Role of a Serotonin Precursor in Development of Gut Microvilli

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Monoamines exert diverse functions in various cells in peripheral organs as well as in the central nervous system. 5-Hydroxy-L-tryptophan (5-HTP) has been simply regarded as a precursor of serotonin, and it is believed that the biological significance of 5-HTP is essentially ascribable to the production of serotonin. Systemic treatment with 5-HTP is often applied to patients with low serotonin levels in the brain. Here we show that endogenous and exogenous 5-HTP but not serotonin induced the development of microvilli in the gut villi epithelium. In contrast, serotonin but not 5-HTP regulated phagocytosis by macrophages. 5-HTP specifically induced actin remodeling and decreased phosphorylation of extracellular signal-regulated kinase (ERK) in the gut, whereas serotonin stimulated actin remodeling and increased ERK phosphorylation in macrophages. Functionally, inhibition of ERK activity promoted the development of microvilli in the gut and ameliorated phagocytosis by macrophages. Thus, 5-HTP and serotonin contribute to distinct cell-type-specific functions via common mediators. Our study might create an opportunity to explore the effects of exogenously applied 5-HTP in humans. (*Am J Pathol* 2008, 172:333–344; DOI: 10.2353/ajpath.2008.070358)

The mature small intestinal surface is covered with abundant finger-like villi that project into the luminal space. The apical surface of the mature intestinal epithelium is covered with dense brush border microvilli. The formation of the microvilli is regulated by the organization of individual cell membrane domains and cell:cell junctions via interaction between membrane proteins and actin cytoskeleton.

Serotonin [5-hydroxytryptamine (5-HT)] has been implicated in a number of physiological and pathological

functions in several peripheral organs and tissues such as liver, platelet, and immune systems.^{1–6} An enormous proportion of 5-HT is produced in the enterochromaffin cells and is stored in the platelets that release 5-HT in multiple peripheral organs. It has been believed that the released 5-HT essentially exerts its biological effects via 5-HT receptors on various cells in a paracrine manner. There are multiple types of 5-HT receptors that are distributed widely among endocrine, cardiovascular, immune, and gastrointestinal tissues. Receptors for 5-HT fall into one of four distinct families (5-HTR1, 5-HTR2, 5-HTR3, 5-HTR4-7), which are characterized by different signal transduction mechanisms and physiological roles. In the gut, 5-HT plays several physiological roles. For example, 5-HT has been reported to increase the rate at which enterocyte precursors proliferate, and the enhancement of enterocyte proliferation by 5-HT might be mediated by a 5-HT2 receptor.⁷

The biosynthesis of 5-HT is accomplished through multistep enzyme reactions. Tryptophan hydroxylase-1 (TPH-1) and TPH-2 catalyze the formation of 5-hydroxy-L-tryptophan (5-HTP) from L-tryptophan, the first and rate-limiting step in the biosynthesis of 5-HT. Subsequently, 5-HT is produced from 5-HTP by aromatic L-amino acid decarboxylase (AADC). Despite the frequent use of 5-HTP to treat metabolic diseases, the biological activities of 5-HTP itself have not been essentially explored. It has been generally assumed that 5-HTP, a 5-HT precursor, is immediately decarboxylated to 5-HT, and consequently little 5-HTP exists in the central nervous system.⁸ However, 5-HTP has been detected biochemically⁹ and 5-HTP immunoreactivity has been visualized in neurons.^{10–12} In addition, a few studies have reported scavenging activity of 5-HTP against reactive oxygen species.^{13,14} These findings raise the possibility that exogenous 5-HTP itself as well as the endogenous 5-HTP produced in the extraneuronal cells function *in vivo*.

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In the present investigation, we found that 5-HTP is produced in the gut epithelial cells, and the endogenous and exogenous 5-HTP contributes to the development of microvilli in the intestine. In contrast, the phagocytic activity of macrophages, which also produce 5-HTP, is not dependent on 5-HTP but on exogenous 5-HT. Either 5-HTP or 5-HT eventuates actin remodeling and ERK in each cell, indicating that 5-HTP and 5-HT dictate cell-type-specific functions through common mediators.

Materials and Methods

Animals

C57BL/6 mice and Wistar rats were obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan). The animals were maintained according to the guidelines of Juntendo University. For pharmacological treatments of animals, *p*-chlorophenylalanine (PCPA) (160 mg/kg, i.p.; Sigma, St. Louis, MO), NSD-1015 (100 mg/kg, i.p.; Nacalai, Kyoto, Japan), and 5-HTP (40 mg/kg, i.p.; Wako, Tokyo, Japan) were injected once a day for 5 days. Reagents were dissolved in 0.9% physiological saline, and the volume of injection was 10 ml/kg. The dose of PCPA (160 mg/kg) was determined essentially according to previous reports,^{15,16} in which 150 mg/kg or 160 mg/kg PCPA was injected into rats. To obtain the intestine, spleen, and peritoneal macrophages, animals were anesthetized with sodium pentobarbital.

Preparation of Anti-TPH-1 and Anti-TPH-2 Antisera

Polyclonal antisera against rat TPH-1 (67-83) and rat TPH-2 (45-58) were raised. The regions close to the N terminus were chosen to be the least homologous between the TPH isoforms and the best conserved among rat, mouse, and human. The corresponding peptides with an additional N-acetylated cysteine at the N terminus were synthesized (Sigma Genosys, Tokyo, Japan) and named pepTPH1 (C+DINREQLNDIFPLLKSH) and pepTPH2 (C+EDKRSGKDTSESSK). They were cross linked to keyhole limpet hemocyanin (KLH) as the hapten carrier protein using sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC; Pierce, Rockford, IL). Animals were immunized with the conjugates pepTPH1-KLH (rabbits and guinea pigs) and pepTPH2-KLH (rabbits). The raised antisera were subjected to affinity purification by the hapten-bound thiopropyl-Sepharose 6B according to the manufacturer's instructions (GE Health Care Bio-Sciences Corp., Piscataway, NJ).

Cell Culture

The human colon adenocarcinoma cell line, Caco-2, was obtained from Dr. Makoto Shimizu, School of Agricultural Life Sciences, The University of Tokyo. Caco-2 cells were cultured on collagen-coated plates in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 4

mmol/L L-glutamine, and a 1% nonessential amino acids solution, at 37°C under 5% CO₂/95% air. Peritoneal macrophages were collected from the peritoneal fluid of C57BL/6 mice 48 hours after intraperitoneal injection of thioglycollate and were cultured in RPMI 1640 medium with 10% fetal calf serum. Cells were treated with 100 μmol/L PCPA, 100 μmol/L 5-HTP, 100 μmol/L serotonin (Sigma), 200 μmol/L NSD-1015, and 10 μmol/L PD98059 (Calbiochem, San Diego, CA).

Determination of 5-HT Levels in Caco-2 Cells

Sepiapterin was purchased from Schircks Laboratory (Jona, Switzerland). The Caco-2 cells were plated on collagen-coated 96-well analytical culture plates (Falcon 3072; BD Biosciences, San Jose, CA) at 5×10^4 cells/well with 200 μl of ordinary culture medium.¹⁷ Under these conditions, cells reached apparent confluence within 2 days, and then the sheet became tighter for the next several days, suggesting that the cells underwent differentiation or maturation. They gradually expressed their characteristic shape and function as the intestinal epithelium. In this experiment, rather immature cells were used 2 to 6 days after plating. The determination of 5-HT and tryptophan levels was performed as described using high performance liquid chromatography with a fluorescence detector^{18,19} capable of quantifying ~40 fmol of 5-HT per application.

Immunostaining

For the immunohistochemistry of the intestine, spleen, and brain, the dissected organs were embedded in OCT compound, and sections (5 μm in thickness) prepared using cryostat were fixed with acetone. Occasionally, the intestine and brain were paraffin-embedded and sections were prepared using a microtome. For immunostaining of antibodies for TPH-1, TPH-2, 5-HTP,¹² and 5-HT (Chemicon, Temecula, CA), sections were treated with 0.3% hydrogen peroxide and blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.05% Tween 20 (BSA-PBST). After three washes with PBS, the sections were incubated with these primary antibodies in BSA-PBST for an hour at room temperature, and were washed three times with PBS. Then, horseradish peroxidase-conjugated anti-rabbit or -rat secondary antibody (DAKO, Carpinteria, CA) in BSA-PBST was applied for an hour. After three washes with PBS, the signals were visualized with diaminobenzidine. Occasionally, the nuclei were counterstained with hematoxylin.

To accomplish single immunofluorescence labeling with Oregon Green 488-labeled phalloidin (Molecular Probes, Eugene, OR), Alexa Fluor 647-labeled phalloidin (Molecular Probes), 5-HTP, 5-HT, TPH-1, and TPH-2, and double-immunofluorescence labeling of the intestine or spleen with anti-TPH-1 antibody together with Alexa Fluor 647-labeled phalloidin (Molecular Probes) or Alexa Fluor 647 or Alexa Fluor 546 (Molecular Probes)-conjugated F4/80 antibody (Dainippon Sumitomo Pharma, Osaka, Japan), sections were fixed with acetone or 4% parafor-

maldehyde in 0.1 mol/L phosphate buffer, pH 7.4, and blocked with BSA-PBST. Occasionally, the sections were incubated with the primary antibodies followed by Cy2-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) or Cy3-conjugated anti-rat IgG (Jackson ImmunoResearch). Each step was followed by three washes with PBS. For dual labeling with guinea pig anti-TPH-1 and rabbit anti-EBP50 (Affinity BioReagents, Golden, CO) antibodies, Cy2-conjugated anti-rabbit (Jackson ImmunoResearch) and Cy5-conjugated anti-guinea pig (Jackson ImmunoResearch) secondary antibodies were used. Occasionally, the nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Labeled cells were visualized with a confocal microscope (LSM510; Zeiss, Thornwood, NY).

Western Blot Analysis

Total cell lysate was obtained essentially as described.²⁰ For the detection of TPH-1 and TPH-2, the GST-rat TPH-1 or GST-rat TPH-2 expression vector (pDEST-GST-TPH-1 or pDEST-GST-TPH-2, gateway system) (Invitrogen, Carlsbad, CA) was transfected into *Escherichia coli*, and the lysate was immunoblotted with the anti-TPH-1 antibody or anti-TPH-2 antibody and horseradish peroxidase-conjugated secondary antibody. The signals were visualized with the enhanced chemiluminescence detection system.

To evaluate the phosphorylation of ERK, Caco-2 cells were cultured for 4 days, and starvation was applied to the cells by eliminating serum from the medium. Then, the cells were stimulated with NSD-1015, NSD-1015 plus 5-HT, or NSD-1015 plus 5-HTP. NSD-1015 was given simultaneously with 5-HT or 5-HTP. Thioglycollate-stimulated peritoneal macrophages were stimulated with 5-HT or 5-HTP. Fifteen and sixty minutes after the stimulation, cells were harvested and Western blotting of the cell lysate was done using anti-phospho-ERK (Cell Signaling, Danvers, MA), anti-ERK (Cell Signaling) and anti-actin primary antibodies. The intensity of the signal of phospho-ERK relative to that of ERK signals was quantified using NIH image (National Institutes of Health, Bethesda, MD).

Phagocytosis

To evaluate the phagocytic activity of splenic macrophages *in vivo*, Cy5 (Molecular Probes)-labeled *Saccharomyces cerevisiae* zymosan (2 mg, Molecular Probes) was intravenously injected into mice. Forty-eight hours after the injection, the spleens were embedded in OCT compound, and the sections (5 μ m in thickness) were prepared using a cryostat. Cy5-positive zymosan captured by splenic macrophages was observed using a confocal microscope (Zeiss LSM510).

Phagocytosis of zymosan by peritoneal macrophages *in vitro* was estimated essentially as described.²¹ The peritoneal macrophages were cultured for 24 hours, and Cy5.5 (Molecular Probes)-labeled zymosan was added to the macrophages for an hour in a final concentration of 100 μ g/ml. The number of zymosan particles in a cell was

quantitatively determined. The sections were co-stained with DAPI.

Isolation of Rat Intestinal Brush Borders

Brush borders were isolated from rat intestinal mucosa essentially according to Fujita and colleagues.^{22,23} The method was based on the fact that brush borders remain intact through careful homogenization of the mucosa under isotonic conditions, and they were sedimented faster than nuclei by mild centrifugation. The small intestine was removed from a rat, opened longitudinally, spread over, and blotted three times on filter paper to clean it thoroughly. Then the mucosae were collected by scraping with the edge of a slide glass. The mucosae were carefully homogenized with 30 strokes of a loose-fitted Dounce-type glass-Teflon homogenizer in 0.25 mol/L sucrose containing 0.5 mmol/L ethylenediaminetetraacetic acid and 5 mmol/L imidazole/HCl buffer (pH 7.0), followed by brief centrifugation at $100 \times g$ for 5 minutes. Taking the sedimented portion, the homogenization and centrifugation were repeated (five to six times) with similar strokes (~ 20) and a stepwise increase in spinning force (up to $200 \times g$) until pure brush borders were isolated. The brush borders paired like a mouth-lip with the inner surfaces attached together. The purified brush borders were kept in an ice-cold 0.25 mol/L sucrose solution until immunostaining and optical examination.

Electron Microscopy

Caco-2 cells were cultured for 7 days. Caco-2 cells and mouse intestine were fixed with 2% glutaraldehyde, 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4. Scanning and transmission electron microscopic analyses were performed using a S-800 hrSEM (Hitachi, Tokyo, Japan) and 1200EX electron microscope (JEOL, Tokyo, Japan) as previously reported.²⁴ The number of microvilli was measured from at least five separate SEM images.

Statistics

Statistical significance was set at $P < 0.05$ using Student's *t*-test or analysis of variance.

Results

TPHs and 5-HTP in the Epithelial Cells of the Intestine

Cells that produce 5-HTP might efficiently take up exogenous 5-HTP and keep it stably inside. To determine the extraneuronal cells expressing TPH-1 and/or TPH-2, we generated specific antibodies against rat TPH-1 and TPH-2. TPH-1 and TPH-2 display high sequence homology and have high sequence identity within the C-terminal catalytic domain.²⁵ Antibodies were raised against the regions close to the N terminus of TPH-1 and TPH-2,

Figure 1. Production of anti-TPH-1 and anti-TPH-2 antibodies. **A:** Amino acid sequences of rat TPH-1 and TPH-2 were compared. To raise specific polyclonal antisera, regions with the least homology between TPH-1 (**top**) and TPH-2 (**bottom**) were searched for. Based on the comparison, DINREQLNDIFPLKSH (**blue line**) and EDKRSKGKDTSESSK (**red line**) were synthesized as the specific hapten of TPH-1 and TPH-2, respectively. **Asterisks** indicate identical amino acid sequences. **B:** Western blotting of the protein from *E. coli* transfected with the GST-rat TPH-1 or TPH-2 expression vector with anti-TPH-1 or anti-TPH-2 antibody. **C:** Immunofluorescence staining of the rat raphe with anti-TPH-1 and anti-TPH-2 antibodies (green). Scale bars = 50 μ m.

the least homologous region between TPH-1 and TPH-2 and the best conserved region among rats, mice, and humans (Figure 1A). The specificity of each antibody was confirmed by the following experiments. First, the protein from *E. coli* transfected with the GST-rat TPH-1 expression vector was immunoblotted with the anti-TPH-1 antibody or anti-TPH-2 antibody (Figure 1B). The anti-TPH-1 antibody recognized the TPH-1 fusion protein with a predicted molecular weight of 80.3 kDa, whereas the anti-TPH-2 antibody did not. Conversely, only the anti-TPH-2 antibody recognized the TPH-2 fusion protein (84.8 kDa). TPH-1 and TPH-2 are strongly expressed in the periphery and brain, respectively.²⁵ Second, we used immunohistochemistry to show that anti-TPH-1 and anti-TPH-2 antibodies stained the intestine (Figure 2A) and the raphe nuclei in the brain (Figure 1C), respectively.

Enormous amounts of 5-HT are produced in the enterochromaffin cells and mast cells and stored in the platelets. The expression of TPHs was, therefore, expected to be confined to enterochromaffin cells and mast

cells in the intestine. Unexpectedly, TPH-1 was also found in other cells. TPH-1 immunoreactivity was found in the epithelial cells of the mucosa (Figure 2A). In addition, TPH-1 was also detected in the muscle and submucosal layers and hematopoietic cells in lamina propria including F4/80-positive macrophages (Figure 2B). The distribution of TPH-1 inside the cells was not uniform. TPH-1 was abundant on the apical side of the villus epithelium as well as in other cytoplasmic regions (Figure 2A, arrow in magnified right panel). TPH-2 showed a more restricted distribution and weaker expression than TPH-1 in the intestine. TPH-2 was also detected on the apical side of villus epithelium (Figure 2A). The localization of TPH-1 and TPH-2 to the apical side of villus epithelium was apparent in rats at embryonic day 20 (Figure 2A, arrows). TPHs were found in the brush border in the gut epithelial cells, by immunohistochemistry with the isolated brush border fraction (Figure 2C). The distribution of endogenous 5-HT overlapped with that of TPHs on the apical side of the villus epithelium (Figure 2D), as evidenced by immunohistochemistry of rat intestine with anti-5-HT antibody.¹² Exogenous 5-HT was also taken up into the gut epithelial cells because the addition of 5-HT resulted in enhanced immunoreactivity for 5-HT in gut epithelial Caco-2 cells when NSD-1015, an AADC inhibitor, was applied (Figures 2E and 4A).

Immunoreactivity for 5-HT was not obtained in the intestinal epithelial cells expressing TPH-1 and TPH-2 (Figure 2D) except for enterochromaffin cells, although the anti-5-HT antibody detected 5-HT in the mucosa of the stomach (unpublished data). Further analysis was performed to detect 5-HT with high performance liquid chromatography. We failed to detect TPH activity in a cell-free enzyme assay using disrupted Caco-2 cells in a complete reaction mixture for 2 hours (data not shown). A prolonged incubation *in vitro* might be required, but it was not practical because the essential co-factor 6R-L-erythro-5,6,7,8-tetrahydrobiopterin (BH4) was not stable enough. Hence the living cells under monolayer culture were used, and *de novo* synthesis of 5-HT was demonstrated as shown in Figure 3. Only a trace amount of 5-HTP, the primary reaction product, was detected (less than 10% of 5-HT, data not shown); most of it might have been converted to 5-HT in Caco-2 cells, which were reported to have AADC activity.²⁶ The following observations suggested that the majority of 5-HT accumulation was dependent on enzymic activity of TPH, although the uptake of 5-HT to a fraction was not excluded from culture additives such as fetal calf serum. The accumulation of 5-HT was significantly inhibited by a classic TPH inhibitor PCPA (Figure 3A). Furthermore, the cellular 5-HT level was dependent on sepiapterin (SP), a precursor of the co-factor BH4 (Figure 3, B and C). However, the amount of 5-HT was very small (~ 0.05 pmol/ μ g); ~ 150 times less than that in RBL2H3 cells (7.6 pmol/ μ g), a 5-HT-producing mast cell line.²⁷

5-HTP Increases the Density of Microvilli

Because TPHs are located in the villi epithelial cells, we attempted to explore the functions of endogenous 5-HTP

Figure 2. Endogenous 5-HTP in the epithelial cells of the intestine. **A:** Immunohistochemistry with anti-TPH-1 and anti-TPH-2 antibodies (brown) in the adult and embryonic day 20 (E20) rat intestine. The nuclei of the adult intestine were counterstained with hematoxylin (blue). **B:** Double-immunofluorescence staining of the lamina propria in the intestine with anti-TPH-1 (green) and anti-F4/80 (red) antibodies. **C:** Immunohistochemistry of the isolated brush border from rat intestine with anti-TPH-1 and -TPH-2 antibodies (brown). **D:** Immunoperoxidase staining of the intestine from adult and E20 rats with anti-5-HTP and anti-5-HT antibodies (brown). The nuclei of the adult intestine were counterstained with hematoxylin (blue). Anti-5-HTP antibody was preabsorbed with 5-HT as previously described.¹² **E:** Immunofluorescence staining of Caco-2 cells with anti-5-HTP antibody (white) after the application of NSD-1015 and 5-HTP plus NSD-1015. **Arrows** indicate the signals of TPHs on the apical side of the epithelial cells. Scale bars = 10 μ m.

Figure 3. 5-HT synthesis in the gut epithelial cells. **A:** 5-HT levels in Caco-2 cells with (closed bars) or without (open bars) PCPA were determined with high performance liquid chromatography. 5-HT content increased by ~ 0.02 pmol/ μ g protein in the cell mainly in the last 2 days in culture. Cells were plated on 96-well analytical culture plate at 2×10^4 cells/well, and adapted for 2 days. Then PCPA was added for 2 or 4 days as indicated. The inhibitory effect of PCPA was significant ($P < 0.05$) on the increase in 5-HT. **B:** Cellular 5-HT levels with (closed bars) or without (open bars) the BH4 precursor sepiapterin (SP). SP was added for 1 or 2 days as indicated after a 4-day adaptation. Only a slight increase in the cellular 5-HT was observed without SP. SP induced a prominent increase in 5-HT content up to 1.95 pmol/ μ g protein (~ 28 -fold within 24 hours). **C:** The effect of SP on the total amount of 5-HT in the medium (open bars) and in the cells (closed bars, taken from **B** and recalculated per well). The cultures were common to those in **B**. The total amount of 5-HT (9.8 ± 2.2 pmol/well, medium plus cell) in the culture with SP was far greater than that in the culture without SP (2.2 ± 1.2 pmol/well, medium plus cell). Average 5-HT concentrations of three experiments are shown. Error bars represent the mean $\pm$ SD. * $P < 0.05$.

as well as exogenously applied 5-HTP using inhibitors of 5-HT synthesis cascade (Figure 4A). Analyses with electron microscopy revealed that the systemic application of 5-HTP increased the density of microvilli in the mouse intestine *in vivo* (135% relative to the control, $P = 0.0001$) (Figure 4B). To exclude the influences of cells other than the epithelial cells, we examined the development of microvilli in Caco-2 cells (Figure 4C). Exogenous 5-HTP, which can enter cells (Figure 2E), increased the density of microvilli (262% relative to the control, $P < 0.0001$), but exogenous 5-HT did not ($P = 0.56$). In addition, when exogenous 5-HTP was applied together with PCPA, the density was also increased ($P < 0.0001$), indicating that exogenous 5-HTP has a role without the effect of endogenous 5-HTP. To confirm the specific role of exogenous 5-HTP in the development of microvilli without the effect of synthesized 5-HT from 5-HTP, we used NSD-1015, an

AADC inhibitor (Figure 4A). NSD-1015 blocks the synthesis of 5-HT and induces the accumulation of 5-HTP (Figure 4A). When 5-HTP was given together with NSD-1015, the density was higher than when NSD-1015 was given alone ($P = 0.0005$). Thus, the effect is dependent on exogenous 5-HTP. We then assessed the roles of endogenous 5-HTP. Application of PCPA resulted in a decrease in the density of microvilli (40% relative to the control, $P = 0.004$). To distinguish 5-HTP from 5-HT, we used NSD-1015. An increase in the density was observed after the application of NSD-1015 ($P = 0.0001$). Furthermore, the addition of PCPA together with NSD-1015 resulted in a lower density than the addition of NSD-1015 alone ($P < 0.0001$). These results suggest a role of endogenous 5-HTP.

Phagocytic Activity of Macrophages Is Not Dependent on 5-HTP but on 5-HT

Given the dominant role of 5-HTP in microvilli development in the intestine, we then tested whether 5-HT and the precursor 5-HTP have differential roles in different types of cells. TPH-1 was detected in macrophages in the lamina propria in the intestine (Figure 2B). In the splenic marginal zone, TPH-1 was expressed in macrophages including F4/80 strong-positive marginal zone macrophages (Figure 5A). In addition, The application of 5-HT induced an increase in macrophage phagocytosis that was blocked by the 5-HT_{1A} receptor antagonist WAY100635,²¹ indicating that exogenous 5-HT contributes to phagocytosis, and the enhancing effect is partly mediated by the 5-HT_{1A} receptor. Thus, macrophages are ideal cells to test the hypothesis. We intravenously injected a fluorescence-labeled *S. cerevisiae* zymosan in mice and evaluated phagocytic activity of macrophages *in vivo*. Forty-eight hours after the injection, fluorescence-labeled zymosan was trapped mainly in the marginal zone. The systemic application of 5-HTP *in vivo* leads to accumulation of both 5-HT and 5-HTP in various cells, and macrophages can respond to 5-HT released from other cells via 5-HT receptors in a paracrine manner. Mice given 5-HTP showed greater phagocytic activity, as evidenced by larger numbers of internalized particles. In contrast, pretreatment with PCPA reduced phagocytic activity (Figure 5B).

Peritoneal macrophages were collected from mice after the stimulation with thioglycollate, an activator of macrophages, and the isolated peritoneal macrophages were found to express TPH-1 and TPH-2 proteins (Figure 5C). Immunofluorescence staining revealed the production of endogenous 5-HTP, and the addition of 5-HTP together with NSD-1015 further enhanced the immunoreactivity for 5-HTP (Figure 5D). Therefore, the phagocytic activity of peritoneal macrophages is suitable for determining whether the activity depends on 5-HTP and/or 5-HT *in vitro*.

Thioglycollate-activated peritoneal macrophages treated with exogenous 5-HTP exhibited essentially a comparable efficiency in the phagocytosis of zymosan ($P = 0.86$) (Figure 5E). To evaluate the actions of endogenous

Figure 4. 5-HTP regulates the formation of microvilli in the intestine. **A:** Schematic representation of the actions of serotonergic inhibitors. **B:** Transmission electron microscopic analysis of the microvilli of intestine in mice treated with 5-HTP. **C:** Scanning electron microscopic analysis of Caco-2 cells treated with 5-HTP, 5-HT, PCPA, 5-HTP plus PCPA, NSD-1015, NSD-1015 plus 5-HTP, and NSD-1015 plus PCPA. The density of microvilli was quantitatively estimated. Analysis of variance revealed a significant difference among the groups [$F(7,76) = 35.0$; $P < 0.0001$]. Post hoc test was also performed to test the significant differences between the two groups of interest. Error bars represent the mean $\pm$ SEM. ** $P < 0.01$. Scale bar = 100 nm.

5-HTP, PCPA was added to the cells. The treatment was ineffective ($P = 0.60$). Thus, the phagocytic activity is not dependent on exogenous and endogenous 5-HTP. Although exogenous 5-HT was effective ($P < 0.0001$) (Figure 5E), the significance of autocrine action of 5-HT could not be obtained.

5-HTP- or 5-HT-Specific F-Actin Expression and ERK Activation

We next examined if 5-HTP in the intestinal epithelial cells and 5-HT in the macrophages induce each effect via common mediators. Actin is abundant on the apical side of the villi epithelium and a major constituent of microvilli, and actin polymerization is involved in phagocytosis by macrophages. When we doubly stained the apical side of the villus epithelium with TPH-1 and phalloidin recognizing F-actin, co-localization was observed (Figure 6A). Ezrin/radixin/moesin (ERM) proteins act as membrane cytoskeleton linkers by binding to the membrane proteins at their NH₂-terminal domains and to F-actin at its COOH-terminal domain.²⁸ ERM-binding phosphoprotein of 50 kDa (EBP-50) also co-localized with TPH-1 (Figure 6B). We then examined the functional link between 5-HTP and actin. Caco-2 cells treated with 5-HTP and NSD-1015 showed an increase in F-actin expression, whereas 5-HT had no effect (Figure 6C). Likewise, the expression of F-actin was enhanced on the apical side in mice treated with

5-HTP together with NSD-1015 (Figure 6D), suggesting an association between 5-HTP and F-actin in the gut. Conversely, 5-HT induced F-actin expression in peritoneal macrophages more efficiently than 5-HTP (Figure 6E).

A second candidate is MAP kinase extracellular signal-regulated kinase (ERK). The Raf-MEK-ERK pathway couples growth factor, mitogenic and extracellular matrix signals to cell fate decisions such as growth, proliferation, migration, differentiation, and survival.²⁹ ERK is expressed in the intestinal brush border.³⁰ Western blotting using anti-phospho-ERK antibody showed stronger expression of phospho-ERK-2 than phospho-ERK-1 in both Caco-2 cells and macrophages (Figure 7A). The phosphorylation of ERK-2 was decreased in Caco-2 cells when 5-HTP was given together with NSD-1015 for 15 and 60 minutes than in cells treated with only NSD-1015 (Figure 7A). The quantitative analysis revealed lower ERK-2 phosphorylation levels relative to the total ERK-2 (41% and 36% relative to the control, respectively). Phospho-ERK-2 levels relative to actin were also lower in cells treated with 5-HTP. Notably, when the same experiment was performed using thioglycollate-stimulated peritoneal macrophages, the phosphorylation levels were efficiently enhanced by the application of 5-HT for 15 and 60 minutes (196% and 152% relative to the control, respectively) rather than 5-HTP (Figure 7A). Again, phospho-ERK-2 levels relative to actin were also higher in cells treated

Figure 5. Phagocytic activity of macrophages is not dependent on 5-HTP. **A:** Double-immunofluorescence staining of the mouse spleen with anti-TPH-1 (red) and anti-F4/80 (green) antibodies. **Arrows** indicate TPH-1-positive and F4/80 strong-positive marginal zone macrophages. **B:** Phagocytosis of Cy5-labeled zymosan (blue) by splenic macrophages in mice treated with vehicle, 5-HTP, or PCPA *in vivo*. **C:** Immunofluorescence staining of the peritoneal macrophages with anti-TPH-1 (green) or anti-TPH-2 (green) antibodies. **D:** Immunofluorescence staining of peritoneal macrophages with anti-5-HTP antibody (red) after the application of 5-HTP and NSD-1015. **E:** Phagocytosis of Cy5.5-labeled zymosan (red) by peritoneal macrophages treated with 5-HT, 5-HTP, or PCPA *in vitro*. The nuclei were counterstained with DAPI (green). **Left:** Representative examples. **Right:** The number of zymosan particles in a cell was counted. We counted all phagocytosed zymosan in the cells of different areas in each preparation. Analysis of variance revealed a significant difference among the four groups [$F(3,36) = 11.2$; $P < 0.0001$]. Post hoc test demonstrated a significant difference between control and 5-HT groups. F, follicle; MZ, marginal zone; RP, red pulp. Error bars represent the mean $\pm$ SEM. ** $P < 0.01$. Scale bars = 50 μ m.

Figure 6. 5-HTP- or 5-HT-specific F-actin expression in the gut and macrophages. **A:** Double-immunofluorescence staining of the mouse intestine with anti-TPH-1 (green) antibody and phalloidin (blue). **B:** Double-immunofluorescence staining of the mouse intestine with anti-TPH-1 (green) and anti-EBP-50 (blue) antibodies. **C:** Fluorescence staining of Caco-2 cells with phalloidin (white) after the application of both NSD-1015 and 5-HTP or 5-HT alone. **D:** Fluorescence staining of the mouse intestine with phalloidin (blue) after the application of NSD-1015 and 5-HTP. **E:** Fluorescence staining of the peritoneal macrophages with phalloidin (white) stimulated with 5-HT or 5-HTP. Scale bars = 20 μ m.

with 5-HT. Thus, the activation pattern of ERK paralleled 5-HT or 5-HTP dependency in each cell.

Raf phosphorylates and activates MEK, and MEK phosphorylates and activates ERK/MAPK.²⁹ Functionally, the MEK inhibitor PD98059 caused an increase in the density of microvilli in Caco-2 cells (223% relative to the control, $P = 0.001$) (Figure 7B). Finally, we assessed the involvement of ERK in 5-HT-induced phagocytosis in the macrophages. PD98059 inhibited the phagocytic activity (55% relative to the control, $P = 0.02$) (Figure 7C). Consistently, the phagocytic activity was also lower than that of treatment with PCPA or 5-HTP (Figure 5E) ($P < 0.05$). The 5-HT-induced augmentation of phagocytosis of zymosan was also reversed by PD98059 (40% relative to 5-HT group, $P = 0.0003$) (Figure 7C). Again, the phagocytic activity was also lower than that of treatment with PCPA or 5-HTP (Figure 5E) ($P < 0.05$). Thus, ERK was

demonstrated to mediate the functions of both 5-HTP and 5-HT.

Discussion

5-HT is present in a variety of peripheral tissues. Evidence suggests that 5-HT modulates immune functions through several 5-HT receptors including 5-HT_{1A} receptor.³² In macrophages, 5-HT regulates phagocytosis through the 5-HT_{1A} receptor.²¹ Our study demonstrated that exogenous 5-HT induces phagocytosis, whereas 5-HTP was ineffective.

In addition, we explored the intrinsic role of endogenous monoamine precursors. A previous report demonstrated that 5-HTP shared the scavenger activity against reactive oxygen species with 5-HT; however, 5-HT was

Figure 7. ERK is a common mediator of 5-HT- and 5-HTP-induced functions. **A:** Western blotting of proteins from Caco-2 cells and peritoneal macrophages with anti-phospho-ERK, anti-ERK, and anti-actin antibodies. The cell lysates were obtained 15 and 60 minutes after the stimulation. Signal intensities of phosphorylated ERK-2 proteins were estimated by densitometric scanning. Each intensity was normalized to the intensity of total ERK as seen in previous reports.³¹ **B:** Scanning electron microscopic analysis of Caco-2 cells treated with the MEK-1 inhibitor PD98059. The density of microvilli was quantitatively estimated. **C:** Effect of a MEK-1 inhibitor PD98059 on 5-HT-induced phagocytosis of zymosan (red) by peritoneal macrophages. The nuclei were counterstained with DAPI (green). The number of zymosan particles in a cell was quantitatively determined. We counted all phagocytosed zymosan in the cells of different areas in each preparation. Error bars represent the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

>10-fold more potent than 5-HTP in protecting NK cells against functional inhibition and apoptosis.¹⁴ Importantly, our data revealed that endogenous 5-HTP but not 5-HT regulates the development of microvilli in the intestinal epithelial cells. The phagocytic activity of macrophages is essentially elicited extemporaneously when pathogens or foreign substances invade. In contrast, intestinal villus

epithelial cells regularly show renewal every 2 or 3 days. Biological information can be expressed by the kinetics of ERK/MAPK activation and its subcellular compartmentalization but can also be interpreted differently in a cell-type-specific context.²⁹ In ovarian cancer cells, the engagement of the hyaluronic acid receptor CD44 induced the formation of an IQGAP1 complex with ERK2/MAPK

and Cdc42, which promoted cell migration through Cdc42-mediated actin binding.³³ 5-HTP, F-actin, and ERK³⁰ are localized to the brush border. In the intestine, the receptor for 5-HTP has not been identified. Rather, it is plausible to postulate intracellular interaction of endogenous 5-HTP with F-actin-regulating molecules and ERK in the brush border. Such local interaction might enable the epithelial cell-specific stable molecular dynamics dictating the continuous development of intestinal epithelial cells. Similarly, 5-HT also exerts a platelet-specific effect inside the cells in a 5-HT receptor-independent manner.¹

We found the expression of TPH protein in the enterocytes. Similar to our results, the differentiated villous epithelial cells displayed an intense immunoreactivity for anti-TPH antibody in the human duodenum.³⁴ Consistently, we found observations suggesting subtle 5-HT synthesis in Caco-2 cells. SP, a BH4 precursor, increases cellular BH4 levels much more effectively than BH4 itself when used as the supplement.³⁵ SP is much more stable than BH4 in the medium and does not serve as a co-factor of TPH. Once it is taken up by the cell, it is converted to BH4 and accumulates beyond a saturating level for the TPH reaction.³⁶ The cellular content of 5-HT increased up to 0.10 pmol/ μ g protein (threefold within the last 24 hours) without SP supplementation, ie, with the endogenous BH4. The low concentration of co-factor might have limited the rate of the TPH reaction. Intracellular levels of BH4 were $\sim$ 15.4 pmol/ 10^6 cells.¹⁷ This was $\sim$ 3.4 μ mol/L based on an estimated single cell volume of 4.6 pL, below the K_m values of TPH-1 and TPH-2.³⁷ Adding the BH4 precursor induced a prominent increase in 5-HT content up to 1.95 pmol/ μ g protein ($\sim$ 28-fold within 24 hours). Hence the SP-dependent increase in cellular 5-HT was strong evidence of TPH function in the cell. The possibility can be excluded that the increase in cellular 5-HT was attributable to uptake from the culture medium: 5-HT was actually included in the culture medium derived mainly from the fetal calf serum. The amount of 5-HT in the medium decreased dramatically after 1 or 2 days of culture, an indication that cells took up and stored or degraded 5-HT, but there was a slight increase in cellular 5-HT (control cultures without SP). When SP was added, cellular 5-HT levels increased prominently as mentioned above. In this case, the total amount of 5-HT (9.8 ± 2.2 pmol/well, medium plus cell) in the culture with SP was greater than that in the culture without SP (2.2 ± 1.2 pmol/well, medium plus cell). Thus it was concluded that Caco-2 cells synthesized very small amounts of 5-HT *de novo*. In the mammary gland, prolactin-induced 5-HT regulates the development of the mammary gland in an autocrine manner specifically during pregnancy and lactation.³⁸ It is still under investigation whether the small amount of 5-HT produced in the gut epithelial cells might contribute to the functions of the gut such as gastrointestinal motility and absorption in autocrine and/or paracrine manners via 5-HT receptors.

The dopamine precursor L-DOPA and the 5-HT precursor 5-HTP are often administered to patients with metabolic disorders. The oral administration of 5-HTP is used in patients with metabolic disorders such as autosomal recessively transmitted deficiencies of BH4 known as

atypical phenylketonuria to correct 5-HT levels. We found that exogenous 5-HTP functions in the intestine. In addition, we have shown that an AADC inhibitor augments the development of microvilli. AADC inhibitors are currently used to treat Parkinson's disease.³⁹ These results suggest effects of the exogenous 5-HT precursor and AADC inhibitors. TPH-1 immunoreactivity is widely observed in various peripheral organs and tissues such as the heart, lung, muscle, and skin (unpublished data). We suggest that monoamine precursors might regulate diverse functions in various cells.

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References

1. Walther DJ, Peter JU, Winter S, Holtje M, Paulmann N, Grohmann M, Vowinkel J, Alamo-Bethencourt V, Wilhelm CS, Ahnert-Hilger G, Bader M: Serotonylation of small GTPases is a signal transduction pathway that triggers platelet alpha-granule release. *Cell* 2003, 115:851–862
2. O'Connell PJ, Wang X, Leon-Ponte M, Griffiths C, Pingle SC, Ahern GP: A novel form of immune signaling revealed by transmission of the inflammatory mediator serotonin between dendritic cells and T cells. *Blood* 2006, 107:1010–1017
3. Lesurtel M, Graf R, Aleil B, Walther DJ, Tian Y, Jochum W, Gachet C, Bader M, Clavien PA: Platelet-derived serotonin mediates liver regeneration. *Science* 2006, 312:104–107
4. Iken K, Cheng S, Fargin A, Goulet AC, Kouassi E: Serotonin upregulates mitogen-stimulated B lymphocyte proliferation through 5-HT1A receptors. *Cell Immunol* 1995, 163:1–9
5. Idzko M, Panther E, Stratz C, Muller T, Bayer H, Zissel G, Durk T, Sorichter S, Di Virgilio F, Geissler M, Fiebich B, Herouy Y, Elsner P, Norgauer J, Ferrari D: The serotonergic receptors of human dendritic cells: identification and coupling to cytokine release. *J Immunol* 2004, 172:6011–6019
6. Abdouh M, Albert PR, Drobetsky E, Filep JG, Kouassi E: 5-HT1A-mediated promotion of mitogen-activated T and B cell survival and proliferation is associated with increased translocation of NF-kappaB to the nucleus. *Brain Behav Immun* 2004, 18:24–34
7. Fiorica-Howells E, Hen R, Gingrich J, Li Z, Gershon MD: 5-HT(2A) receptors: location and functional analysis in intestines of wild-type and 5-HT(2A) knockout mice. *Am J Physiol* 2002, 282:G877–G893
8. Hamon M, Glowinski J: Regulation of serotonin synthesis. *Life Sci* 1974, 15:1533–1548
9. Tappaz ML, Pujol JF: Estimation of the rate of tryptophan hydroxylation in vivo: a sensitive microassay in discrete rat brain nuclei. *J Neurochem* 1980, 34:933–940
10. Touret M, Kitahama K, Geffard M, Jouvett M: 5-Hydroxytryptophan (5-HTP)-immunoreactive neurons in the rat brain tissue. *Neurosci Lett* 1987, 80:263–267
11. Geffard M, Touret M, Kitahama K: First characterization of 5-hydroxytryptophan in rat brain by using specific antibodies. *Brain Res* 1987, 426:191–196
12. Fujimiya M, Okumiya K, Nakazawa M, Kitahama K, Kimura H, Maeda T: Effect of reserpine on 5-hydroxytryptophan (5HTP)-immunoreactive neurons in the rat brain. *Histochemistry* 1994, 101:21–26
13. Christen S, Peterhans E, Stocker R: Antioxidant activities of some tryptophan metabolites: possible implication for inflammatory diseases. *Proc Natl Acad Sci USA* 1990, 87:2506–2510
14. Betten A, Dahlgren C, Hermodsson S, Hellstrand K: Serotonin protects NK cells against oxidatively induced functional inhibition and apoptosis. *J Leukoc Biol* 2001, 70:65–72
15. Fletcher PJ, Selhi ZF, Azampanah A, Sills TL: Reduced brain serotonin activity disrupts prepulse inhibition of the acoustic startle reflex.

- Effects of 5,7-dihydroxytryptamine and p-chlorophenylalanine. *Neuropsychopharmacology* 2001, 24:399–409
16. Prinssen EP, Assie MB, Koek W, Kleven MS: Depletion of 5-HT disrupts prepulse inhibition in rats: dependence on the magnitude of depletion, and reversal by a 5-HT precursor. *Neuropsychopharmacology* 2002, 26:340–347
17. Sawabe K, Suetake Y, Nakanishi N, Wakasugi KO, Hasegawa H: Cellular accumulation of tetrahydrobiopterin following its administration is mediated by two different processes; direct uptake and indirect uptake mediated by a methotrexate-sensitive process. *Mol Genet Metab* 2005, 86(Suppl 1):S133–S138
18. Hasegawa H, Ichiyama A: Tryptophan 5-mono-oxygenase from mouse mastocytoma: high-performance liquid chromatography assay. *Methods Enzymol* 1987, 142:88–92
19. Hasegawa H, Yanagisawa M, Inoue F, Yanaihara N, Ichiyama A: Demonstration of non-neural tryptophan 5-mono-oxygenase in mouse intestinal mucosa. *Biochem J* 1987, 248:501–509
20. Ono M, Okada H, Bolland S, Yanagi S, Kurosaki T, Ravetch JV: Deletion of SHIP or SHP-1 reveals two distinct pathways for inhibitory signaling. *Cell* 1997, 90:293–301
21. Freire-Garabal M, Nunez MJ, Balboa J, Lopez-Delgado P, Gallego R, Garcia-Caballero T, Fernandez-Roel MD, Brenlla J, Rey-Mendez M: Serotonin upregulates the activity of phagocytosis through 5-HT_{1A} receptors. *Br J Pharmacol* 2003, 139:457–463
22. Fujita M, Ota H, Kawai K, Matsui H, Nakao M: Differential isolation of microvillous and basolateral plasma membranes from intestinal mucosa: mutually exclusive distribution of digestive enzymes and ouabain-sensitive ATPase. *Biochim Biophys Acta* 1972, 274:336–347
23. Fujita M, Ohta H, Uezato T: Characterization of brush borders purified in iso-osmotic medium and microvillar membranes subfractionated from mouse small intestine. *Biochem J* 1981, 196:669–673
24. Inokuchi S, Sakai T, Shirato I, Tomino Y, Koide H: Ultrastructural changes in glomerular epithelial cells in acute puromycin aminonucleoside nephrosis: a study by high-resolution scanning electron microscopy. *Virchows Arch A Pathol Anat Histopathol* 1993, 423:111–119
25. Walther DJ, Peter JU, Bashammakh S, Hortnagl H, Voits M, Fink H, Bader M: Synthesis of serotonin by a second tryptophan hydroxylase isoform. *Science* 2003, 299:76
26. Vieira-Coelho MA, Teixeira VL, Guimaraes JT, Serrao MP, Soares-da-Silva P: Caco-2 cells in culture synthesize and degrade dopamine and 5-hydroxytryptamine: a comparison with rat jejunal epithelial cells. *Life Sci* 1999, 64:69–81
27. Hasegawa H, Kojima M, Oguro K, Nakanishi N: Rapid turnover of tryptophan hydroxylase in serotonin producing cells: demonstration of ATP-dependent proteolytic degradation. *FEBS Lett* 1995, 368:151–154
28. Mangeat P, Roy C, Martin M: ERM proteins in cell adhesion and membrane dynamics. *Trends Cell Biol* 1999, 9:187–192
29. Kolch W: Coordinating ERK/MAPK signalling through scaffolds and inhibitors. *Nat Rev Mol Cell Biol* 2005, 6:827–837
30. Boucher MJ, Rivard N: Regulation and role of brush border-associated ERK1/2 in intestinal epithelial cells. *Biochem Biophys Res Commun* 2006, 311:121–128
31. Lan M, Kojima T, Murata M, Osanai M, Takano K, Chiba H, Sawada N: Phosphorylation of ezrin enhances microvillus length via a p38 MAP-kinase pathway in an immortalized mouse hepatic cell line. *Exp Cell Res* 2006, 312:111–120
32. Mössner R, Lesch KP: Role of serotonin in the immune system and in neuroimmune interactions. *Brain Behav Immun* 1998, 12:249–271
33. Bourguignon LY, Gilad E, Rothman K, Peyrollier K: Hyaluronan-CD44 interaction with IQGAP1 promotes Cdc42 and ERK signaling, leading to actin binding. Elk-1/estrogen receptor transcriptional activation, and ovarian cancer progression. *J Biol Chem* 2005, 280:11961–11972
34. Meyer T, Brinck U: Differential distribution of serotonin and tryptophan hydroxylase in the human gastrointestinal tract. *Digestion* 1999, 60:63–68
35. Yamamoto K, Nagano M, Nakanishi N, Hasegawa H: A comparison of sepiapterin and tetrahydrobiopterin uptake by RBL2H3 cells. *Pteridines* 1996, 7:154–156
36. Hasegawa H, Oguro K, Naito Y, Ichiyama A: Iron dependence of tryptophan hydroxylase activity in RBL2H3 cells and its manipulation by chelators. *Eur J Biochem* 1999, 261:734–739
37. McKinney J, Knappskog PM, Haavik J: Different properties of the central and peripheral forms of human tryptophan hydroxylase. *J Neurochem* 2005, 92:311–320
38. Matsuda M, Imaoka T, Vomachka AJ, Gudelsky GA, Hou Z, Mistry M, Bailey JP, Nieport KM, Walther DJ, Bader M, Horseman ND: Serotonin regulates mammary gland development via an autocrine-paracrine loop. *Dev Cell* 2004, 6:193–203
39. Holm KJ, Spencer CM: Entacapone. A review of its use in Parkinson's disease. *Drugs* 1999, 58:159–177