5 receptors. Normal bile ducts and cholangiocytes express the message and protein for NPY and secrete NPY, secretion that

Table 1. Measurement of body weight, liver weight, liver-to-body weight ratio, and %positive cholangiocytes and IBDM in liver sections from normal and BDL rats treated with vehicle or NPY for 1 wk

<table>
<thead>
<tr>
<th>Groups</th>
<th>Normal + Rats + Vehicle</th>
<th>n</th>
<th>Normal + Rats + NPY</th>
<th>n</th>
<th>BDL + Rats + Vehicle</th>
<th>n</th>
<th>BDL + Rats + NPY</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>185.20 ± 2.94</td>
<td>21</td>
<td>178.82 ± 4.58</td>
<td>21</td>
<td>148.54 ± 3.51</td>
<td>21</td>
<td>140.26 ± 3.04</td>
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<td>Liver wt, g</td>
<td>8.01 ± 0.28</td>
<td>21</td>
<td>6.70 ± 0.32</td>
<td>21</td>
<td>8.16 ± 0.32</td>
<td>21</td>
<td>7.27 ± 0.32</td>
<td>21</td>
</tr>
<tr>
<td>Liver-to-body weight ratio, %</td>
<td>4.34 ± 0.16</td>
<td>21</td>
<td>3.75 ± 0.21</td>
<td>21</td>
<td>5.50 ± 0.18a</td>
<td>21</td>
<td>5.20 ± 0.21b</td>
<td>21</td>
</tr>
<tr>
<td>SGOT, U/l</td>
<td>136.22 ± 9.28</td>
<td>9</td>
<td>97.75 ± 25.2</td>
<td>5</td>
<td>1,192.1 ± 294.4a</td>
<td>9</td>
<td>552.5 ± 406.5b</td>
<td>5</td>
</tr>
<tr>
<td>SGPT, U/l</td>
<td>62.56 ± 6.1</td>
<td>9</td>
<td>38.75 ± 15.5</td>
<td>5</td>
<td>374.88 ± 105.25a</td>
<td>9</td>
<td>175.5 ± 122.5b</td>
<td>5</td>
</tr>
</tbody>
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Data are means ± SE of 7 evaluations; n, no. of rats. IBDM, intrahepatic bile ductal mass; NPY, neuropeptide Y; BDL, bile duct ligation; SGOT, glutamic oxaloacetic transaminase; SGPT, glutamate pyruvate transaminases. *P < 0.05 vs. the corresponding value of normal wild-type (WT) rats. **P < 0.05 vs. the corresponding value of WT rats with BDL for 7 days.
decreased in serum and supernatant of cholangiocyte cultures following BDL. NRICC secrete NPY at the basolateral and apical domains. Chronic administration of NPY to BDL rats decreased intrahepatic bile ductal mass (IBDM), whereas anti-NPY antibody increased IBDM compared with BDL rats treated with saline. Data are means ± SE. Ten nonoverlapping fields were evaluated in 3 slides. Yellow arrows represent cytokeratin-19 immunoreactivity. *P < 0.05 vs. the corresponding value of BDL rats treated with saline. 

Fig. 3. Modulation of NPY activity alters cholangiocyte proliferation in vivo. A: administration of NPY to BDL rats decreased intrahepatic bile ductal mass (IBDM), whereas anti-NPY antibody increased IBDM compared with BDL rats treated with saline. Data are means ± SE. Ten nonoverlapping fields were evaluated in 3 slides. Yellow arrows represent cytokeratin-19 immunoreactivity. *P < 0.05 vs. the corresponding value of BDL rats treated with saline. 

Cholangiocytes

The BDL model is characterized by increased expression of a number of neuroendocrine factors and transporters and enhanced biliary secretion in response to gastrointestinal hormones (2, 4, 5, 12). There is growing information regarding the autocrine regulation of biliary mass by factors such as VEGF, serotonin, melatonin, and NGF (5, 12, 13, 23, 28). Local targeting of autocrine factors is indeed an important approach for managing liver disorders. For example, autocrine and paracrine VEGF signaling promotes the growth of liver cysts in Pkd2KO mice (10, 33). Little information exists regarding the autocrine/paracrine role of NPY in the management of cholestatic disorders. For example, decreased orexigenic response to NPY has been shown in cholestatic rats (29). Amelioration of portal hypertension and the hyperdynamic circulatory syndrome has been observed in cirrhotic rats by NPY via pronounced splanchnic vasoaction (25).

The NPY serum levels observed in our normal rats are similar to those found in other studies in rats (32) and slightly higher than those observed in humans (35, 36). We propose that the decrease in serum NPY levels observed in BDL rats may be due to reduced secretion of NPY by the neural tissue of the central and peripheral nervous system (38). The decrease in NPY serum levels observed in BDL rats was paralleled by
enhanced IBDM in cholestatic rats, consistent with the antiproliferative effect of NPY on biliary growth. In support of our findings, a study has demonstrated that decreased plasma NPY levels are correlated with the severity of liver damage (similar to BDL) (2), which may be the reason for hemodynamic and ascitic formation changes in liver cirrhosis patients (19). On the other hand, increased plasma levels of NPY were detected in patients with hepatorenal syndrome (36). No difference in circulating NPY levels was observed between normal patients and patients with fulminant hepatic failure (34).

The presence of NPY mRNA in cholangiocytes and the secretion of NPY (decreased after BDL) by the apical domain of cholangiocytes explain its presence in the bile of rats. The decrease of NPY secretion in proliferating BDL cholangiocytes rats is consistent with the concept that NPY is a local antiproliferative factor. A similar local mechanism has been detected in biliary tumors, where we demonstrated that NPY expression is upregulated in cholangiocarcinoma, which exerts local control on tumor cell proliferation and invasion (6). High NPY concentrations were detected in tissue extracts of liver, gallbladder, cystic, and bile ducts (1, 8). In addition to a paracrine pathway, local modulation of NPY biliary expression/secretion may be important for modulating the selective growth/damage of the biliary epithelium.

However, while some studies demonstrated the growth-promoting effects of NPY (27, 30), other studies demonstrate antiproliferative effects. For example, NPY inhibits vascular smooth muscle proliferation at low cell density and serum content (39). Treatment of colon carcinoma cells reduced their invasion potential in vitro (26). Also, NPY inhibits cholangiocarcinoma growth in both in vivo and in vitro models (6).

A shortcoming of our study is that the specific Y receptor involved in NPY inhibition of biliary hyperplasia was not identified due to the lack of reliable specific antagonists for Y5 and Y4. However, because we have shown that BIIE 0246 (a specific inhibitor of Y2) (6, 7) blocks the in vitro inhibitory effect of NPY on cholangiocarcinoma growth, we speculate that Y2 may partly regulate NPY inhibition of biliary hyper-

Fig. 4. Modulation of NPY activity alters cholangiocyte proliferation in vitro. A: NPY (1.0 μmol/l) decreased NRICC proliferation (by PCNA immunoblots) after incubation for 72 h and 7 days. Data are means ± SE of 8 immunoblots. *P < 0.05 vs. the corresponding basal value. B: cell cycle progression was assessed by flow cytometry, and the percentage of cells in the G0/G1, S, and G2 phases was determined. Representative traces from basal and 7 days after NPY treatment are shown. Analysis of the cell cycle progression demonstrated that there was a concomitant increase in the percentage of cells in the G0/G1 phase and a reduction in the percentage of cells in the G2/M phase after 7 days of incubation with NPY (1.0 μmol/l). C: in vitro treatment of NRICC with anti-NPY antibody decreased cell proliferation [by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assays] compared with the corresponding basal value. Data are means ± SE of 7 experiments. *P < 0.05 vs. the corresponding basal value.

G0/G1 S G2/M
basal 22.3 15.6 62
24 hr 21.4 18.8 59.8
48 hr 27.8 21 50.5
72 hr 23.8 17.2 57.8
7 days 51.8 33.2 14.2

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plasia. Further experiments (e.g., gene silencing of Y receptors with small-interfering RNAs) are warranted to determine the specific Y receptors modulating biliary hyperplasia. In addition, our data indicate that the expression of Y3 mRNA is increased in BDL cholangiocytes compared with normal rats, and it would therefore be tempting to speculate that this receptor be involved in the NPY-mediated effects observed here; however, this did not correlate with an increase in Y3 protein expression, suggesting that there may be a posttranscriptional control of Y3 expression. Such posttranscriptional regulation of NPYRs, in particular NPY Y1, has previously been demonstrated (22), although whether this occurs in cholangiocytes after BDL is unknown and warrants further investigation.

In summary, we have demonstrated that NPY inhibits biliary proliferation both in vivo and in vitro by paracrine and autocrine interactions with Y receptors. Modulation of circulating and/or local levels of NPY may be important for maintaining biliary homeostasis.

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DISCLOSURES

All authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS


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