Exogenous Serotonin Regulates Proliferation of Interstitial Cells of Cajal in Mouse Jejunum Through 5-HT$_{2B}$ Receptors

MIRA M. WOUTERS,*‡ SIMON J. GIBBONS,*‡ JAIME L. ROEDER,*‡ MARNE DISTAD,*‡,§ YIJUN OU,*‡ PETER R. STREGE,*‡ JOSEPH H. SZURSZEWSKI,*‡,§ and GIANRICO FARRUGIA*‡,§

*Enteric Neuroscience Program, ‡Division of Gastroenterology and Hepatology, §Department of Physiology and Biomedical Engineering, Mayo Clinic College of Medicine, Rochester, Minnesota

**Background & Aims:** Interstitial cells of Cajal (ICC) are required for normal gastrointestinal motility. Loss of ICC is associated with several motility disorders. The mechanisms modulating ICC survival and proliferation are poorly understood. This study aimed to establish whether 5-hydroxytryptamine (5-HT) plays a role in regulating ICC proliferation.

**Methods:** Expression of 5-HT receptor mRNA was investigated in muscle strips, in purified populations of ICC, and in identified single cells. The effect of 5-HT$_{2B}$ receptor ligands on ICC numbers was studied in primary cell cultures. Proliferation of ICC was determined by counting Ki67-positive cells in culture.

**Results:** Of the 5-HT receptors known to be involved in proliferation, 5-HT$_{2B}$ receptor mRNA was detected by reverse transcriptase–polymerase chain reaction (RT-PCR) in jejunal muscle, whereas 5-HT$_{1A}$, 5-HT$_{1D}$, and 5-HT$_{2C}$ receptor mRNAs were not. 5-HT$_{2B}$ receptor mRNA was found in single ICC and cells purified by flow cytometry. Exogenous 5-HT (1 µmol/L) increased (66% ± 9%, $P < .005$) ICC numbers in culture. The 5-HT$_{2}$ receptor antagonist, ritanserin, and the 5-HT$_{2B}$ receptor antagonist, SB204741, inhibited the effect of 5-HT. The 5-HT$_{2B}$ receptor agonist BW 723C86 induced a concentration-dependent increase in ICC number (50% ± 6% at 50 nM, $P < .04$) and increased ICC proliferation (25% ± 3% vs 19 ± 1% in controls, $P < .03$).

**Conclusions:** These studies establish that 5-HT$_{2B}$ receptors are expressed on ICC. Exogenous 5-HT regulates ICC numbers through 5-HT$_{2B}$ receptors in part by increasing ICC proliferation. The 5-HT$_{2B}$ receptor may serve as a novel pathway to regulate ICC numbers.

The majority of serotonin (5-hydroxytryptamine, 5-HT) in the body is produced in the gastrointestinal tract, where it functions as a neurotransmitter, a neuromodulator, and a paracrine factor. 5-Hydroxytryptamine is produced and released in the gastrointestinal tract by enterochromaffin cells in the mucosa and from a subset of enteric nerves. Platelets and mast cells can take up and deliver 5-HT. 5-Hydroxytryptamine acts on multiple distinct 5-HT receptors. Of the 7 classes of 5-HT receptors, 5-HT$_{1A}$, 5-HT$_{1D}$, 5-HT$_{2C}$, 5-HT$_{4}$, and 5-HT$_{7}$ receptors are known to be expressed in the gastrointestinal tract and to affect gastrointestinal motor function. The role of 5-HT in regulating gastrointestinal motility by mediating the transduction of luminal signals across the mucosa and by participating in enteric neurotransmission is well established. There is however significant evidence, mostly from outside the gastrointestinal tract but also in studies on enteric nervous system development, that shows that 5-HT can also regulate cell survival and proliferation. Four serotonin receptors have been implicated in transduction of the 5-HT signal to regulate cell survival and proliferation: 5-HT$_{1A}$, 5-HT$_{1D}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors. 5-Hydroxytryptamine$_{1A}$ and 5-HT$_{2C}$ receptor activation results in increased numbers of newly formed neurons in rat brain, whereas 5-HT$_{1A}$ and 5-HT$_{1D}$ receptors regulate mitogenesis in human small lung cell carcinoma. 5-Hydroxytryptamine$_{1A}$ receptors are also involved in proliferation of T cells and B cells and rat blood lymphocytes. A role for 5-HT$_{3B}$ receptors in cell differentiation and proliferation has been shown in mouse enteric neurons and hepatocytes. Activation of the 5-HT$_{2B}$ receptor by 5-HT also induces proliferation of mouse cardiomyocytes and retinal cells in Xenopus, indicating a conserved role for 5-HT$_{2B}$ in regulating cell survival and proliferation in different species.

Interstitial cells of Cajal (ICC) are the “pacemaker cells” in the gastrointestinal tract; one of their functions is to generate slow waves. Therefore, ICC determine the frequency of contractions. Additionally, ICC act as amplifiers of neuronal signals and as mechanosensors, and to set the smooth muscle membrane potential gradient. A decreased number of ICC or a disrupted ICC network is associated with pathologic conditions such as...
slow transit constipation, diabetic gastroparesis, and pseudo-obstruction. Understanding the factors that underlie ICC survival and proliferation is important in determining how ICC networks are maintained and how to limit ICC loss or induce recovery in disease states associated with loss of ICC. Recent observations indicate that turnover of ICC occurs in immature and adult ICC. However, the factors that regulate this turnover have not been established except that the effects of the receptor tyrosine kinase c-Kit and its ligand, stem cell factor, are known to be required for ICC maintenance by a still-undefined mechanism.

Given the roles that 5-HT and 5-HT receptors play in regulating cell survival and proliferation on other cell types and the availability of 5-HT in the gastrointestinal tract, the aim of this study was to determine the potential role of 5-HT and 5-HT receptors in regulating ICC survival and proliferation.

Materials and Methods

Animals

Mice were maintained and experiments performed with approval from the Institutional Animal Care and Use Committee of Mayo Clinic College of Medicine. c-Kit dominant white spotting/viable white spotting locus (W/ W') and their respective controls, and BALB/c mice were obtained from Jackson Laboratory (Bar Harbor, ME) and Harlan-Sprague-Dawley (Indianapolis, IN) respectively. Three-day-old mice were killed by CO2 inhalation and decapitation. One-month-old mice were killed by CO2 inhalation. The fundus and jejunum were quickly dissected out, flushed with ice-cold calcium-free Hanks' balanced salt solution (Invitrogen, Carlsbad, CA), and pinned onto a sylgard-lined petri dish; and for all experiments the mucosa and mesentery were removed using a binocular microscope.

Reverse Transcription–Polymerase Chain Reaction

To determine the expression of the different 5-HT receptors, polymerase chain reaction (PCR) was performed on adult mouse jejunal muscle strips. Adult mouse brain served as a positive control. Whole mouse brain was quickly dissected out on ice. Total RNA from jejunal muscle strips and brain was isolated using RNA-bee (Tel-Test, Friendswood, TX) according to the manufacturer’s instructions. Reverse transcription (RT) was performed using Powerscript reverse transcriptase (Clontech Laboratories Inc., Palo Alto, CA) as described by the manufacturer. Polymerase chain reaction for 5-HT, 5-HT, 5-HT, and 5-HT receptor message was performed on 50 ng of sample cDNA using 300 nmol/L of gene specific primers (Table 1) and AmpliTaq Gold (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Twenty-five-microliter PCRs were set up by dividing all solutions by 2. Thermal cycler conditions were identical for all primer sets in the first PCR, namely, 95°C for 10 minutes, 35 cycles of 95°C for 20 seconds, 60°C for 20 seconds, and 72°C for 30 seconds. For 5-HT, 5-HT, and 5-HT receptors, a nested PCR was performed on 1 µL of the first PCR using the following program: 95°C for 10 minutes and 35 cycles of 95°C for 20 seconds, 61°C for 20 seconds, and 72°C for 30 seconds.

Enzymatic Dissociation of ICC

Freshly dispersed cells were obtained from mouse small intestine. Intestinal muscle strips from 3 mice were pooled for each experiment. BALB/c mice of either sex between 2 and 4 days old were used. The small intestine was removed and pinned out in a Sylgard-lined dish containing Hanks' calcium-free buffer and 1% antibiotic-antimycotic (Invitrogen; Carlsbad, CA). The muscularis propria was placed in a collagenase-based dissociation cocktail. The cocktail contained 2500 U collagenase (Worthington Biochemical Company, Lakewood, NJ), 20 mg bovine serum albumin (Sigma, St Louis, MO), 20 mg trypsin inhibitor (Sigma), and 5 mg adenosine triphosphate (Sigma) in 10 mL of calcium-free Hanks' balanced salt solution. The pH was adjusted to 7.0 with 0.1 mol/L NaOH. After 15 minutes of incubation at 32°C in a gently shaked water bath, the tissue was triturated and

<table>
<thead>
<tr>
<th>Gene</th>
<th>5′-3′</th>
<th>3′-5′</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Kit</td>
<td>GGACCTGGATGATTTGCTGA</td>
<td>GGAGAAGAGCCTCCAGAGGA</td>
</tr>
<tr>
<td>c-Kit nested</td>
<td>CTTCCATCGAGTGCCAAGAG</td>
<td>ACGAGCCTGAAAAGCTCTC</td>
</tr>
<tr>
<td>5-HT2b</td>
<td>AAGCCAAATCGAAGGCAATC</td>
<td>GGGGCAAATCAAGGACAAA</td>
</tr>
<tr>
<td>5-HT2b nested</td>
<td>ATTCAGCGAATCAGTGCAA</td>
<td>CTTGAGACCCATCAGCAATC</td>
</tr>
<tr>
<td>5-HT3a</td>
<td>CTGTTGTATGCCCTGTATGTT</td>
<td>CCATCCTCGTGCCATCATCCTT</td>
</tr>
<tr>
<td>5-HT3a nested</td>
<td>ACTGTCCCAATCTCGCACTG</td>
<td>GATGTTGACCCCTGCTCT</td>
</tr>
<tr>
<td>5-HT1D</td>
<td>TCGTGGTCTGTCATACACTG</td>
<td>GAGCTGGCCAAAGGTTCCAG</td>
</tr>
<tr>
<td>5-HT1D nested</td>
<td>GCTCCAAACAGTCCTGAA</td>
<td>TGCAGCGTCTGAGTCAAGA</td>
</tr>
<tr>
<td>5-HT2c</td>
<td>GCTCAGTCTCTGTCGACCTA</td>
<td>CTTAGCTCCGGAATTGACCC</td>
</tr>
<tr>
<td>5-HT2c nested</td>
<td>GCCATTGTTGGCCAGTGC</td>
<td>TGCCCTATGTTGCAATGATT</td>
</tr>
<tr>
<td>Actg2</td>
<td>CATGACCATCATCACACCTTG</td>
<td>GACCTCATGCCAATGAGGAAA</td>
</tr>
<tr>
<td>Actg2 nested</td>
<td>CCCCCTAACCACCCAAGCAA</td>
<td>AAGGCGCTGCGGCAAAATG</td>
</tr>
<tr>
<td>β-actin</td>
<td>CGTGCCTGACATCAAGAGA</td>
<td>TTTGCGATCACATCTGCTG</td>
</tr>
</tbody>
</table>

Table 1. Primers used for PCR
spinned down at 2100g for 10 minutes. Cells were resuspended in 2 mL Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 1% sodium pyruvate, and 1% antibiotic-antimyocytic.

**Mouse ICC and Murine Fibroblast Cocultures**

Freshly dispersed cells obtained from the mouse small intestine were cultured on 22-mm glass coverslips covered with mouse fibroblasts genetically engineered to produce murine stem cell factor as previously described. Briefly, SI/SI4 mSCF248, murine stem cell factor-secreting fibroblasts (provided by Dr. David Williams, Indianapolis, IN) were plated on 22-mm plain glass coverslips at 4.5 × 10⁴ cells per coverslip in high-glucose Dulbecco’s modified Eagle medium containing 10% fetal bovine serum, 1% sodium pyruvate, and 1% antibiotic-antimycotic (all from Invitrogen). After 30 hours, the fibroblast cell division was arrested by irradiation at 16000 Gy. After a 24-hour recovery period, 250 µL of suspensions containing cells freshly dissociated from mouse jejunum were plated onto the SI/SI4 mSCF248 fibroblasts at a cell density of approximately 3 × 10⁵ cells per coverslip. Cells were allowed to incubate for 30 minutes at 37°C/5% CO₂ before adding 2 mL of the culture medium to the well. These culture conditions result in cell cultures highly enriched in interstitial cells of Cajal.

**Enrichment of ICC**

Interstitial cells of Cajal were selected on the basis of detection of the ICC marker c-Kit. This procedure was carried out on ice. Freshly dissociated cells were stained for c-Kit expression with the phycoerythrin-labeled antimouse CD117 (eBioscience, San Diego, CA) diluted at 1:300 (final vol/vol) in flow cytometry staining buffer (eBioscience). Cells were incubated for 20 minutes in the dark on a shaker and washed three times with ice-cold staining buffer. The total volume was adjusted to 1 mL in cold flow cytometry staining buffer and filtered through a 40-µm mesh filter before sorting. Enrichment of ICC was performed using a Becton Dickinson Immunocytometry Systems (San Jose, CA) FACSVantage Sorter. c-Kit–expressing cells were sorted on the basis of phycoerythrin-ACK2 immunolabeling and collected in sterile polystyrene culture tubes (Fisher Scientific, Eagan, MN). The nonlabeled, nonfluorescent cell population that includes smooth muscle cells and enteric neurons as well as other cell types was also collected and served as a negative control. Both the phycoerythrin-ACK2–labeled cells and the nonlabeled cells were collected by centrifugation at 3000g for 10 minutes and immediately processed for RNA extraction.

**Isolation of Single ICC**

c-Kit–immunolabeled cells were prepared as described previously. Single ICC were selected on the basis of phycoerythrin-ACK2 immunolabeling and collected by gentle aspiration into a 30-µm-wide patch clamp pipette tip. The cell was lifted out of the chamber and immediately expelled into a 200-µL tube on dry ice as described previously. For controls single cells not labeled with phycoerythrin-ACK2 were collected in each tube.

**Single-Cell PCR**

Per isolated cell, 0.5 µg yeast tRNA (Ambion, Austin, TX) and 10 µg proteinase K (Roche Diagnostics GmbH, Mannheim, Germany) were added. The sample was centrifuged at 3300g at 4°C for 30 seconds to release RNA. Nucleases and proteinases were destroyed by incubation at 90°C for 10 minutes, 55°C for 30 minutes, and 90°C for 10 minutes.

Two-step reverse transcription and PCR were performed using the TaqMan Gold RT-PCR kit (Applied Biosystems) as described by the manufacturer. By dividing all solutions by 225 µL, PCR reactions were set up. For the first PCR reaction, cDNA of three collected cells was pooled and used as template. Three microliters of the first PCR reaction (94°C for 10 minutes, 25 times 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and 72°C for 10 minutes) was used for the nested PCR (94°C for 10 minutes, 35 times 94°C for 30 seconds, 61°C for 30 seconds, 72°C for 30 seconds, and 72°C for 10 minutes).

**Chemicals**

Ritanserin and 5-HT were obtained from Sigma (St. Louis, MO), and BW 723C86 and SB204741 were purchased from Tocris Cookson Inc. (Ellisville, MO). Before use, 5-HT was freshly dissolved in distilled water. The BW 723C86 (100 mmol/L), SB204741 (100 mmol/L), and ritanserin (33.3 mmol/L) stock solutions were made in DMSO. Further dilutions were made fresh on the experimental day in distilled water. All dilutions were maintained in solution using a 37°C water bath. Primary cell cultures were treated every 12 hours with 5-HT and every 20 hours with BW 723C86, ritanserin, or SB204741 for 2.5 days.

**Immunohistochemistry**

After 2.5 days in culture, ICC were immunolabeled using the rat monoclonal anti-c-Kit antibody ACK2 (eBioscience) as previously described. Briefly, acetone-fixed coverslips (4°C, 10 min) were washed with phosphate-buffered saline (PBS), incubated with 10% normal donkey serum (NDS, Jackson Immunoresearch Laboratories, West Grove, PA) and 0.2% Triton-X-100 (Sigma) in PBS for 1 hour to minimize nonspecific antibody binding and then incubated with primary antibody (1/150 in 5% NDS) overnight at 4°C. Next, the sections were rinsed in PBS and incubated in the dark for 1 hour at room temperature with donkey anti-rat IgG conjugated to CY3 (Jackson Immunoresearch Laboratories, 1/800 dilution in 2.5% NDS).
Ki67 was used as a proliferation marker. After the ACK2 staining procedure, coverslips were postfixed with 4% paraformaldehyde for 10 minutes, washed with PBS, and incubated with Ki67 (1/500 in 5% NDS; Abcam Inc, Cambridge, MA) overnight at 4°C. After washing, the coverslips were incubated for 1 hour with donkey antirabbit conjugated to fluorescein isothiocyanate (1/200 dilution in 2.5% NDS).

4′,6-Diamidino-2-phenylindole (DAPI) (1/3000 for 5 minutes; Invitrogen) was used as a nuclear counterstain. Coverslips were mounted with 30% glycerol (Sigma).

Immunostained cultures were examined for c-Kit-positive cells with the use of a fluorescent microscope (Olympus BX51WI; Olympus America Inc., Center Valley, PA). A times 20 objective (numerical aperture = 0.5) objective was used to count the number of c-Kit-positive cells per high-power field. One high-power field covered 0.94 mm². At least 35 high-power fields were counted per culture. This number was chosen on the basis of results from prior culture experiments and is the optimal number to minimize field-to-field variation while not overcounting fields.

Data Analysis

All data analysis was carried out on the raw non-normalized data. Statistical analysis was performed using the unpaired t test with Welch correction. For cell culture experiments, a paired t test was performed to compare 2 groups of data. All data for these experiments are expressed as the mean ± SEM where n represents the number of experiments.

Results

5-HT Increased ICC Numbers

Exogenous-applied 5-HT induced a dose-dependent saturable increase in ICC number in primary culture (Figure 1). At 1 nmol/L 5-HT, no effect was observed on ICC numbers versus control (3.8 ± 1.1 ICC per field vs 4.2 ± 0.4). An increase in number was first detected at 10 nmol/L 5-HT (5.1 ± 1.2 ICC per field versus 4.2 ± 0.4) and 100 nmol/L 5-HT (6.8 ± 0.7 ICC per field versus 4.2 ± 0.4). The maximal effect was observed at 1 μmol/L 5-HT. At this drug concentration, ICC increases were counted per culture. This number was chosen on the basis of results from prior culture experiments and is the optimal number to minimize field-to-field variation while not overcounting fields.

Effect of 5-HT on ICC number normalized to control (% change) vs log [5-HT] (M)

Figure 1. 5-HT increased ICC numbers in primary cell cultures. 5-HT had a dose-related effect on ICC numbers. Initial effects were seen at 10 nmol/L 5-HT, and the maximum effect was reached at 1 μmol/L. At higher doses, less or no effect was observed. Data are shown as means ± SEM; n = 4–6.

Single ICC expressed 5-HT<sub>2B</sub> receptor mRNA. Cell sorting results in an enriched population of ICC. However, the sample is contaminated with c-Kit–positive cells that are not ICC, such as mast cells, whereas in the sorted, nonlabeled cell population c-Kit–negative ICC may still be present. To determine the expression of 5-HT<sub>2B</sub> receptor mRNA on single ICC, freshly dispersed single ICC from the small intestine muscle of neonatal...
mice were labeled with phycoerythrin-ACK-2 and collected. For each experiment, 3 ICC were pooled. Using nested PCR, products of the expected size were amplified for c-Kit (218 nt) and the 5-HT2B receptor (392 nt) from single ICC (Figure 3C and D). This experiment was carried out 3 times. In 2 of the 3 experiments, both c-Kit and 5-HT2B message was found. In one experiment, neither message for c-Kit or 5-HT2B was found, perhaps reflecting degraded RNA. Single-cell ICC did not express enteric smooth muscle actin mRNA (Actg2, 440 nt) (Figure 3E) or PGP 9.5 mRNA (data not shown), suggesting that there was no contamination of the 3 pooled ICC with smooth muscle cells or nerves.

**Figure 2.** 5-HT2B receptor mRNA—but not 5-HT1A, 5-HT1D, or 5-HT2C receptor mRNA—was expressed in adult mouse jejunum muscle strips. 5-HT1A (panel A), 5-HT1D (panel B), or 5-HT2C receptor mRNA (panel D) was expressed in brain but not in jejunal muscle strips. The expected amplicons contained 231, 316, and 148 nucleotides, respectively. 5-HT2B receptor mRNA was expressed in brain and jejunal muscle strips (panel C). The amplicon contained 543 nucleotides. All PCR products were confirmed by sequencing. –RT, minus reverse transcriptase; ladder, DNA molecular weight marker XIV (Roche).

**5-HT2B Receptor Agonists and Antagonists Modulated ICC Numbers in Primary Cell Cultures**

To determine whether the 5-HT2B receptor on ICC mediates the effects on ICC numbers observed with 5-HT, ligands selective for 5-HT2B receptors were used on ICC cultures from mouse small intestine. A dose of 5-HT (100 nmol/L) that induced 60% of the maximal effect of 5-HT was used to study the effect of 5-HT receptor antagonists on ICC numbers. The nonspecific 5-HT2 receptor antagonist ritanserin partly blocked the effect of 100 nmol/L 5-HT. 5-HT (100 nmol/L) alone increased ICC numbers by 78% ± 24% compared with 22% ± 17% when ritanserin was added (n = 4 experiments; control, 8.3 ± 1.5; 5-HT, 12.4 ± 0.6, ritanserin, 9.1 ± 1.2 ICC per field; P < .02) (Figure 4). The specific 5-HT2B receptor antagonist SB204741 completely blocked the effect of 5-HT on ICC numbers. In the presence of SB204741, 5-HT did not increase ICC numbers (44 ± 10% increase for 100 nmol/L 5-HT compared with no change in the presence of SB204741 (n = 6 experiments; control, 7.8 ± 0.7; 5-HT, 11.1 ± 0.6; SB204741, 7.5 ± 0.6 ICC per field; P < .004) (Figure 5).

The 5-HT2B receptor agonist BW 723C86 increased ICC numbers in a concentration-dependent manner (Figure 6). Initial effects were seen at 1 nmol/L and maximal effects seen at 50 nmol/L with a 50 ± 6% increase in ICC numbers versus control (n = 7 experiments; control, 5.4 ± 0.6; 50 nmol/L BW 723C86, 8.0 ± 1.1 ICC per field; P < .04). Similar to the 5-HT data, the dose response was bell shaped, with 100 nmol/L resulting in no increase in ICC numbers.

**Activation of the 5-HT2B Receptor Increased ICC Proliferation**

To determine if the increase in ICC numbers evoked by activation of the 5-HT2B receptor was caused by an increase in proliferation, the number of proliferating cells was compared in control versus BW 723C86-treated cells. Colocalization of nuclear Ki67, a marker for dividing cells, and c-Kit immunoreactivity were used to identify proliferating ICC (Figure 7A). In control, 19 ± 1% of ICC were Ki67-positive. In the presence of the 5-HT2B agonist BW 723C86 (50 nmol/L), significantly more ICC were Ki67 positive (25 ± 3%, n = 6, P < .03) (Figure 7B).

antagonist SB204741 completely blocked the effect of 5-HT on ICC numbers. In the presence of SB204741, 5-HT did not increase ICC numbers (44 ± 10% increase for 100 nmol/L 5-HT compared with no change in the presence of SB204741 (n = 6 experiments; control, 7.8 ± 0.7; 5-HT, 11.1 ± 0.6; SB204741, 7.5 ± 0.6 ICC per field; P < .004) (Figure 5).

The 5-HT2B receptor agonist BW 723C86 increased ICC numbers in a concentration-dependent manner (Figure 6). Initial effects were seen at 1 nmol/L and maximal effects seen at 50 nmol/L with a 50 ± 6% increase in ICC numbers versus control (n = 7 experiments; control, 5.4 ± 0.6; 50 nmol/L BW 723C86, 8.0 ± 1.1 ICC per field; P < .04). Similar to the 5-HT data, the dose response was bell shaped, with 100 nmol/L resulting in no increase in ICC numbers.

**Activation of the 5-HT2B Receptor Increased ICC Proliferation**

To determine if the increase in ICC numbers evoked by activation of the 5-HT2B receptor was caused by an increase in proliferation, the number of proliferating cells was compared in control versus BW 723C86-treated cells. Colocalization of nuclear Ki67, a marker for dividing cells, and c-Kit immunoreactivity were used to identify proliferating ICC (Figure 7A). In control, 19 ± 1% of ICC were Ki67-positive. In the presence of the 5-HT2B agonist BW 723C86 (50 nmol/L), significantly more ICC were Ki67 positive (25 ± 3%, n = 6, P < .03) (Figure 7B).
In this study, we showed that exogenous applied 5-HT increased ICC numbers in vitro, that the effect of 5-HT on ICC numbers was mediated through 5-HT2B receptors expressed on ICC and that the effect was mediated in part by increasing proliferation of ICC.

Increasing evidence suggests that ICC numbers are not static and that there is considerable ICC turnover in both health and in motility disorders such as slow transit constipation. Our understanding of the control of ICC numbers was fairly limited until recently. We now know that there are several substances that help modulate ICC numbers in the gastrointestinal tract. The role of a functional c-Kit–stem cell factor pathway in the development and maintenance of ICC is well established. It recently became apparent that in a mouse gastroparesis model, a reduced insulin/insulin growth factor I (IGF-I) signaling pathway may lead to ICC depletion by causing smooth muscle atrophy and reduced SCF production. Also the protective role of neuronally derived nitric oxide on ICC numbers and network volume in the mouse gastric body was recently established. Our data suggest that another potential regulator of ICC

![Figure 3. ICC expressed 5-HT2B receptor mRNA. (A) c-Kit mRNA was expressed in the sorted enriched ICC population but was absent in sorted nonlabeled cells (NLC). The expected amplicon contained 218 nucleotides. (B) 5-HT2B receptor message was present in both the sorted enriched ICC population and the NLC. The amplicon contained 375 nucleotides. (C) c-Kit mRNA was expressed by single ICC. The expected amplicon contained 218 nucleotides. The other PCR product contained approximately 450 nucleotides and was due to nonspecific amplification. (D) 5-HT2B receptor mRNA was expressed by single ICC. The amplicon contained 375 nucleotides. (E) Enteric smooth muscle actin, Actg2, mRNA was detected in single smooth muscle cells (SMC) but not in ICC. The two amplicons both represent Actg2 (568 and 440 nt). (F) β-Actin mRNA was amplified in all isolated cells (453 nt). In the SMC, genomic DNA for β-actin was also amplified (673 nt). All PCR products were confirmed by sequencing. ICC, interstitial cells of Cajal; −RT, minus reverse transcriptase; ladder, DNA molecular weight marker XIV (Roche).](image1)

![Figure 4. The 5-HT2 receptor antagonist ritanserin reduced the effect of 5-HT on ICC numbers. Ritanserin (3 nmol/L) partly blocked the effect of 5-HT on ICC numbers in primary culture. (Mean ± SEM; * P < .02 by paired t test; n = 4.)](image2)

![Figure 5. The 5-HT2B receptor antagonist SB204741 blocked the effect of 5-HT on ICC numbers in primary cell cultures. SB204741 (2 nmol/L) completely blocked the increase in ICC numbers induced by 5-HT. (Mean ± SEM; * P < .004 by paired t test; n = 6.)](image3)

**Discussion**

In this study, we showed that exogenous applied 5-HT increased ICC numbers in vitro, that the effect of 5-HT on ICC numbers was mediated through 5-HT2B receptors expressed on ICC and that the effect was mediated in part by increasing proliferation of ICC.
numbers is 5-HT through the 5-HT_{2B} receptor. The intracellular mechanism by which this effect is mediated is not yet known. Binding of 5-HT to the Gq-coupled 5-HT_{2B} receptor is known to activate phospholipase C, which initiates a rapid release of inositol trisphosphate and increases intracellular calcium. Also the 5-HT_{2B} receptor is involved in 5-HT–induced mitogenesis in which c-src is required for cell cycle progression via the mitogen-activated protein kinase pathway. Through its PDZ (postsynaptic density protein [PSD95], Drosophila disc large tumor suppressor [DlgA], and zonula occludens-1 protein [zo-1]) domain, the 5-HT_{2B} receptor also activates nitric oxide synthesis, another cytoprotective molecule for ICC. Which, if any, of these mechanisms is operative in ICC remains to be established.

The current study does establish that the increase in ICC induced by activation of the 5-HT_{2B} receptor in vitro results, at least in part, from an increase in proliferation of ICC. The demonstration that the nuclear proliferation marker Ki67 is expressed in ICC is a novel observation. The Ki67 antigen labels the granular components of the nucleolus during late G1, S, G2, and M phases. Of ICC on vehicle-treated control cover slips, 19% expressed this marker. When the 5-HT_{2B} receptor agonist BW 723C86 was used, ICC proliferation was increased to 25%. Therefore c-Kit–positive ICC with a mature morphology are capable of proliferating in culture and 5-HT increases proliferation of mature ICC. It is not known whether c-Kit–negative precursor cells express the 5-HT_{2B} receptor and also proliferate and differentiate into mature ICC after 5-HT_{2B} receptor activation.

Primary cell cultures from 3-day-old mice were used to determine the effect of exogenous-applied 5-HT on ICC numbers. In a dose-dependent manner, 5-HT induced increased ICC numbers compared with control. The maximum effect was reached at 1 μmol/L. At higher doses, 5-HT had no effect on ICC numbers. This lack of effect at higher concentrations may be due to receptor internalization or desensitization or is possibly due to an effect of 5-HT on other receptors.

Interstitial cells of Cajal are known to express 5-HT_{3} and 5-HT_{4} receptors as well as several other receptor classes. It is possible that activation of these receptors by 5-HT at higher concentrations may reverse the proliferative effects of 5-HT seen at lower concentrations. The concentration of 5-HT that induced an increase in ICC numbers is similar to that described in literature for regulation of proliferation in 5-HT_{2B} receptor–transfected mouse fibroblast cell line deficient in thymidine kinase (LMTK–).

To date, 5-HT_{3} and 5-HT_{4} receptors were the only 5-HT receptors described on ICC. Outside the gastrointestinal tract, 5-HT receptors, in particular 5-HT_{1A}, 5-HT_{1D}, 5-HT_{2B}, and 5-HT_{2C} receptors, have been shown to mediate cell survival and proliferation. The expression and function of these 5-HT receptors in ICC was unknown. Data presented in this study suggest that of these 5-HT receptors only 5-HT_{2B} is expressed on ICC as 5-HT_{1A}, 5-HT_{1D}, and 5-HT_{2C} mRNA could not be detected in preparations from adult mouse jejunum in which 5-HT_{2B} receptor mRNA could be amplified. Previous studies have
shown that the 5-HT<sub>2B</sub> receptor is also expressed on neurons and smooth muscle cells.\textsuperscript{15,16,61} We know that the 5-HT<sub>2B</sub> receptor in the rodent gastrointestinal tract is involved in the maturation of neurons\textsuperscript{15,29} and in rodent fundus contraction,\textsuperscript{62} suggesting a more generalized role for the 5-HT<sub>2B</sub> in the control of cell number and function.

The 5-HT<sub>2B</sub> receptor expressed on ICC, as demonstrated by amplification of 5-HT<sub>2B</sub> receptor mRNA from sorted and single ICC, appears to be involved in regulating ICC numbers, as activation of the receptor increased ICC numbers, and the nonspecific and specific 5-HT<sub>2B</sub> receptor antagonists ritanserin and SB 204741 reduced the effect of 5-HT on ICC numbers at concentrations consistent with the known affinity of these compounds for the 5-HT<sub>2B</sub> receptor.\textsuperscript{63} This and the dose-dependent effects of the agonists 5-HT and BW 723C86 indicate that the compounds are producing a specific receptor-mediated increase in ICC number rather than having a nonspecific protective effect due to the known antioxidant activity of 5-HT and similar compounds.\textsuperscript{64}

The known expression of 5-HT<sub>3</sub> receptors on subsets of ICC and of 5-HT<sub>4</sub> receptors together with the data presented here that show expression of 5-HT<sub>2B</sub> receptors on ICC suggest that 5-HT may have several modulatory effects on ICC. Additional experiments need to address the possible physiological role of endogenously released 5-HT on ICC. Also, the source of 5-HT is not clear. In vivo, in the mouse small intestine ICC are located at the level of the deep muscular plexus, with another population of ICC forming a network surrounding the myenteric ganglia. In larger animals ICC are also distributed within the muscle layers. In nondiseased tissue it is unlikely that ICC respond to 5-HT released by the enterochromaffin cells in the mucosa, because the 5-HT transporters present in serotonergic neurons and in mucosal and submucosal cells will rapidly take up 5-HT.\textsuperscript{65,66} However, a subset of intrinsic primary afferent neurons located in the submucosal plexus do project to the myenteric plexus and are activated by mucosal 5-HT.\textsuperscript{51,22} These submucosal to myenteric projections may explain the ability of mucosal 5-HT release to indirectly activate myenteric neurons and ICC. In inflammation, 5-HT transporters have been shown to be down-regulated\textsuperscript{67,68} and 5-HT production increased,\textsuperscript{67} making it more likely that mucosal-generated 5-HT reaches ICC. Another possible source of 5-HT is the serotonergic interneurons, which represent about 2% of the total number of enteric neurons.\textsuperscript{4,69} However, although the myenteric plexus interneurons are in close apposition to ICC, they do not come into direct contact with ICC and therefore would be unlikely to provide sufficient 5-HT to activate ICC 5-HT receptors. Another source for 5-HT is mast cells. It has been well established that rodent mast cells can synthesize and release 5-HT to the appropriate target cells.\textsuperscript{8,9} Although it is generally accepted that human mast cells do not contain 5-HT, recent data suggest that human mast cells are capable of serotonin synthesis and release,\textsuperscript{10} albeit to a lower extent than mouse mast cells. Mast cells are known to be in close apposition to ICC and may therefore be one of the sources of 5-HT. It is currently unknown whether platelet-derived 5-HT can activate ICC.\textsuperscript{7,70}

In conclusion, we show that exogenous-applied 5-HT induces an increase in ICC number in vitro, that ICC express the 5-HT<sub>2B</sub> receptor, and that activation of the 5-HT<sub>2B</sub> receptor increases ICC proliferation. The 5-HT<sub>2B</sub> receptor may therefore serve as a novel pathway to regulate ICC numbers.

References

13. Craig DA, Clarke DE. Pharmacological characterization of a neuronal receptor for 5-hydroxytryptamine in guinea pig ileum with properties similar to the 5-hydroxytryptamine receptor. J Pharmacol Exp Ther 1999;252:1378–1386.


Received November 22, 2006. Accepted May 31, 2007.
Address requests for reprints to: Gianrico Farrugia, MD, Mayo Clinic, Division of Gastroenterology and Hepatology, 200 First Street SW, Rochester, Minnesota 55905. e-mail: farrugia.gianrico@mayo.edu; fax: (507) 284-0266.
Supported by National Institute of Health grants DK52766 and DK57061.
The authors thank Gary Stoltz for tissue dissection and Kristy Zodrow for secretarial assistance.