Serotonin inhibits the influx of iron into cells by decreasing the expression of SLC11A2 in Caco-2 cells

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Abstract. Iron is an essential trace element for biological functions and its homeostasis is strictly regulated. Intestinal iron absorption primarily occurs in the duodenum, and the iron transporter solute carrier family 11 member 2 (SLC11A2; also known as divalent metal transporter 1) is crucial in this process. Notably, iron absorption is regulated by iron stores in the body and hormones; however, the effects of serotonin on iron transport are poorly understood. The present study aimed to analyze the effects of serotonin on the expression and function of SLC11A2 using human colon carcinoma-derived Caco-2 cells. SLC11A2 mRNA and protein levels were measured using reverse transcription-quantitative PCR and western blotting, respectively. To investigate the effect of serotonin on intracellular iron concentrations, iron levels were measured in cells treated with a soluble iron salt. The results revealed that serotonin decreased SLC11A2 mRNA levels to ~25% and protein levels to ~35% of control values ($P<0.01$). A soluble iron salt markedly increased the intracellular iron concentration in Caco-2 cells; however, this increase was suppressed by serotonin treatment ($P<0.01$). To investigate the mechanism of serotonin action, Caco-2 cells were treated with BW723C86, a selective 5-hydroxytryptamine (5-HT) 2B receptor agonist. BW723C86 also decreased SLC11A2 mRNA levels to ~25% and protein levels to ~60% of control values ($P<0.01$ and $P<0.05$). Furthermore, BW723C86 suppressed the increase in intracellular iron concentration induced by a soluble iron salt ($P<0.01$). In addition, the serotonin-induced decrease in SLC11A2 mRNA levels was inhibited by SB204741, a selective antagonist of the 5-HT2B receptor ($P<0.05$). These findings suggested a novel link between serotonin signaling and iron absorption, which may inform future therapeutic approaches for iron deficiency anemia.

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Introduction

Iron deficiency anemia (IDA) is a prevalent health concern, particularly in children, women, and elderly populations. IDA is the most common type of anemia and has a multifactorial etiology, including insufficient iron intake, impaired absorption, increased demand, and excessive loss (1,2). Oral iron supplementation is a standard treatment; however, its low absorption rate and gastrointestinal side effects often pose challenges. Intravenous iron administration is an alternative; however, its long-term use raises concerns regarding adverse effects, such as atherosclerosis and an increased risk of infections (3,4). Therefore, identifying molecular regulators of intestinal iron absorption could reduce reliance on high-dose supplementation and lower adverse risks.

Iron absorption primarily occurs in the duodenum, where iron transporters, such as solute carrier family 11 member 2 (SLC11A2; also known as divalent metal transporter 1), solute carrier family 40 member 1 (SLC40A1; also known as ferroportin), and solute carrier family 46 member 1 (SLC46A1; also known as heme carrier protein 1), play key roles (5-7). Understanding the regulatory mechanisms of these transporters is essential to improving IDA treatment strategies.

Serotonin is widely recognized as a neurotransmitter; however, studies conducted in the 1960s have suggested its potential involvement in iron absorption (8). Recently, abnormal peripheral serotonin levels have been linked to dysregulation of iron homeostasis in patients with myelodysplastic syndromes (9). Serotonin has also been demonstrated to regulate erythropoiesis (10) and may influence iron metabolism via adenosine monophosphate-activated protein kinase (AMPK)-dependent pathways (11). Elevated peripheral serotonin levels have been reported in pediatric patients with IDA, suggesting that serotonin may contribute to its pathophysiology (12). In addition, serotonin is secreted by enterochromaffin cells in the gut, where it regulates intestinal motility, secretion, and epithelial cell function (13,14). This suggests that serotonin influences iron transport in the intestine. However, no studies have directly examined how serotonin affects specific intestinal iron transporters such as SLC11A2. Addressing this gap may reveal new regulatory pathways for optimizing iron supplementation.

In this study, we investigated the effects of serotonin on the expression and function of iron transporters. Specifically, we used Caco-2 cells, a widely used intestinal epithelial cell

model, to analyze the effect of serotonin on iron-ion transport mediated by SLC11A2. We hypothesize that serotonin modulates intestinal iron absorption by regulating SLC11A2 expression and function.

Materials and methods

Chemicals and reagents. Serotonin and receptor ligands were obtained from Nacalai Tesque Inc. (Kyoto, Japan), Cayman Chemical Co. (Ann Arbor, MI, USA) and Abcam (Cambridge, UK). Antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), Proteintech Group, Inc. (IL, USA) and R&D Systems Inc. (MN, USA). Other general reagents used in the biochemical experiments were obtained from Wako (Osaka, Japan). All antibodies and reagents used were of analytical grade. All primers were obtained from FASMAC (Kanagawa, Japan). The primer sequences are listed in Table I.

Cell culture. Human colon carcinoma Caco-2 cells were maintained in Dulbecco's modified Eagle's medium (Wako) supplemented with 10% heat-inactivated fetal bovine serum (Equitech-Bio, Inc., TX, USA) and 1X MEM non-essential amino acid solution, with 5% CO_2 at 37˚C. For assays, cells were cultured for 21 days after reaching confluence to allow differentiation into enterocyte-like monolayers. The cells were used between passages 10 and 20 and seeded at $2x10^5$ cells/dish in a 35 mm dish for all experiments. Caco-2 cells were chosen as they are a validated intestinal epithelial model for iron transport studies.

cDNA synthesis from total RNA. Caco-2 cells were treated with 100, 200, 300, 400 or 500 μg/ml serotonin for 6 h prior to RNA extraction. In another experiment, Caco-2 cells were treated with serotonin (500 μg/ml) for 1, 3, 6, 9 or 24 h. Untreated cells, which received culture medium without serotonin, were included as controls. Total RNA was extracted from cultured Caco-2 cells using ISOGEN II (Nippon Gene, Tokyo, Japan), and cDNA was synthesized using the ReverTra Ace® qPCR RT Master Mix (TOYOBO, Osaka, Japan). In brief, RNA (0.5 μg) was combined with 2 μl of 5X RT Master Mix and incubated at 37˚C for 15 min. The reverse transcription reaction was terminated by heating at 98˚C for 5 min to inactivate the enzyme.

Reverse transcription (RT)-PCR and RT-quantitative PCR (RT-qPCR). RT-PCR was carried out using serotonin receptor-specific forward and reverse primers (Table I) with Takara Ex Taq reagent (Takara, Shiga, Japan). The thermal cycling conditions consisted of an initial denaturation at 94˚C for 30 sec, followed by 30 cycles of denaturation at 98˚C for 10 sec, annealing at 55˚C for 30 sec, and extension at 72˚C for 1 min. The PCR products were separated via electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

RT-qPCR was performed using SLC11A2-specific forward and reverse primers (Table I) and THUNDERBIRD® Next SYBR™ qPCR Mix (TOYOBO). The reaction protocol involved an initial denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 34 sec. Expression levels of SLC11A2 mRNA were quantified using a standard curve and normalized to GAPDH expression within the same sample.

Western blotting. Caco-2 cells were treated with serotonin (500 μg/ml) for 6 or 24 h prior to protein extraction. Untreated cells, which received culture medium without serotonin, were included as controls. Total proteins extracted from Caco-2 cells (5 μg per sample) were separated via SDS-PAGE (4-15% gradient gel, 200 V, 30 min) and then blotted onto a PVDF membrane at 0.35 A for 1 h. The membrane was blocked with 1% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) for 1 h and then incubated overnight at 4˚C with an anti-NRAMP2 (another name of SLC11A2) polyclonal antibody (1:5,000). After washing with TBS-T, the membrane was incubated with HRP-conjugated anti-mouse IgG polyclonal antibody (1:5,000) for 2 h at room temperature. Detection was carried out using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA), and signal intensities were quantified using the Fusion-FX imaging system (Vilber, Marne-la-Vallée, France). Protein expression levels of SLC11A2 were normalized to GAPDH levels in the same sample.

Measurement of intracellular iron concentration (Ferrozine assay). Caco-2 cells treated with serotonin (500 μg/ml) for 6 or 24 h were administered 10 mM ammonium iron (II) sulfate hexahydrate as a soluble iron salt. After 30 min of incubation, cells were washed with phosphate-buffered saline (-), and lysates were prepared using RIPA buffer. Untreated cells, which received culture medium without serotonin and a soluble iron salt, were included as controls. Protein content (50 μg) from each lysate was mixed with an acidic solution (pH 2.5) of HCl. Intracellular Fe^{2+} levels were quantified using a ferrozine-based iron assay kit (Metallogenics, Chiba, Japan), according to the manufacturer's instructions (15). Ferrozine specifically forms a magenta complex with ferrous iron, exhibiting a characteristic absorbance peak at approximately 562 nm, which enables selective spectrophotometric detection of Fe^{2+} (16). Therefore, absorbance at 562 nm, corresponding to the Fe^{2+}-ferrozine complex, was measured to determine iron concentration.

Statistical analysis. All experimental data were obtained from four independent replicates and are expressed as means ± standard error (SE). Normality of the data was assessed using the Shapiro-Wilk test in JMP Pro 17 (SAS Institute). Comparisons between groups were conducted using one-way ANOVA followed by Tukey-Kramer post hoc test. Effect sizes (Cohen's d) and 95% confidence intervals were calculated where appropriate. Statistical analyses were performed using JMP Pro 17, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of serotonin on SLC11A2 mRNA and protein expression. First, the effect of serotonin treatment on SLC11A2 mRNA expression in Caco-2 cells was examined via RT-qPCR. As shown in Fig. 1A, when Caco-2 cells were treated with serotonin (100-500 μg/ml) for 6 h, SLC11A2

Table I. Primers designed for RT-PCR and RT-qPCR.

Gene	Forward, 5'-3'	Reverse, 5'-3'
GAPDH	GCGAGATCCCTCCAAAATCA	ATGGTTCACACCCATGACGA
SLC11A2	AGTCATCCTGTGGCTGAT	GTATCTGCAATGGTGATGAG
5-HT1A receptor	ACAACACCACATCACCACC	AGCAGCAGAGAGGTGATCA
5-HT1B receptor	ATGGCGCAGAGAAACCTT	TGTGGCTTGACAATCGCT
5-HT2A receptor	TCAGCTCTTGCATGCAGT	TTCTGTGACTCGCTGCAT
5-HT2B receptor	TGCTGGATGGTTCTCGAA	CGTTGGAAATGGTCTGCA
5-HT2C receptor	ACCGCTGACGATTATGGT	TCTTGCAGCACTTCAGGA
5-HT3 receptor	AACATCTCTTTGTGGCGC	TGCTGAACTCCCGAAAGT
5-HT4 receptor	TTCACCTGAGGCTTTCCGTC	AGGAGGCCATTATGTCCCCT
5-HT5 receptor	ACCTCCTTTTCCCTCTCCA	ATAAGCATCCGAAGACCG
5-HT6 receptor	TACCTGCAGGGATCATAGC	CAACATCCAGAGTCCTCCA
5-HT7 receptor	AGGGTCTCTGTGATTTCCC	TCTCAAGACCCTTCAGAGC

RT-PCR, reverse transcription-PCR; RT-qPCR, RT-quantitative PCR; SLC11A2, solute carrier family 11 member 2; 5-HT, serotonin.

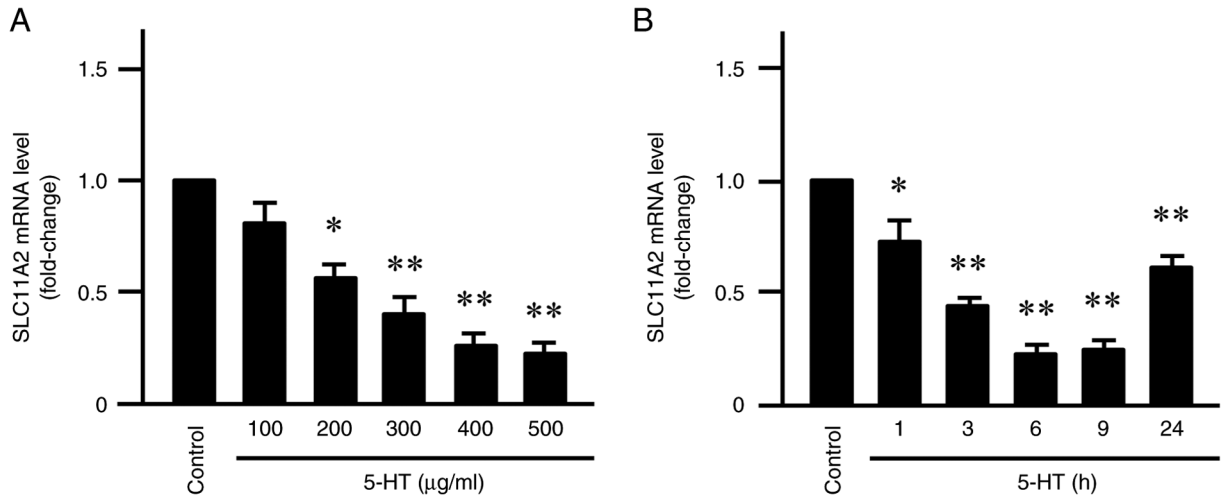

Figure 1. Effect of serotonin on the mRNA expression of SLC11A2 in Caco-2 cells. (A) Cells were treated with 5-HT at the indicated concentrations for 6 h. (B) Cells were treated with 500 μg/ml 5-HT for the indicated times. The mRNA levels of SLC11A2 were measured using reverse transcription-quantitative PCR and normalized to that of GAPDH. Results show the mean ± standard error (n=4). Statistical significance was determined using one-way ANOVA followed by the Tukey-Kramer post hoc test. *P<0.05; **P<0.01 vs. control. Exact P-values: (A) 0.014, <0.001, <0.001, <0.001; (B) 0.013, <0.001, <0.001, <0.001, <0.001. SLC11A2, solute carrier family 11 member 2; 5-HT, serotonin.

mRNA levels decreased in a dose-dependent manner. The most significant reduction was observed at 500 μg/ml, where mRNA levels were ~0.25-fold lower compared to those in the control group (P<0.01; Fig. 1A). Furthermore, time-course analysis showed that SLC11A2 mRNA levels remained low for up to 24 h after serotonin (500 μg/ml) treatment, showing a significant decrease of ~0.25-fold at 6 and 9 h (P<0.01; Fig. 1B). In addition, serotonin treatment reduced the mRNA levels of SLC40A1 and SLC46A1 (Fig. S1).

SLC11A2 protein expression levels were evaluated via western blotting. SLC11A2 protein expression decreased significantly, by ~0.35-fold at 6 h and ~0.62-fold at 24 h after serotonin treatment (P<0.01; Fig. 2A). The image in Fig. 2A presents a typical result from biological replicates obtained from four independent experiments.

Effect of serotonin on iron-ion transport into Caco-2 cells. Intracellular iron concentrations were measured using the ferrozine assay. Cells treated with ammonium iron (II) sulfate hexahydrate (hereafter, 'Fe reagent') was 230 μg/dl, approximately 80 times higher than that in control cells (2.96 μg/dl). When serotonin-treated Caco-2 cells were subsequently exposed to Fe reagent, intracellular iron concentrations decreased to 132 μg/dl after 6 h and 176 μg/dl after 24 h of the treatment (P<0.01; Fig. 2B). Fig. 2B shows intracellular iron concentrations in control and serotonin-treated cells, with error bars representing SE of four independent biological replicates.

Expression of serotonin receptors in Caco-2 cells. The mRNA expression of serotonin receptor subtypes in Caco-2 cells was analyzed using RT-PCR. Fig. 3 shows the presence or

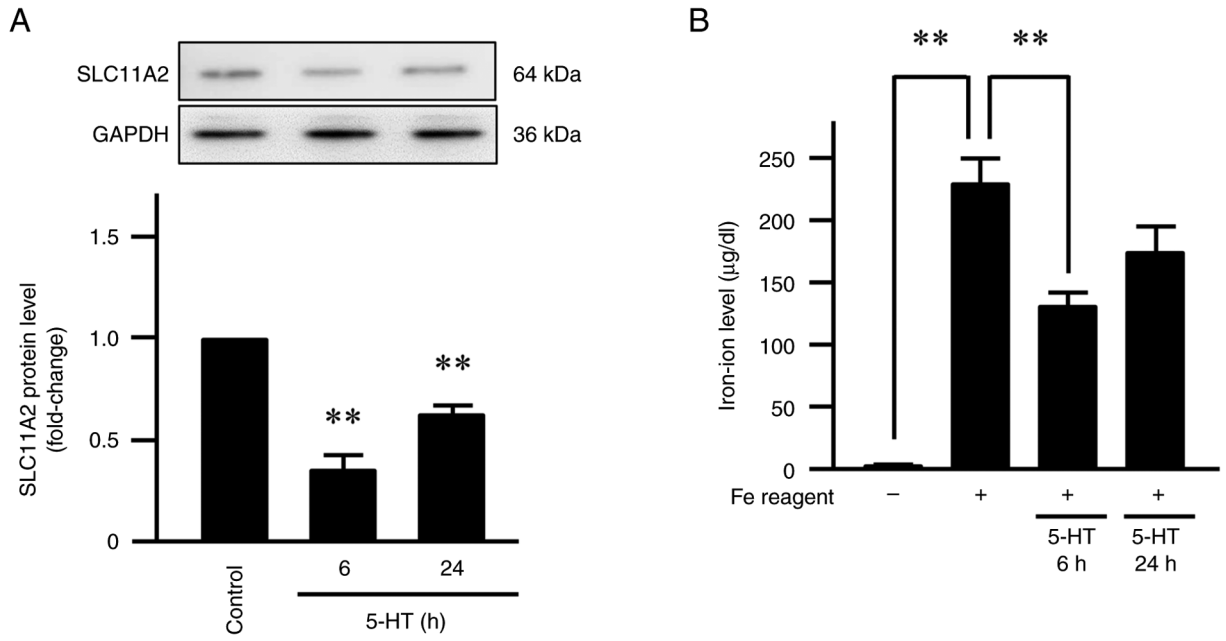

Figure 2. Effect of serotonin on the protein expression of SLC11A2 and the intracellular iron-ion concentration in Caco-2 cells. (A) Cells were treated with 500 μg/ml 5-HT for the indicated times. Western blotting was conducted to detect SLC11A2 protein. Panels show the western blotting results indicating the SLC11A2 expression of cells. Bar graph indicates the relative intensity of SLC11A2 signals shown at panels, quantified with a densitometer. Result shows the mean ± SE (n=4). Statistical significance was determined by one-way ANOVA followed by the Tukey-Kramer post hoc test. **P<0.01 vs. control. Exact P-values: <0.001, <0.001. (B) Cells were treated with 500 μg/ml 5-HT for 6 h or 24 h and then exposed to 10 mM ammonium iron (II) sulfate hexahydrate for 30 min. Intracellular iron concentration was determined by ferrozine assay. Result shows the mean ± standard error (n=4). Statistical significance was determined using one-way ANOVA followed by the Tukey-Kramer post hoc test. **P<0.01. Exact P-values: <0.001, <0.001. SLC11A2, solute carrier family 11 member 2; 5-HT, serotonin.

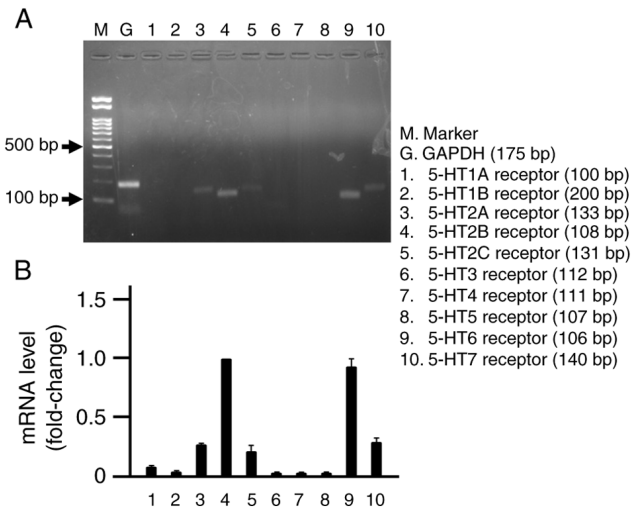

Figure 3. mRNA expression of serotonin receptor subunits in Caco-2 cells. The mRNA expression of each serotonin receptor in the cells was examined by reverse trasnscription-PCR. (A) Agarose gel electrophoresis of the PCR amplified products of GAPDH (lane G) and serotonin receptor subtypes (lane 1-10). Lane M represents a 100-bp DNA ladder. (B) The relative intensity of the bands was quantified by densitometry using ImageJ software, and the mRNA levels were normalized to GAPDH. Data are expressed relative to the maximum expression level, which was arbitrarily set to 1.0. Results are representative of four independent experiments. 5-HT, serotonin.

absence of each receptor subtype. The results showed mRNA expression of 5-HT2A, 5-HT2B, 5-HT2C, 5-HT6, and 5-HT7 receptors; however, 5-HT1A, 5-HT1B, 5-HT3, 5-HT4, and 5-HT5 receptors were not expressed (Fig. 3).

Involvement of 5-HT2B receptor in serotonin actions. 5-HT2B receptor is widely expressed in peripheral tissues such as vascular and gastrointestinal smooth muscles (17,18), making it the most likely candidate for regulating SLC11A2 expression in Caco-2 cells. Therefore, we treated Caco-2 cells with BW723C86 (100 μM). This resulted in a significant reduction in SLC11A2 mRNA expression by ~0.31-0.45-fold (P<0.01; Fig. 4A), as well as a decrease in protein expression by ~0.65-fold (P<0.05; Fig. 4B). Intracellular iron concentration also decreased from 237 μg/dl (cells treated only with Fe reagent) to 149 μg/dl after 6 h and to 192 μg/dl after 24 h of BW723C86 treatment (P<0.01; Fig. 4C). Furthermore, treatment with SB204741 (100 μM) inhibited the serotonin-induced reduction in SLC11A2 mRNA expression (P<0.05; Fig. 5). Figs. 4 and 5 summarize representative results from four independent biological replicates, with error bars indicating SE and asterisks denoting statistically significant differences (P<0.05).

Additionally, to examine whether other serotonin receptor subtypes might also be involved, we tested TCB-2, a selective 5-HT2A receptor agonist, and MK-212, a selective 5-HT2C receptor agonist. TCB-2 reduced the mRNA levels of SLC11A2 (Fig. S2).

Discussion

Iron ion absorption primarily occurs in the small intestine. Therefore, this study required a cultured cell line that closely resembled human intestinal epithelial cells. Consequently, we used Caco-2 cells, a human colorectal cancer-derived cell line with a morphology and structure similar to those of

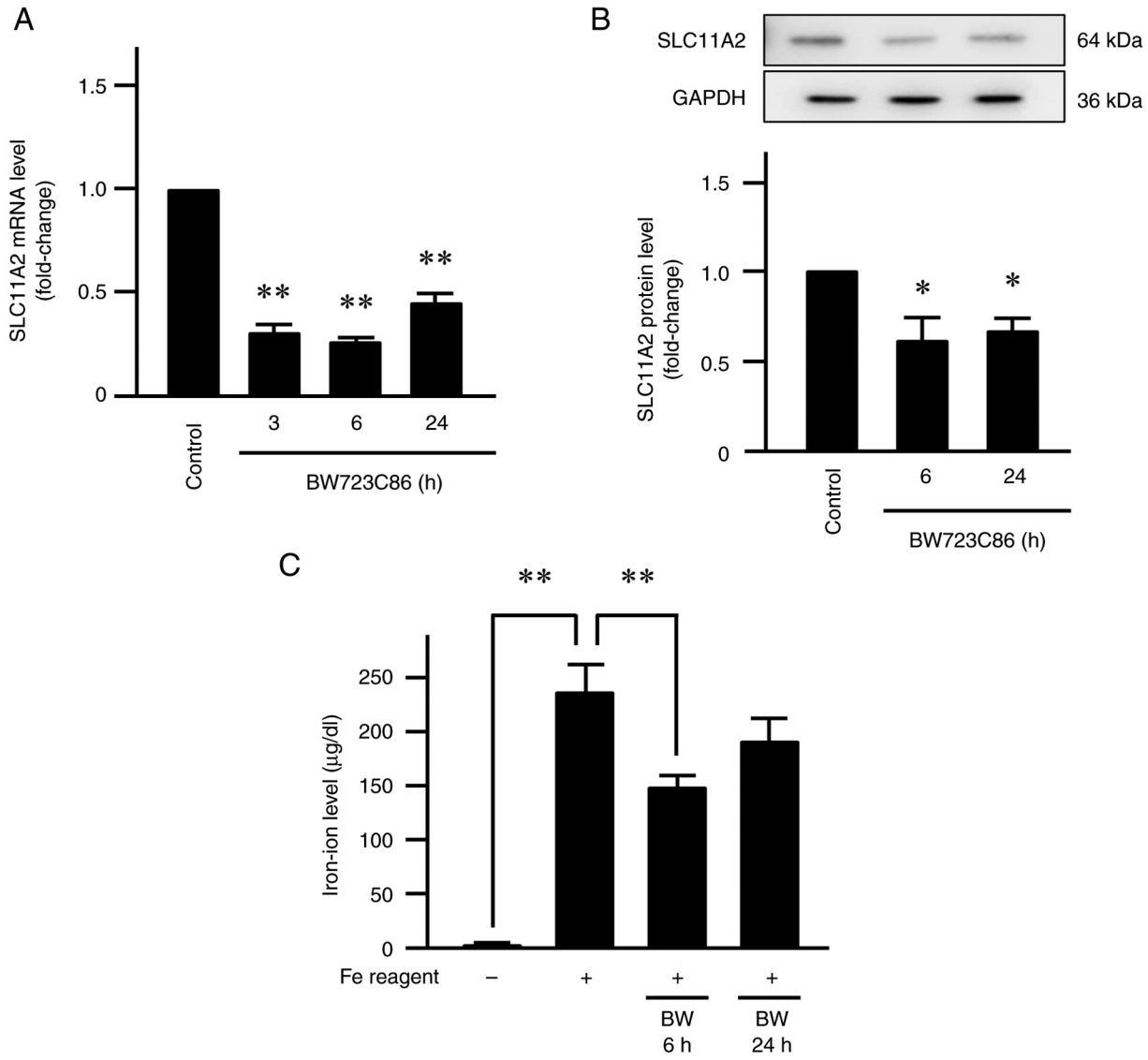

Figure 4. Effect of BW on the mRNA and protein expression of SLC11A2 and the intracellular iron-ion concentration in Caco-2 cells. (A) Cells were treated with 100 μM BW for the indicated times. The mRNA levels of SLC11A2 were measured by reverse transcription-quantitative PCR and normalized to that of GAPDH. Results show the mean ± SE (n=4). Statistical significance was determined using one-way ANOVA followed by the Tukey-Kramer post hoc test. **P<0.01 vs. control. Exact P-values: <0.001, <0.001, <0.001. (B) Cells were treated with 100 μM BW for the indicated times. Western blotting was conducted to detect SLC11A2 protein. Panels show the western blotting results indicating the SLC11A2 expression of cells. Bar graph indicates the relative intensity of SLC11A2 signals shown at panels, quantified with a densitometer. Result shows the mean ± SE (n=4). Statistical significance was determined by one-way ANOVA followed by the Tukey-Kramer post hoc test (*P<0.05 vs. control). Exact P-values: 0.039, 0.043. (C) Cells were treated with 100 μM BW for 6 h or 24 h and then exposed to 10 mM ammonium iron (II) sulfate hexahydrate for 30 min. Intracellular iron concentration was determined by ferrozine assay. Result shows the mean ± SE (n=4). Statistical significance was determined using one-way ANOVA followed by the Tukey-Kramer post hoc test. **P<0.01. Exact P-values: <0.001, 0.009. BW, BW723C86; SLC11A2, solute carrier family 11 member 2; SE, standard error.

human intestinal epithelial cells (19,20). Previous studies have reported that Caco-2 cells can be used to evaluate the cellular uptake of drugs and ions, and that they possess sufficient amounts of various iron transport-related factors, including iron transporters (21,22). Based on these characteristics, we determined that Caco-2 cells are the most suitable model for this study.

In this study, we selected SLC11A2 as the target iron transporter. SLC11A2 is primarily responsible for divalent cation transport, and its expression in the brush border membrane of intestinal epithelial cells fluctuates depending on iron deficiency in the body, such as in IDA (23,24). Therefore, it is reasonable to hypothesize that SLC11A2 plays a crucial role in the response to changes in systemic iron levels. Identifying the factors that influence SLC11A2 expression and function could elucidate the mechanisms of iron absorption and provide insights into new therapeutic targets for anemia caused by iron deficiency.

Based on these considerations, we examined the effects of serotonin on the expression of SLC11A2 and iron-influx in Caco-2 cells. As a result, serotonin consistently reduced SLC11A2 expression at both the mRNA and protein levels, accompanied by decreased intracellular iron accumulation. These results suggest that serotonin suppresses SLC11A2 expression, thereby affecting iron transport capacity. Although measurement methods using this study do not fully

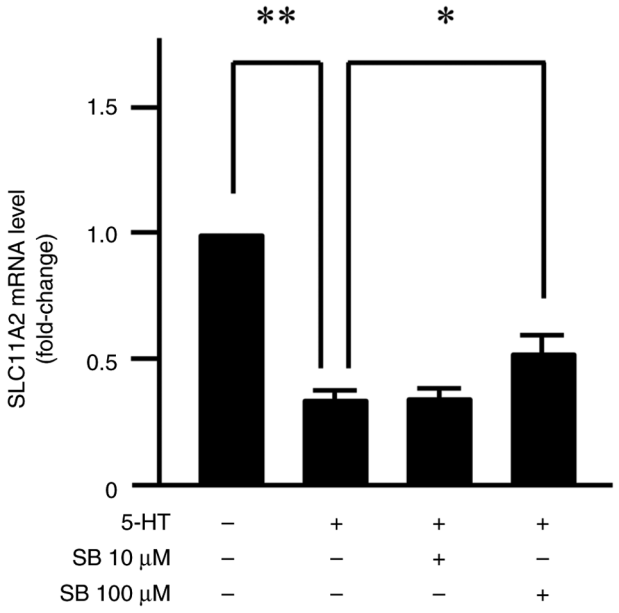

Figure 5. Effect of SB204741 on the serotonin-induced reduction in SLC11A2 mRNA expression in Caco-2 cells. Cells were treated with SB at the indicated concentrations for 24 h, followed by 500 μg/ml 5-HT for 6 h. The mRNA levels of SLC11A2 were measured using reverse transcription-quantitative PCR and normalized to that of GAPDH. Results show the mean ± standard error (n=4). Statistical significance was determined using one-way ANOVA followed by the Tukey-Kramer post hoc test. *P<0.05; **P<0.01. Exact P-values: <0.001, 0.036. SLC11A2, solute carrier family 11 member 2; 5-HT, serotonin; SB, SB204741.

replicate the polarized intestinal epithelium, previous studies have validated Caco-2 cells as a reliable in vitro model for investigating intestinal iron absorption and transporter function (25).

Serotonin receptors are classified into seven subtypes, each containing multiple variants (26,27). We found that 5-HT2A, 2B, 2C, 6, and 7 receptor mRNAs were involved in the serotonin-mediated effects. Among these, 5-HT2A, 2C, 6, and 7 receptors are primarily expressed in the central nervous system, whereas 5-HT2B receptors are widely expressed in peripheral tissues, including gastrointestinal smooth muscle (17,26). Therefore, we investigated the involvement of 5-HT2B receptors in mediating the effects of serotonin.

BW723C86 consistently reduced SLC11A2 expression at both the mRNA and protein levels, and decreased intracellular iron accumulation. Moreover, SB204741 suppressed serotonin-induced reduction in SLC11A2 mRNA levels. These results suggest that serotonin suppresses SLC11A2 expression and iron influx through the 5-HT2B receptors. However, partial inhibition by SB204741 suggests the involvement of additional receptor subtypes such as 5-HT2A, 5-HT2C, 5-HT6, or 5-HT7, which were also expressed in Caco-2 cells. TCB-2 also reduced the mRNA levels of SLC11A2. Thus, further investigations are warranted to elucidate their potential involvement in iron absorption.

This work focused on SLC11A2 as a primary iron transporter. However, serotonin treatment reduced the mRNA levels of SLC40A1 and SLC46A1 in Caco-2 cells. These results suggest that serotonin may influence SLC11A2 as well as other iron transporters, regulating both intracellular iron influx and extracellular iron export. Detailed analyses of these transporters are necessary to gain a comprehensive understanding of the effects of serotonin on iron metabolism.

Serotonin is abundant in the gastrointestinal tract, where it regulates motility, secretion, and epithelial renewal. Previous clinical reports have shown altered serotonin levels in patients with iron deficiency anemia and chronic inflammatory disorders such as inflammatory bowel disease, which are frequently associated with disturbed iron absorption (12,28,29). Our findings therefore provide a potential mechanistic link between peripheral serotonin signaling and intestinal iron metabolism. Whereas, this study is limited by the use of supraphysiological serotonin concentrations, reliance on an in vitro model, and lack of in vivo validation. Future work should address whether physiological serotonin levels exert comparable effects in animal models and humans.

The findings of this study contribute to the understanding of the pathophysiology of IDA. Notably, we demonstrated that 5-HT2B regulates iron influx by modulating SLC11A2 expression, suggesting that this receptor may be a novel therapeutic target for IDA. However, therapeutic targeting of this receptor requires caution. Both 5-HT2B agonists and antagonists have been implicated in cardiac valvulopathy and other cardiovascular risks (30,31). Further validation using animal models is essential to determine whether 5-HT2B receptor ligands can serve as novel therapeutic strategies for enhancing iron absorption.

In summary, our findings support 5-HT2B-mediated regulation of intestinal iron transport, pointing to serotonin signaling as a previously underrecognized modulator of iron homeostasis. Future in vivo studies will be necessary to clarify its physiological relevance and therapeutic potential in IDA.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YS conceptualized the study, designed the methodology, performed formal data analysis, managed the overall progress of the project (project administration), prepared figures and graphical representations (visualization), and wrote the original draft of the manuscript. MA contributed to formal data analysis and experimental investigations, and assisted in the preparation of figures (visualization). NK contributed to formal data analysis and experimental investigations, and assisted in figure preparation (visualization). SN conceptualized the study, supervised the research activities, managed the project (project administration), and revised and edited the

manuscript. YS and SN confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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