Serotonin Augments Gut Pacemaker Activity via 5-HT_3 Receptors

Hong-Nian Liu^1, Susumu Ohya^2, Yuji Nishizawa^2, Kenta Sawamura^1, Satoshi Iino^4, Mohsin Md Syed^1*, Kazunori Goto^1, Yuji Imaizumi^3, Shinsuke Nakayama^1*

1 Department of Cell Physiology, Nagoya University Graduate School of Medicine, Nagoya, Japan, 2 Department of Anatomy and Cell Biology, Nagoya University Graduate School of Medicine, Nagoya, Japan, 3 Department of Molecular and Cellular Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan, 4 Department of Anatomy, Faculty of Medical Sciences, University of Fukui, Fukui, Japan

Abstract

Serotonin (5-hydroxytryptamine: 5-HT) affects numerous functions in the gut, such as secretion, muscle contraction, and enteric nervous activity, and therefore to clarify details of 5-HT’s actions leads to good therapeutic strategies for gut functional disorders. The role of interstitial cells of Cajal (ICC), as pacemaker cells, has been recognized recently. We thus investigated 5-HT actions on ICC pacemaker activity. Muscle preparations with myenteric plexus were isolated from the murine ileum. Spatio-temporal measurements of intracellular Ca^{2+} and electric activities in ICC were performed by employing fluorescent Ca^{2+} imaging and microelectrode array (MEA) systems, respectively. Dihydropyridine (DHP) Ca^{2+} antagonists and tetrodotoxin (TTX) were applied to suppress smooth muscle and nerve activities, respectively. 5-HT significantly enhanced spontaneous Ca^{2+} oscillations that are considered to underlie electric pacemaker activity in ICC. LY-278584, a 5-HT_3 receptor antagonist suppressed spontaneous Ca^{2+} activity in ICC, while 2-methylserotonin (2-Me-5-HT), a 5-HT_3 receptor agonist, restored it. GR113808, a selective antagonist for 5-HT_2A and O-methyl-5-HT (O-Me-5-HT), a non-selective 5-HT receptor agonist lacking affinity for 5-HT_3 receptors, had little effect on ICC Ca^{2+} activity. In MEA measurements of ICC electric activity, 5-HT and 2-Me-5-HT caused excitatory effects. RT-PCR and immunostaining confirmed expression of 5-HT_3 receptors in ICC. The results indicate that 5-HT augments ICC pacemaker activity via 5-HT_3 receptors. ICC appear to be a promising target for treatment of functional motility disorders of the gut, for example, irritable bowel syndrome.

Introduction

Special interstitial cells with abundant c-Kit receptors on their surface are distributed throughout the gastrointestinal tract. These cells are referred to as interstitial cells of Cajal (ICC) due to the histological features of the network [1–3]. It is now considered that ICC in the myenteric region act as pacemaker cells, and produce gut movements in concert with enteric neurones and smooth muscle cells [4–6]. Numerous neurotransmitters and hormones are likely to affect ICC activity, and thereby regulate gut motility.

Serotonin (5-hydroxytryptamine: 5-HT), well known for mood control in the brain, also plays a crucial role in cellular signalling in the gut. Indeed, enterochromaffin cells release the majority (>90%) of 5-HT in the human body in response to the pressure of intraluminal content and other noxious stimuli [7]. Some enteric neurones in the descending peristaltic reflex pathway also release 5-HT as a neurotransmitter [8–10]. Since enteric neurones and smooth muscle cells express various 5-HT receptors depending upon cell type and location of the cell, and their functions are critically affected by this signalling molecule [11,12], 5-HT receptors are key targets in pharmacological interventions of gut functional disorders, as well as psychiatric disorders of the brain.

It is thought that oscillations of the intracellular (cytosolic) Ca^{2+} concentration ([Ca^{2+}]) in ICC cells underlie gut pacemaker activity. Namely, periodic activation of Ca^{2+}-sensitive ion channels in the plasma membrane generates pacemaker potentials [13,14]. Previously, we demonstrated that spontaneous electrical activity occurs in synchrony with [Ca^{2+}] oscillations in ICC, and that coordinated actions of intracellular Ca^{2+} release channels and transmembrane Ca^{2+} influx pathways underlie ICC [Ca^{2+}] oscillations [15–17].

In the present study, we provide evidence that 5-HT regulates ICC pacemaker activity. We performed Ca^{2+} imaging and potential mapping of ICC pacemaker activity using fluorescent Ca^{2+} probes and microelectrode array (MEA), respectively, and found that 5-HT enhances both Ca^{2+} and electric activities of ICC via 5-HT_3 receptors, which are nonselective cation channels permeable to Ca^{2+}. We also carried out RT-PCR and immunostaining to confirm the expression of 5-HT_3 receptors in ICC. Our findings suggest that 5-HT modulation of ICC activity should also be considered for gut motility disorders, for example, irritable bowel syndrome.
bowel syndrome with a prevalence of around 10% [18]. Interestingly, this disease is known to be frequently complicated by psychiatric illness and mood disorders.

**Materials and Methods**

**Animals**

Animals used in the present study were treated ethically. All procedures were approved by the Institutional Animal Care and Use Committee. BALB/c (wild-type) and W/Hi* mice (3–6 weeks after birth) were killed by cervical dislocation, after being anaesthetized with diethyl ether. Unless otherwise stated, BALB/c mice were used in all experiments.

**Ca\(^{2+}\) imaging**

Cell cluster preparations were used in Ca\(^{2+}\) imaging [16,19]. Although we detected 5-HT-augmentation of ICC Ca\(^{2+}\) activity in isolated ileal muscle segments containing the myenteric plexus (Figure S1; Video S1 and Video S2), we used cell cluster preparations in Ca\(^{2+}\) imaging to examine numerous drugs related to 5-HT, because it was difficult to stably load Ca\(^{2+}\) indicators in many intact muscle segments.

The musculature along with the myenteric plexus were carefully dissected from the ileum, and incubated in Ca\(^{2+}\)-free Hanks’ solution containing collagenase (1 mg/ml, Wako Chemical, Osaka, Japan), trypsin inhibitor (2 mg/ml, type I-S, Sigma, St Louis, MO, USA), ATP (0.3 mg/ml, Seikagaku Kogyo, Tokyo, Japan), and bovine serum albumin (2 mg/ml, Sigma) for 40 min at 37°C. The musculature preparation was then triturated with fire-blunted glass pipettes. The resultant cell clusters were plated onto a lab-made culture dish, and kept in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% foetal bovine serum (Sigma) and antibiotics (30 μg/ml streptomycin and 30 units/ml penicillin; Sigma) at 37°C for 2–3 days. The cultured cell cluster preparations were incubated in ‘normal’ solution containing approximately 8 μM Fluo-3-AM (acetoxymethyll ester of Fluo-3: Dojindo, Kumamoto, Japan) and detergents (0.02% Phloronic F-127: Dojindo; 0.02% cremophor EL, Sigma) for 3–4 h at room temperature. A CCD camera system (Argus HISCA, Hamamatsu Photonics, Hamamatsu, Japan) was used to continuously monitor digital images of Fluo-3 emission light. The cell clusters were illuminated at 488 nm and emission light of 515–565 nm was detected. The temperature of the recording chamber was kept at 35°C using a micro-warm plate system (MP10DM, Kiitazato Supply, Fujimiyama, Japan). Digital images (0.963 μm/pixel) were normally collected at 300 ms intervals. Changes in fluorescence emission intensity (F) were expressed as F/F\(_{0}\) where F\(_{0}\) is the basal fluorescence intensity. Ratio-images were constructed by dividing each Ca\(^{2+}\) image with a Ca\(^{2+}\) image acquired at a basal [Ca\(^{2+}\)] time after subtracting background fluorescence. The frequency of spontaneous [Ca\(^{2+}\)] oscillations in the presence of nifedipine (and TTX) did not differ from that of spontaneous oscillatory inward currents previously measured by the patch clamp technique [20]. We thus judged that the procedure for loading Fluo-3 was appropriate, and the spontaneous [Ca\(^{2+}\)] activity reflected pacemaker activity in ICC. This notion also agrees well with previous reports in which dihydropyridine (DHP) Ca\(^{2+}\) antagonists selectively suppress [Ca\(^{2+}\)] activity in smooth muscle by blocking L-type Ca\(^{2+}\) channels [13,19].

In preliminary experiments, we checked the effects of several concentrations of 5-HT receptor agonists and antagonists in order to assess which subtypes of 5-HT receptors are responsible for the augmentation of [Ca\(^{2+}\)], oscillations in ICC. First, 5-HT was examined at 1, 3, 10 and 50 μM (n = 3–4). The active area of [Ca\(^{2+}\)], oscillations was nearly the same (102% of the control) at 1–3 μM, and increased to ~145% at 10 μM and to ~149% at 50 μM. We thus compared the effects of 5-HT and other 5-HT receptor agonists at 10 μM (Fig. 1 and 2). LY-278584, a 5-HT\(_{3}\) antagonist, did not significantly suppress [Ca\(^{2+}\)], oscillations below 10 μM in 10 min. In order to minimize the deterioration of fluo-3 fluorescence during illumination, 10 μM of LY-278584 was normally applied (Fig. 2A–B). Also, according to previous experiments in enteric neurones and ICC, 10 μM GR113808 [21] and 40 μM SK&F96365 [17] were used to inhibit 5-HT\(_{4}\) receptors and Ca\(^{2+}\)-permeable transmembrane channels, respectively (Fig. 2C–D).

**Electrical recording**

An 8×8 planar microelectrode array (150 μm in polar distance) connected to a 64-channel amplifier (MED 64 System: Alpha Med Science, Osaka, Japan) was used to simultaneously record electrical field potentials of ~1 mm\(^2\) square [22,23]. Ileal muscle segments (~5 mm×20 mm) containing the myenteric plexus were fixed using a brain slice anchor (SDH series, Harvard Apparatus, MA, USA) in order to isolate ICC pacemaker activity by suppressing smooth muscle and neural activities. Slow electrical potentials were recorded by applying a high-pass filter of 0.1 Hz to stabilize the baseline drift [24]. A sampling rate of 20 kHz was applied.

In field potential data processing, the digital resolution was reduced to 50 ms (20 Hz) by thinning out the recording points at 1:1000, and an FFT-based digital band-pass filter (0.04–0.5 Hz) was additionally applied. The effects of 5-HT and 2-Me-5-HT on ICC pacemaker activity were evaluated using a power spectrum (9.4–27.0 cm\(^2\)). Two-dimensional field potential images were constructed by calculating the values at the desired location via spline interpolation (with 50 points between each electrode), using the MATLAB software package (Mathworks: Natick, MA, USA) [25].

**RT-PCR**

A longer (50 min) enzymatic incubation and more complete trituration with glass pipettes were performed to obtain isolated cells. The digestive enzymes used were the same as described for cell cluster preparations. The resultant cell suspension was incubated with 10–20 μM nifedipine (Sigma) and TTX (LKT Laboratories: St Paul, MN, USA) in order to isolate ICC pacemaker activity by suppressing smooth muscle and neural activities. Slow electrical potentials were recorded by applying a high-pass filter of 0.1 Hz to stabilize the baseline drift [24]. A sampling rate of 20 kHz was applied.

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The following PCR primers were used: 5-HT3A (NM_013561, 363-463, amplicon = 101 bp): 5'-GATGAAACCGTGGCATGATCAGGAG-3' (s) and 5'-CGCCATCGTTTTCAGTGAGA-3' (c); 5-HT3B (NM_020274, 607-728, amplicon = 122 bp): 5'-ACTTCCCAGCAGTGGCAGAGATTG-3' (s) and 5'-GAGTCCGGAGGTCCTGCAATG-3' (c); 5-HT4A (NM_008313, 1100-1222, amplicon = 123 bp): 5'-GTTGTCCGTGGCTTGGCTATCA-3' (s) and 5'-GTGTTTTGTC-
AGCGCTCATCATCAC-3; c-Kit (Y00864, 2156-2256, amplicon = 101 bp): 5-GAGCCTTCCTGTGACAGTTCAAA-3 (+) and 5-TCTATTCTTGCGGATCTCCTCTTG-3 (-); glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (M32599, 730–833, amplicon = 104 bp): 5-CATGGCCTTCCGTGTTCCT-3 (+) and 5-CCTGCTTCACCACCTTCTTGA-3 (-); CD68 (a marker for mast cells) (NM_009853, 574-830, amplicon = 257 bp): 5-CACCTGTCTCTCTCATTTCC-3 (+) and 5-CTTAGAGAGAGCAGGTGAAG-3 (-); Cma1 (a marker for macrophages) (NM_010780, 332–668, amplicon = 337 bp): 5-CGGGAAGGTCTATAACAGTCCTCC-3 (+) and 5-CTGGTGAAGTGTTTGCAGGCT-3 (-). The pair of primers for 5-HT4 was also designed to cover 5-HT4A, 5-HT4B, 5-HT4E, and 5-HT4F.

Immunohistochemistry

Small segments (10 mm × 20 mm) of smooth muscle layers (including the myenteric plexus) isolated from the mouse ileum, were fixed with 4% paraformaldehyde (4°C) for 30 min, and permeabilized with 0.1% triton X-100 and 5% BSA (bovine serum albumin, fraction V; Sigma) for 1 h. The tissue was double stained sequentially with anti-5-HT3 antibody [SR-3 (H-138) sc-28958: Santa Cruz Biotechnology, Santa Cruz, CA, USA] and anti-mouse CD117 (c-Kit) antibody (ACK4: Acris antibodies, Germany) in 100 mM PBS (phosphate buffered-saline solution) overnight. The PBS contained 1% BSA in order to block nonspecific reactions. This was followed by incubation with secondary antibodies, Alexa Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 555-conjugated anti-rat IgG (Molecular Probes, Eugene, OR, USA) at a concentration of 15 μg/ml for 1 h. Double-stained small segments were mounted on a slide glass with an anti-fading agent (ProLong: Molecular Probes) and scanned using a confocal microscope (TCS-SP2: Leica Microsystems, Tokyo, Japan). Controls were prepared by omitting the primary antibodies. The reactivity was negligible in network-forming cells in the myenteric plexus (i.e. ICC). The antibody used for staining the 5-HT3 receptor (sc-28958: Santa Cruz Biotechnology) is a rabbit polyclonal antibody raised against amino acids 341–478 mapping at the C-terminus of 5-HT3A of human origin, and is used in human, mouse and rat specimens.

Solutions and drugs

The composition of the ‘normal’ extracellular solution used in [Ca2+]i imaging and electrical recording was (in mM): NaCl, 125; KCl, 5.9; MgCl2 1.2; CaCl2 2.4; glucose 11; Tris-HEPES 11.8 (pH 7.4). Nifedipine, LY-278584, 2-methyl-5-HT (maleate salt), GR113808 and O-methyl-5-HT (hydrochloride) were purchased from Sigma. SK&F96365 was from Calbiochem (San Diego, CA, U.S.A.). Stock solutions of nifedipine were prepared by dissolving the drug in ethanol, while other drugs were dissolved in dimethyl sulfoxide (DMSO). The working concentrations of the solvents were less than 1%. In preliminary experiments, we observed that applications of this concentration of either solvent alone had little effect on [Ca2+]i oscillation in cell cluster preparations. DMEM and other reagents for cell culture were purchased from Sigma.

Statistics

Numerical data are expressed as mean±SD. Differences between means were evaluated by paired t-tests. The probability (P<0.05) was taken as a statistically significant difference.
Results

5-HT augments \([\text{Ca}^{2+}]\), activity in ICC

ICC generate pacemaker activity employing \([\text{Ca}^{2+}]\) oscillations \([13,14]\). To examine the effect of 5-HT on \([\text{Ca}^{2+}]\) activity in ICC, we used cell cluster preparations (100–200 μm in diameter) obtained from the mouse ileum. This preparation contains ICC, smooth muscle cells and enteric neurons, and is considered to be an integrated model system to investigate gut pacemaker activity. Dihydropyridine (DHP) \([\text{Ca}^{2+}]\) antagonists can selectively suppress \([\text{Ca}^{2+}]\), and contractile activities of smooth muscle cells by blocking L-type \([\text{Ca}^{2+}]\) channels, while pacemaking \([\text{Ca}^{2+}]\),
oscillations in ICC persist [19]. We therefore carried out [Ca^{2+}]_i imaging in the presence of nifedipine.

A typical effect of 5-HT is shown in Fig. 1A. In the continuous presence of nifedipine (1 mM), spontaneous [Ca^{2+}]_i oscillations were observed in a small limited area (near (x)) ([control: Panels (a–b) in Fig. 1A]. Application of 5-HT (10 μM) enlarged the region showing spontaneous [Ca^{2+}]_i oscillations [Panels (c–d)]. The time course of changes in [Ca^{2+}]_i (F/F_0) is plotted in Fig. 1B. In control conditions (a), only the region of interest (ROI) (x) shows [Ca^{2+}]_i oscillations with an amplitude of more than 1.2 (F/F_0), while such activity was negligible in ROI (y) and (z). After application of 5-HT, the area yielding spontaneous [Ca^{2+}]_i oscillations enlarged, covering all three regions (x–z) synchronously. The peak amplitude in ROI (y) reached 80–90% of that in (x). On average, the area yielding [Ca^{2+}]_i oscillations increased from 11.5±6.0 to 42.5±10.3% (n = 22), while the frequency was little affected (19.4±1.2 in normal vs. 19.5±1.7 cycles/min in the presence of 5-HT, n = 22) (Fig. 1C–E). Essentially similar effects of 5-HT were observed even in the presence of TTX as well as nifedipine (n = 5), suggesting that 5-HT augmented ICC pacemaker activity, but not through neural activity.

An interesting response was observed in preparations showing intermittent [Ca^{2+}]_i oscillations. 5-HT (10 μM) caused the activity towards a continuous oscillation pattern, with a significant enlargement of the oscillation active area (n = 6) (Figure S2).

When [Ca^{2+}]_i imaging was carried out in ileal cell cluster preparations from W/Wv mice, which have few pacemaking interstitial cells in this part of the small intestine (Fig. 1F–G) [27–29], no [Ca^{2+}]_i oscillation was observed irrespective of 5-HT application (in the presence of 1 μM nifedipine, n = 4) (Fig. 1H). This result confirms that ICC are responsible for 5-HT-mediated enhancement in normal mice.

5-HT receptor agonists and antagonists

Next, we assessed which receptors are responsible for 5-HT-mediated pacemaker [Ca^{2+}]_i activity. In these experiments, we used preparations showing synchronous [Ca^{2+}]_i oscillations (like the preparation shown in Fig. 1). Application of LY-278584 (10 μM), an antagonist for type 3 5-HT receptors (5-HT_3), significantly decreased the amplitude of the oscillations, and the intervals between them often became irregular (middle traces in Fig. 2A–B). Almost complete recovery was achieved by additional application of 10 μM 2-methyl-5-HT (2-Me-5-HT), a selective agonist for 5-HT_3 receptors (F/F_0: 1.27±0.06 in control vs 1.25±0.06 with 2-Me-5-HT, n = 5) (the right trace in Fig. 2A). Also, in order to confirm the target cells, 2-Me-5-HT (10 μM) was applied in cell cluster preparations from W/Wv mice, but no [Ca^{2+}]_i oscillation was induced (n = 5).

O-methyl-5-HT (O-Me-5-HT) is a known non-selective 5-HT receptor agonist, but lacking affinity for 5-HT_3 receptors. Application of O-Me-5-HT (10 μM) caused no recovery of spontaneous [Ca^{2+}]_i activity in the presence of LY-278584 (10 μM)(n = 4, Fig. 2B). Essentially the same results as shown in Fig. 2A–B were obtained in the presence of TTX (250 nM), indicating that there is no contribution of neural activity: Namely these drugs work through action potential-independent mechanisms.

In contrast to the effects of 5-HT_3 antagonists, application of 10 μM GR113808, a selective antagonist for 5-HT_4, had little effect on spontaneous [Ca^{2+}]_i oscillations (like [Ca^{2+}]_i activity in ICC is generated by endogenous 5-HT via 5-HT_3 receptors. 5-HT influx sensitive to SK&F96365 plays an essential role in generating ICC pacemaker [Ca^{2+}]_i activity, presumably coordinating with intracellular Ca^{2+} release channels [16,17]. In order to assess whether this Ca^{2+} influx pathway contributes to the pacemaker activity mediated via 5-HT_4 receptors, the effect of 2-Me-5-HT was examined in the presence of SK&F96365. Application of 40 μM SK&F96365 suppressed pacemaker [Ca^{2+}]_i activity in ICC as observed previously [17], but subsequent

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Figure 3. RT-PCR examinations for 5-HT receptors. A) RNA samples were obtained from isolated c-kit-immunopositive cells (ICCs) or smooth muscle cells (SMCs). NTC represents ‘no template control’. RT-PCR was performed for 5-HT_2b, 5-HT_3a, c-kit and GAPDH (an index of proper amplification). Numbers on the right of each gel indicate the size marker (bp). B) Examples of RT-PCR detection of mast cell markers (CD68 and Cma1) in spleen (35 cycles) and c-kit-immuopositive cells (50 cycles). doi:10.1371/journal.pone.0024928.g003
application of 2-Me-5-HT caused no recovery (n = 5) (Fig. 2D). The responses of pacemaker [Ca\textsuperscript{2+}] activity to the drugs relating to 5-HT receptors and Ca\textsuperscript{2+} influx are summarized in Fig. 2E.

5-HT\textsubscript{3} receptors in ICC

To confirm expression of 5-HT receptors in ileal ICC, we performed RT-PCR. After enzymatic dispersion, c-Kit immunopositive interstitial cells (equivalent to ICC) and smooth muscle cells were individually collected. A transcript for 5-HT\textsubscript{3A} was detected only in ICC, while 5-HT\textsubscript{2B} was detected in both ICC and smooth muscle cells (Fig. 3A). We also confirmed that neither CD68 nor Cma1 (mast cell markers) was detected in c-Kit-immunopositive interstitial cells (n = 4), unlike in spleen samples (n = 4) (Fig. 3B). In addition, transcripts for 5-HT\textsubscript{3B} and 5-HT\textsubscript{4} were detected in ICC, but not in smooth muscle cells (not shown).

Immunohistochemistry was also performed. Double-labelled immunostaining with anti-c-Kit and anti-5-HT\textsubscript{3} antibodies (Fig. 4) revealed that network-forming interstitial cells expressing both 5-HT\textsubscript{3} and c-Kit (orange cells in left panel) exist near the myenteric plexus, which contains neurons with only 5-HT\textsubscript{3} immunoreactivity (green). In addition, these network-forming cells have large nuclei (a single cell is shown expanded in the right panels). The network-like structure and large nuclei are known features of ICCs [2,6].

Augmentation of electrical activity

In order to confirm the excitatory effect of 5-HT on ICC pacemaker activity, we next measured electrical activity. Isolated musculature of the mouse ileum was placed on an 8 x 8 microelectrode array (MEA) with a polar distance of 150 μm, and field potentials of a ~1 mm\textsuperscript{2} area were simultaneously monitored through a multi-channel amplifier and recording system (See Materials and Methods). To suppress smooth muscle and neural activities, extracellular solution contained nifedipine (1 μM) and TTX (250 nM), respectively.

A potential map was constructed by using the simultaneous recordings at 64 channels and spline interpolation (Fig. 5A–B). In this preparation, ICC electrical activity propagated from the left bottom to the right top in normal solution (A), and application of 5-HT (10 μM) potentiated ICC electrical activity (B). Representative field potentials of three channels (Fig. 5C–D) show ICC electrical activities for a long duration. 5-HT significantly increased the amplitude of the field potentials, but did not alter the frequency (Video S3 and Video S4). Also, power spectra over the recording area were constructed from field potentials of all 64 channels for approximately 40 s. This analysis shows a marked increase in the spectral power without shifting the peak frequency.

The effect of 2-Me-5-HT (10 μM), a 5-HT\textsubscript{3} receptor agonist (Figure S3), on ICC electrical activity was also examined. Essentially similar enhancement was observed. Graphs in Fig. 6 summarise the enhancement of 5-HT and 2-Me-5-HT on ICC electrical activity. The spectral power between 9.4-27.0CPM (P\textsubscript{9.4-27.0CPM}) was used to evaluate the ICC electrical activity, based on a comparison between wild-type and W/W\textsuperscript{v} mice [25]. In the control of normal ileum, P\textsubscript{9.4-27.0CPM} (from 64 channels) ranged from 1.0 to 19.0 x 10\textsuperscript{-3} μV\textsuperscript{2} (n = 24). Application of 5-HT increased P\textsubscript{9.4-27.0CPM} to 168 ± 45% (P<0.01, n = 12), and 2-Me-5-HT increased it to 153 ± 53% (P<0.01, n = 12).

Discussion

ICC act as gut pacemaker cells. Moreover, fairly recent studies suggest that these cells also coordinate peristaltic movements through their network-forming structure [30]. In the light of the important roles of ICC, any hormones and neurotransmitters that modulate ICC activity are considered to have a significant influence upon gut motility. The present finding that 5-HT augments ICC activity implies that this signalling molecule in particular plays a crucial role in regulating gut motility, because the gut contains a majority of 5-HT in the body [8,11].

The fact that 2-Me-5-HT causes similar effects to 5-HT implies that 5-HT\textsubscript{3} receptors are responsible for the 5-HT-mediated enhancement of ICC Ca\textsuperscript{2+} activity. Lines of studies have suggested that the primary pacemaking activity is the spontaneous [Ca\textsuperscript{2+}]i oscillations in ICC [13,15,31]. Namely, [Ca\textsuperscript{2+}]i oscillations in ICC periodically activate plasmalemmal Ca\textsuperscript{2+}-activated ion channels: Ca\textsuperscript{2+}-activated Cl\textsuperscript{–} channels [32–35], and/or Ca\textsuperscript{2+}-activated non-selective cation channels [36–38]. Ca\textsuperscript{2+} release from the intracellular Ca\textsuperscript{2+} stores, presumably endoplasmic reticulum (ER), appears to be the major Ca\textsuperscript{2+} source of [Ca\textsuperscript{2+}]i oscillations.
in ICC, while Ca\(^{2+}\) influx from the extracellular space is required to maintain this [Ca\(^{2+}\)]\(_i\) activity (periodic Ca\(^{2+}\) release) [13], although co-ordinating mechanisms for these Ca\(^{2+}\) pathways are not yet understood. Since 5-HT\(_3\) receptors are ligand-gated non-selective cation channels, permeable to Ca\(^{2+}\) [39,40], this channel is likely to act as a Ca\(^{2+}\) influx pathway to enhance pacemaker activity in ICC (Figure S4). This notion is supported by the fact that SK&F96365, which is known to block a broad range of Ca\(^{2+}\)-permeable non-selective cation channels [41–43], terminates ICC pacemaker Ca\(^{2+}\) activity even in the presence of 2-Me-5-HT (Fig. 2D). Also, ICC is known to express DHP-insensitive voltage-gated Ca\(^{2+}\)-permeable channels [44,45]. This transmembrane

![Figure 5. MEA measurements of ICC electrical activity in the presence of nifedipine (1 μM) and TTX (250 nM). A-B) Field potential images constructed at 200 ms. The top and bottom of the images correspond to the oral and anal ends of the preparation, respectively. C-D) Representative field potential recordings at three channels in the same preparation. The dotted lines indicate the period of the potential images (PI) acquired. E-F) Power spectra constructed from field potential recordings at all 64 channels. A, C and E: control; B, D and F: during application of 5-HT. doi:10.1371/journal.pone.0024928.g005](https://doi.org/10.1371/journal.pone.0024928.g005)
Ca\(^{2+}\) pathway may simultaneously contribute to the enhancement of ICC [Ca\(^{2+}\)], activity.

Five subunits (i.e. 5-HT\(_{3A-E}\)) are known to form the 5-HT\(_3\) receptor complex, and changes in the composition alter Ca\(^{2+}\)-permeability of this channel [46]. In future studies, it would be of interest to elucidate the composition of 5-HT\(_{3A-E}\) receptor subtypes and how 5-HT\(_3\) receptors are coupled to Ca\(^{2+}\) release channels in intracellular Ca\(^{2+}\) stores to generate pacemaker [Ca\(^{2+}\)]\(_i\) activity. Polymorphism of these 5-HT\(_3\) receptor subunit genes seems likely to affect gut motility by modulating ICC as well as neuronal activities [12], and underlies some functional disorders [47]. Furthermore, RT-PCR examinations detected transcripts of other 5-HT receptor genes in ICC i.e. 5-HT\(_2\) and 5-HT\(_4\) receptor genes [48,49]. Recent studies have shown that 5-HT\(_{3}\) receptor antagonists reduced proliferation of cultured ICC, and that the small intestine of mice lacking 5-HT\(_{2B}\) receptors contains less ICC in the myenteric and deep muscular plexuses, although intestinal transit is not significantly slowed [50,51]. On the other hand, 5-HT\(_3\) receptor antagonists impair the regeneration of enteric neurons after surgical operation and their development in gut-like organs derived from mouse embryonic stem cells, with indistinguishable changes in the ICC network [21,52]. It is likely that 5-HT causes numerous effects via these different 5-HT receptors, depending on cell type, location of the gut, and the stage of development and aging.

The scenario for 5-HT augmentation of ICC activity is possibly modified by the roles of adjacent cells in the actual gut. In the present study, we applied nifedipine and TTX to clearly demonstrate the effect of 5-HT on ICC; however, smooth muscle cells and enteric neurons suppressed by these inhibitors may also be involved, because coordinated actions of these cells produce gut motility [4]. For example, as seen in Fig. 5, ICC pacemaker activity propagates on the luminal plane. Indeed, electric conduction of gut pacemaker activity along the musculature can be detected magnetically [53]. It is thought that ICC and smooth muscle cells are electrically connected [54]. Therefore, under normal conditions (without DHP Ca\(^{2+}\) antagonists), in addition to network-forming processes in ICC, the smooth muscle bundle conducts a part of electric current generated by a group of ICC, and amplifies pacemaker activity in adjacent ICC, because it is thought that ICC possess a mechanism to transform depolarisation in the plasma membrane into activation of [Ca\(^{2+}\)], oscillations for pacemaking [55,56]. Also, some populations of enteric neurones may release activators for ICC pacemaker activity in response to 5-HT. In the myenteric plexus, serotonergic neurones are involved in descending contraction [9,10], and it is known that ICC express numerous receptors for excitatory neurotransmission, e.g. purinoceptors, neuromedin and acetylcholine receptors [57–60]. These molecules may access ICC to activate in parallel.

The present finding on augmentation of ICC activity via 5-HT\(_3\) receptors implies pharmacological interventions on gut motility disorders. For example, irritable bowel syndrome, classified into two types, i.e. diarrhoea- and constipation-dominant IBS (d-IBS and c-IBS), is known to involve 5-HT-related mechanisms along with infectious and inflammatory changes. Excess 5-HT due to impairment of reuptake transport is ascribed to some populations of d-IBS [61–63]. Also, antineoplastic drugs, e.g. cisplatinum, stimulate 5-HT release [64–66]. In such cases, it is rational to suppress ICC pacemaker activity in addition to nervous activities by blocking 5-HT\(_3\) receptors. It is speculated that stimulation of 5-HT\(_3\) receptors in enteric neurons and ICC synergically facilitates gut contractility and afferent neural activity toward the brain. Thereby, 5-HT\(_3\) receptors in the gut may contribute to the gut-brain axis. As seen in murine ileal ICC, we have also observed that 5-HT\(_3\) receptor agonists potentiate, while antagonists suppress both Ga\(^{3+}\) and electric pacemaker activities in the murine stomach in preliminary experiments. Although extensive studies are required in model animals and humans, 5-HT is likely to enhance ICC pacemaker activity throughout the gastrointestinal tract.

Similar regulatory mechanisms may underlie other peripheral spontaneous activities. Evidence is being accumulated that ICC-like interstitial cells ubiquitously exist in many organs and tissues that are effectors of the autonomic nervous system, such as the ureter, urinary bladder, urethra, uterus, lymph ducts, veins, etc, suggesting their possible contribution to spontaneous activity [67–71]. Also, in some ICC-like cells, spontaneous [Ca\(^{2+}\)] and electric activities have already been demonstrated. In the light of regulatory mechanisms of ICC and ICC-like cells, investigating functional disorders related to a wide range of peripheral spontaneous rhythmicity, e.g. irritable bladder, is merited.

In summary, the present study has shown 5-HT augmentation of ICC pacemaker activity via 5-HT\(_3\) receptors. Since 5-HT\(_3\) receptors are Ca\(^{2+}\)-permeable nonselective cation channels, this effect on ICC activity is presumably through enhancement of Ca\(^{2+}\) influx from the extracellular space, through itself and simultaneous activation of voltage-gated Ca\(^{2+}\)-permeable channels. ICC appear to be a promising target in functional motility disorders in the gut.

**Supporting Information**

**Figure S1** An example of 5-HT-augmentation of ICC pacemaker [Ca\(^{2+}\)], oscillations in an ileal musculature preparation. Ileal musculature segments (~5 mm×20 mm) containing the myenteric plexus, the same preparation used in 8 MEA measurements, were loaded with Fluo-3AM. Fluorescence images were continuously monitored the same as cell cluster experiments. The extracellular solution contained 1 μM nifedipine and 250 nM TTX. A) A control fluorescent image. B and C) Series of ratio images of a [Ca\(^{2+}\)], oscillation cycle in control and in the presence of 5-HT (10 μM), respectively. Each image was acquired at 300 ms intervals. Video S1 and Video S2 correspond to ratio images shown in B and C, respectively. Note that green represents the F\(_i\)/F\(_0\) ratio of ~1 in cell cluster preparations (Fig. 1A) to
display the size of the preparation, while black represents the ratio of ~1 in muscle preparations, because the size of preparations were larger than the frame of the image. After 5-HT application the active area markedly increased.

**Figure S2** Regular occurrence of pacemaker [Ca$^{2+}$]i oscillations after application of 5-HT. A) Ca$^{2+}$ images acquired from a cell cluster preparation in control (a–c) and 5 min after 5-HT (10 µM) application (d, e). This preparation showed intermittent [Ca$^{2+}$]i oscillations in control condition. B) Time course of pacemaker [Ca$^{2+}$]i activity recorded in the square (x) indicated in A: control (left) and 5 min after application of 10 µM 5-HT (right). Dotted lines correspond to the times when images (a–e) were acquired. G–E) Bar graphs showing changes in the peak amplitude (C), frequency (D), and active area (E) of spontaneous pacemaker [Ca$^{2+}$]i activity (Mean ± S.D., n = 6).

**Figure S3** An example of the effect of 2-Me-5-HT (10 µM) on field potentials. Field potential images in control (A) and during application of 2-Me-5-HT (B) are displayed at 200 ms intervals. Note, 2-Me-5-HT-enhanced ICC electrical activity, as seen during application of 2-Me-5-HT (B) are displayed at 200 ms intervals. Field potential images in control (A) and during cluster preparation in control (a–c) and 5 min after 5-HT (10 µM) (right). Dotted lines correspond to the times when images (a–e) were acquired. G–E) Bar graphs showing changes in the peak amplitude (C), frequency (D), and active area (E) of spontaneous pacemaker [Ca$^{2+}$]i activity (Mean ± S.D., n = 6).

**Figure S4** Possible underlying mechanisms for 5-HT-enhancement of gut pacemaker activity and contractility. It is thought that intracellular Ca$^{2+}$ release channels, i.e. ryanodine receptors (RyR) and InsP$_3$ receptors (IP$_3$R) in ICC are periodically activated by the support of a Ca$^{2+}$ influx pathway across the plasma membrane, although mechanisms underlying the coordinated actions of intracellular and plasma membrane ion channels are not yet known. 5-HT augments ICC [Ca$^{2+}$]i oscillations presumably (1) by facilitating Ca$^{2+}$ influx via 5-HT$_3$ receptors and (2) simultaneous activation of voltage-gated Ca$^{2+}$-permeable insensitive to DHP Ca$^{2+}$ antagonists (DHP(-)VGCC). In ICC, [Ca$^{2+}$]i oscillations periodically activate Ca$^{2+}$-activated ion channels in the plasma membrane, i.e. Ca$^{2+}$-activated Cl$^{-}$ channels (Cl$_{Ca}$) and/or Ca$^{2+}$-activated nonselective cation channels (NSCC$_{Ca}$), and thereby generate electric pacemaker activity. In smooth muscle (SM) cells, conducted pacemaker activity via gap junction (GJ) channels activates DHP-sensitive voltage-gated Ca$^{2+}$ channels (DHP(+)VGCC), i.e. L-type Ca$^{2+}$ channels, causing periodic contraction. In the present study, to differentiate ICC activity, all experiments were carried out in the presence of a DHP Ca$^{2+}$ antagonist, nifedipine.
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