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**Histamine uptake mediated by plasma membrane monoamine
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Abstract

The resources of released histamine from activated mast cells, as initial effectors of allergic disease, include not only endogenous prepackaged histamine and newly synthesized histamine, but also histamine that is obtained through the *de novo* reuptake pathway. To investigate the *de novo* histamine production pathway, a mast cell line, RBL-2H3 Sc98 in which endogenous histamine production is lacking and only the *de novo* histamine release pathway via transporters is maintained, was used to dissect histamine reuptake in the present study. Histamine content measurements indicated that RBL-2H3 Sc98 cells took up extracellular histamine for storage in granules and subsequent release after stimulation by an antigen. Profiling and inhibition analysis of possible transporters suggested that the plasma membrane monoamine transporter and organic cation transporter 1 may be candidate transporters for histamine uptake from extracellular spaces, and that vesicular monoamine transporter 2 was responsible for intracellular vesicle uptake. These results may provide the foundation to understand the contribution of exogenous histamine to outward histamine release that is mediated by mechanisms other than conventional exocytosis.

Keywords: RBL-2H3 cells, OCT, PMAT, histamine release, histamine uptake

1. Introduction

Biogenic amine histamine is synthesized from L-histidine by histidine decarboxylase (HDC). Histamine is involved in a wide variety of pathological and physiological processes including allergic reactions, inflammation, immune responses, gastric secretion, and neurotransmission. Intracellularly synthesized histamine is stored in cytoplasmic granules of mast cells and basophils, two of the major producers of histamine. Basophilic leukemia (RBL)-2H3 cells, a tumor analog of rat mast cells, is considered to be a convenient model to study immunoglobulin E (IgE)-mediated degranulation of mast cells, because they express the IgE receptor, store mediators in granules, and release mediators, such as histamine, β -hexosaminidase, and serotonin, after crosslinking IgE (Maeyama et al., 1986; Gilfillan and Beaven, 2012).

Mast cells isolated from mice and rats are able to take up histamine from the microenvironment (Day and Green, 1962; Cabut and Haegermark, 1966). It has been considered that the uptake of extracellular histamine into bone marrow-derived mast cell granules is mediated by two membrane transporters, vesicular monoamine transporter 2 (VMAT-2), which is expressed on the membrane of exocytotic vesicles, and an unidentified transporter that must be located on the plasma membrane (Ohtsu et al. 2002). Because the histamine concentration in the microenvironment around mast

cells may reach millimolar levels owing to histamine release, low affinity transporters are assumed to play a role in its uptake via the plasma membrane. Although the mechanism of histamine transport from the cytosol into granules has been elucidated, its uptake from the microenvironment into mast cells and vice versa is still not fully understood.

The actions of released monoamine neurotransmitters are terminated by cellular uptake through specific monoamine transporters, such as Na^+ - and Cl^- -dependent, high affinity serotonin transporter (SERT), dopamine transporter, and norepinephrine transporter, which is referred to as the uptake-1 system (Husztí 2003; Perdan-pirkmajer et al. 2013; Sakurai et al. 2006; Tuomisto et al. 1980). In contrast, uptake-2 system transporters, such as bidirectional, Na^+ - and Cl^- -independent, and low-affinity-high-capacity transporters, possess numerous characteristics and share a broad range of substrate specificities. These transporters, which are located in the plasma membrane, belong to solute carrier (SLC) 22 and SLC29 families, respectively, and have the ability to transport various organic cations including endogenous biogenic amines and drugs (Grundemann et al., 1999; Engel and Wang, 2005; Duan and Wang, 2010; Koepsell, 2013; Duan et al., 2015). Schneider et al. (2005) showed that murine basophils take up histamine from the microenvironment through organic cation

transporter 3 (OCT3). However, there is insufficient data to verify whether histamine uptake by mast cell is also mediated by OCT3.

We identified a mutant RBL-2H3 cell line, RBL-2H3 Sc98, with a highly reduced histamine level compared with wild-type cells. The low level of endogenous histamine in this subclone allowed direct monitoring of histamine uptake. Using RBL-2H3 Sc98 cells, we demonstrated that mast cells took up histamine from the microenvironment via uptake-2 transporters. Furthermore, various inhibitors of each transporter were used to distinguish possible candidates responsible for histamine uptake via the plasma membrane.

2. Material and methods

2.1 Chemicals and drugs

Eagle's minimum essential medium (MEM) and antibiotics (penicillin G sodium and streptomycin sulfate) were purchased from Gibco Life Technologies (Grand Island, NY, USA). Fetal calf serum was obtained from JRH Bioscience (Lenexa, KS, USA). Bovine serum albumin (BSA) was purchased from Millipore (Kankakee, IL, USA). Dinitrophenylated BSA (DNP-BSA) as an antigen and monoclonal IgE against DNP-BSA purified from the supernatant of IgE-producing hybridomas were prepared in our laboratory. Decynium-22 (D22), lopinavir, desipramine, cimetidine, corticosterone, phorbol myristate acetate (PMA), reserpine, and α -fluoromethyl histidine (α -FMH) were purchased from Sigma-Aldrich (St Louis, MO, USA). FFN206 [4-(2-aminoethyl)-7-(methylamino)-2H-chromen-2-one trifluoroacetate], rabbit polyclonal antibodies against OCT1 and OCT3, as well as rabbit monoclonal anti-OCT2 and mouse monoclonal anti-PMAT antibodies were purchased from Abcam (Cambridge, UK). Secondary antibodies against horseradish peroxidase-conjugated rabbit and mouse IgG for western blotting and secondary antibodies against rabbit and mouse IgG labeled with Alexa Fluor 488 for immunocytochemistry were obtained from Invitrogen (Carlsbad, CA, USA).

Histamine phosphate, serotonin-creatinine sulfate, *o*-phthalaldehyde, and *p*-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside were purchased from Wako Pure Chemical Co., (Osaka, Japan). Piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES) and ethylenediaminetetraacetic acid (EDTA, free acid) were purchased from Dojindo (Kumamoto, Japan).

2.2 Culture and vesicle observation of RBL-2H3 and RBL-2H3 Sc98 cells

RBL-2H3 and RBL-2H3 Sc98 cells were cultured in MEM containing 15% fetal calf serum and antibiotics in a flask exposed to a humidified atmosphere with 5% CO₂ at 37°C (Barusmian et al., 1981).

For fluorescence microscopy of FFN206, a functional indicator of VMAT-2, monolayered cells in a culture dish (35, 0/10 mm, Greiner Bio-One, Frickenhausen, Germany) were washed with phosphate-buffered saline and incubated with 2 μ M FFN206 for 30 min. Fluoromicroscopic observation was performed using an ImageXpress Micro microscope (Molecular Devices, Sunnyvale, CA, USA) equipped with a filter cube (excitation and emission wavelengths: 350 and 425–475 nm, respectively).

2.3 Histamine measurements

Before cells were disseminated into 24-well culture plates, they were sensitized with monoclonal IgE as described in section 2.5. After washing twice with PIPES buffer, the cells were stimulated by DNP-BSA in 200 μ l PIPES buffer. For the concentration response evaluation of DNP-BSA, concentrations between 0.2 and 200 ng/ml were added to the cells, followed by incubation at 37°C for 30 min.

Histamine was measured using a high performance liquid chromatography (HPLC)-fluorometry method as described previously (Yamatodani et al., 1985). Briefly, histamine was eluted with 0.25 M potassium phosphate, post-labeled with *o*-phthalaldehyde under alkaline conditions, and then detected using a fluorescence detector (F1080 Fluorometer, Hitachi, Tokyo, Japan) at excitation and emission wavelengths of 350 and 460 nm, respectively.

Net and spontaneous releases were calculated using the following equations:

$$\text{Net release (\%)} = \frac{(HA_s - HA_u)}{(HA_t - HA_u)} \times 100\%$$

$$\text{Spontaneous release (100\%)} = \frac{HA_u}{HA_t} \times 100\%$$

Where HA_s is the histamine content in the supernatant of stimulated cells, HA_u is the histamine content in the supernatant of unstimulated cells, and HA_t is the total histamine content. Finally, the rate of histamine release was calculated as the sum of

the net and spontaneous releases.

2.4 HDC activity assay

For DNP-BSA assays, cells were sensitized with 0.5 $\mu\text{g/ml}$ monoclonal IgE overnight, DNP-BSA (20 ng/ml) or PMA (50 nM) was added to each well, and then the cells were incubated at 37°C for 4 h. The cells were washed twice and then HDC buffer consisting of 0.1 M potassium phosphate buffer (pH 6.8) containing 0.2 mM dithiothreitol, 0.01 mM pyridoxal-5'-phosphate, 1% polyethylene glycol, and 100 $\mu\text{g/ml}$ phenylmethylsulfonyl fluoride was added. The cells were sonicated for 20 s and then centrifuged at 10,000 g for 10 min. The supernatant was dialyzed thrice against 30 volumes of HDC buffer. The dialyzed enzyme solution was incubated with 0.25 mM histidine at 37°C for 2 h in 0.25 ml HDC solution. The reaction was stopped by adding 10 μL of 60% perchloric acid. After centrifugation at 10,000 g for 5 min, histamine content was determined by HPLC-fluorometry, and the total protein quantity was measured using Bio-Rad protein assay dye (BioRad Laboratories, Mississauga, Canada). HDC activity is expressed as the generated histamine (pmol) per minute per mg protein (Maeyama et al., 1988).

2.5 Histamine and serotonin uptake

After the cells had been disseminated in a 24-well culture plate, 100 μ M histamine was added exogenously, and the cells were incubated for 12 h. Various concentrations of histamine were used for the concentration response experiment. For the time course experiment, 100 μ M histamine was added exogenously, and the cells were incubated at 37°C with 5% CO₂ for various times (1–24 h). For the histamine uptake inhibition experiment, inhibitors were added at 15 min before exogenous histamine addition, and then the cells were incubated for the indicated times at 37°C. After incubation, the medium was removed, and the cells were washed twice with 750 μ l PIPES buffer at pH 7.2 (25 mM PIPES, 119 mM sodium chloride, 5 mM potassium chloride, 5.6 mM glucose, 1 mM calcium chloride, 40 mM sodium hydroxide, and 0.1% BSA). For cell reserpinization, the cells were preincubated reserpine (10 nM) was with for 15 min before addition of any other inhibitor. Then, the cells were sonicated, and the histamine content of the supernatant was determined by HPLC-fluorometry as described above.

For the serotonin uptake measurement, serotonin (100 mM) was added exogenously, and the cells were incubated for 12 h. To measure the total serotonin content, 500 μ l PIPES buffer was added to each well, and the cells were sonicated. Then, 100 μ L cell homogenate was mixed with 150 μ l of 5% perchloric acid and 25 μ l

of 2 M KOH/1 M KH₂PO₄. The mixture was centrifuged at 10,000 *g* for 5 min at 4°C, and the serotonin content was determined by HPLC with electrochemical detection as described previously (Yosida et al. 2006). A 200 µl aliquot of the supernatant was injected directly into a column (6.0 mm i.d. × 15 cm length) packed with TSKgel SP-2SW cation exchanger, and the bound materials were eluted with 0.22 M lithium propionate buffer (pH 6.4 adjusted with lithium hydroxide containing 3.4 mM EDTA) at a flow rate of 0.85 ml/min. The eluted serotonin was detected using an electrochemical detector (ALS/HCH electrochemical analyzer model 800, ALS Co. Ltd. Tokyo, Japan) at 0.75 V for the working electrode potential.

2.6 *β*-Hexosaminidase activity assay

A 50 µl aliquot of the same DNP-BSA-stimulated supernatant described for the histamine release experiment was collected. The enzymatic reaction was initiated by addition of 100 µl of 2.5 mM *p*-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside in 50 mM citrate buffer (pH 4.5) as the substrate, and the mixture was incubated at 37°C for 90 min. The reaction was subsequently stopped by adding 15 µl of 2 M KOH/1 M KH₂PO₄, and the absorbance of the reaction mixture was measured colorimetrically at 405 nm (Maeyama et al., 1991).

2.7 Inhibition assay of transporters

RBL-2H3 Sc98 cells were seeded in 96-well culture plates (Falcon, Corning, NY, USA) at a density of 5×10^4 cells/0.2 ml medium per well. The cells were preincubated with various concentrations of the transporter inhibitor at 37°C for 15 min and then loaded with 5 μ M FFN206 for 1 h. After incubation, the cells were washed, and trapped FFN206 in the cells was measured using a fluorometric imaging plate reader (FlexStation II, Molecular Devices) at excitation and emission wavelengths of 350 and 460 nm, respectively.

Cell viability was determined using water-soluble tetrazolium (WST)-1 cell proliferation assay reagent (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's protocol. The absorbance was measured colorimetrically at 450 nm using a Bio-Rad 550 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.8 Immunoprecipitation and western blotting

Immunoprecipitation and western blotting were performed according to a previously described method (Liu et al., 2014). In brief, RBL-2H3 and RBL-2H3 Sc98

cells were lysed in ice-cold lysis buffer (10 mM EDTA, 50 mM 2-amino-2-hydroxymethylpropane-1,3-diol-hydrochloride, 150 mM NaCl, 1% Triton X-100, 2 mM phenylmethylsulphonyl fluoride, 2 mM sodium orthovanadate, 10 mg/mL leupeptin, and 2 mg/ml aprotinin). For immunoprecipitation, 3 µg rabbit polyclonal anti-OCT1 antibody, rabbit monoclonal anti-OCT2 antibody, rabbit polyclonal anti-OCT3 antibody, or mouse monoclonal anti-PMAT antibody were added to 300 µl cell extract, followed by incubation for 1 h at 4°C. Next, 30 µl of a 1:1 slurry of protein G Sepharose 4B beads was added to the antibody-extract mix, followed by incubation for 1 h at 4°C. The beads were washed thrice for 10 min each with binding buffer, and proteins were released from the beads by addition of 30 µl sodium dodecyl sulfate loading buffer. Aliquots (25 µl) of the solution were loaded onto 12% denaturing polyacrylamide gels and transferred onto a polyvinylidene difluoride membrane. The blots were blocked with 5% non-fat dry milk and then incubated overnight with the same primary antibodies against each transporter. After another series of washes, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG for 1 h at 25°C. The blots were then treated with a chemiluminescent substrate (enhanced chemiluminescence ECL prime western blotting detection reagent, GE Healthcare, UK

Ltd., England, UK).

2.9 Immunofluorescence analysis

RBL-2H3, RBL-2H3 Sc98, and rat peritoneal mast cells were purified using the Percoll gradient method (Inagaki et al., 2001), plated onto BD BioCoat Cell ware poly-L-lysine-coated coverslips, and immersed in a 50:50 mixture of ice-cold methanol:acetone for 5 min. After blocking with 3% BSA in phosphate-buffered saline, the cells were incubated with a rabbit polyclonal anti-OCT1 antibody, rabbit monoclonal anti-OCT2 antibody, rabbit polyclonal anti-OCT3 antibody, or mouse monoclonal anti-PMAT antibody at 4°C for overnight. The cells were then incubated with Alexa Fluor 488-conjugated anti-rabbit IgG or anti-mouse IgG as secondary antibodies at room temperature for 1 h. After rinsing, the cells were mounted using ProLong™ Gold Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI). Images were captured under a fluorescence microscope. After background subtraction, the positive area was segmented from the background image, and the average intensity was quantified by ImageJ. More than 25 cells for per sample were used for semiquantitative analysis. The average intensity was divided by the number of cells to determine the average intensity per cell.

2.10 Data analysis

Results are expressed as the mean $\pm$ standard error of the mean (S.E.M). Inhibition curves were fitted by non-linear regression using Prism (GraphPad Software Inc., La Jolla, CA, USA). The sample distributions were analyzed by the Kolmogorov-Smirnov test. Pair-wise comparisons were performed using the two-tailed Student's *t*-test to evaluate the statistical significance of differences in serotonin and histamine uptake of RBL-2H3 Sc98 cells, inhibition of histamine uptake by uptake-2 transporter inhibitors, and expression of transporters detected by western blotting. Two-factor factorial analysis of variance followed by the Scheffé F-test were used to compare the dose response curves of increased antigen stimulation to mediator releases, and time-dependent cell viability of D-22 treatment at the indicated dosages to account for unequal variance. $P < 0.05$ was considered as significant.

3. Results

3.1 Characteristics of RBL-2H3 Sc98 cells

RBL-2H3 Sc98 cells had a small amount of endogenous histamine, which was 0.43% of that in RBL-2H3 cells (Table 1). In RBL-2H3 cells, the basal HDC activity level was $21.68 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, which increased by 1.80- and 3.38-fold after stimulation with DNP-BSA and PMA, respectively. However, HDC activity was not detected without DNP-BSA and PMA stimulation or even after treatment of RBL-2H3 Sc98 cells with DNP-BSA or PMA. These results suggested that the low content of histamine in RBL-2H3 Sc98 cells is due to the lack of HDC activity.

3.2 Potential uptake and exocytotic abilities of RBL-2H3 Sc98 cells

Next, we examined histamine uptake by RBL-2H3 Sc98 cells. For the concentration response experiments, histamine at a concentration range between 0 and $100 \text{ } \mu\text{M}$ was added exogenously, and then the cells were incubated overnight. Fig. 1A shows that, without exogenous histamine treatment, the histamine content of RBL-2H3 Sc98 cells was very low. After exogenous histamine loading, RBL-2H3 Sc98 cells took up histamine in an exogenous histamine concentration-dependent manner. The calculated half maximal effective concentration (EC_{50}) value was $24.46 \pm 1.61 \text{ } \mu\text{M}$. A

time course experiment was conducted by exogenous addition of 100 μ M histamine to the cells, followed by incubation for various times. These experiments also showed that histamine taken up by RBL-2H3 Sc98 cells was increased with the incubation time (Fig. 1B). The histamine uptake almost reached a plateau after 20 h of incubation. These results suggested that RBL-2H3 Sc98 cells have the ability to take up histamine from the microenvironment.

Next, we examined whether the exocytotic process occurs normally in RBL-2H3 Sc98 cells after uptake and storage of histamine in cytoplasmic granules. Histamine and β -hexosaminidase release induced by DNP-BSA stimulation as an antigen was measured in cells sensitized with a monoclonal IgE against DNP-BSA. In RBL-2H3 Sc98 cells without exogenous histamine treatment, the percentage of histamine release after stimulation was very low owing to the low content of histamine in these cells (Fig. 1C). However, once extracellular histamine was taken up into the cells and stored in granules, histamine release occurred following stimulation with DNP-BSA in a concentration-dependent manner. We confirmed the exocytotic process in these mutant cells by measuring β -hexosaminidase release. As shown in Fig. 1D, release of β -hexosaminidase was observed both with and without addition of exogenous histamine. These results suggested that, although RBL-2H3 Sc98 cells had lost their

ability to synthesize histamine, the exocytotic process still occurred after stimulation. However, the percentage of histamine released after exogenous histamine addition and β -hexosaminidase released from RBL-2H3 Sc98 cells was lower than those released from RBL-2H3 cells. The maximum releases from RBL-2H3 Sc98 cells were $26.07\% \pm 0.13\%$ for histamine release after exogenous histamine addition, $11.56\% \pm 1.91\%$ without exogenous histamine addition, and $16.15\% \pm 2.73\%$ for β -hexosaminidase release after exogenous histamine addition. In contrast, wild-type cells showed maximum histamine and β -hexosaminidase releases of $40.50\% \pm 2.54\%$ and $26.45\% \pm 0.58\%$, respectively.

Wild-type RBL-2H3 cells have also shown the ability to take up serotonin from the microenvironment (Botchway et al., 2008). In this study, we determined whether RBL-2H3 Sc98 cells could also take up serotonin. We anticipated that serotonin and histamine might be transported into RBL-2H3 Sc98 cells by the same transporter. Therefore, we measured serotonin and histamine uptake by RBL-2H3 Sc98 cells in the presence of both compounds at the same concentration ($100 \mu\text{M}$) (Fig. 1E & 1F). The serotonin and histamine contents of the cells in the absence of exogenous addition were very low. The cells took up both serotonin and histamine after exogenous addition of a single compound (i.e., serotonin or histamine alone). Serotonin and

histamine uptake was decreased when both compounds were added together with 27.4% and 63.6% suppression of serotonin and histamine uptake, respectively. Although further kinetic analysis needs to be carried out, our results clearly show that histamine and serotonin compete for the same transporter.

3.3 Inhibition of histamine uptake by uptake-2 transporter inhibitors

3.3.1 Inhibition of histamine uptake by D-22

We examined the effect of D-22 on histamine uptake by RBL-2H3 Sc98 cells. D-22 is a well-known uptake-2 transporter inhibitor/blocker, especially for OCT-3 and PMAT. We evaluated the cytotoxicity of D-22 in RBL-2H3 Sc98 cells. The cells maintained their viability after only a short incubation period of approximately 60 min in the presence of a low concentration (0.01 μ M) of D-22 (Fig. 2A). Incubation times of more than 60 min or in the presence of D-22 at concentrations greater than 0.01 μ M decreased cell viability. Therefore, to investigate inhibition of histamine uptake by D-22, we treated the cells with a single concentration (0.01 μ M) and preincubation time of 15 min, followed by addition of histamine and incubation for a further 30 min. Using these experimental conditions, D-22 was found to inhibit histamine uptake in RBL-2H3 Sc98 by up to 30.07% $\pm$ 11.43% (Fig. 2B).

3.3.2 Inhibition of histamine uptake by other uptake-2 transporter inhibitors

We investigated the effects of other drugs that are known to be more potent or selective for inhibiting the activity of each uptake-2 transporter. These agents were desipramine, cimetidine, corticosterone, and lopinavir as inhibitors of OCT1, OCT2, OCT3, and PMAT, respectively. As shown in Fig. 2C, histamine uptake in RBL-2H3 Sc98 cells was inhibited most potently by lopinavir ($IC_{50} = 3.78 \mu M$) and less potently by desipramine ($IC_{50} = 20.66 \mu M$). Cimetidine and corticosterone showed no inhibitory effects on histamine uptake.

In addition to histamine uptake, the kinetics of FFN206 uptake was investigated in RBL-2H3 Sc98 cells. The inhibitory effects of the selected transporter inhibitors on the uptake of FFN206 were examined. Lopinavir and desipramine showed inhibitory effects with IC_{50} values of 29.14 and 15.32 μM , respectively, but cimetidine and corticosterone displayed no inhibitory effects (Fig. 2D). These results supported the trend observed in histamine uptake assays.

3.3.3 Blockade of histamine uptake by lopinavir and desipramine in reserpinized cells

To eliminate VMAT2-dependent transport activity and show that desipramine and

lopinavir only inhibit the transport process of the plasma membrane transporter and not the combination of both plasma and vesicle membrane transporters, we applied reserpinized RBL-2H3 cells and RBL-2H3 Sc98 cells to histamine uptake assays. Because reserpine is a VMAT2 inhibitor, pretreatment of cells with reserpine blocks the vesicle membrane transporter and prevents the storage process of histamine in the cellular vesicles.

In RBL-2H3 Sc98 cells, 100 nM reserpine preincubation was performed for 3–4 hours prior to the histamine uptake assay. As shown in Fig. 2E, the histamine content in reserpinized RBL-2H3 Sc98 cells was significantly increased after exogenous histamine addition, indicating histamine uptake into the cells through the plasma membrane transporter. Furthermore, this increase in histamine content was suppressed after lopinavir and desipramine treatments. Because VMAT2 was blocked by reserpine, these results suggest that the histamine uptake suppression was due to inhibition of plasma membrane transporters by lopinavir and desipramine.

RBL-2H3 cells have a very high HDC activity. To block histamine synthesis in the cells, we added α -FMH as a HDC inhibitor in addition to reserpine treatment. As shown in Fig. 2F, the histamine content in RBL-2H3 cells was decreased after reserpine treatment, indicating that the VMAT2 transporter was blocked by reserpine.

After lopinavir and desipramine treatments, the histamine content was increased, suggesting that the plasma membrane transporters were blocked for transport of histamine outside of the cells. These results demonstrated bidirectional histamine transport through the plasma membrane transporter in the RBL-2H3 cell line.

Taken together, these results suggest that uptake-2 transporters are responsible for extracellular histamine uptake, and that VMAT2 contributes to vesicle uptake of intracellular histamine in mast cells.

3.4 Expression profile of an uptake-2 transporter in RBL-2H3 Sc98 cells

To identify potential candidate uptake-2 transporters for extracellular histamine uptake, the expression profiles of OCT1, OCT2, OCT3, and PMAT were assessed in RBL-2H3 Sc98 and wild-type RBL-2H3 cells (Fig. 3). Western blot analysis following immunoprecipitation (Fig. 3A and 3B) showed that OCT1 and PMAT were expressed at relatively high levels in both wild-type and RBL-2H3 Sc98 cells, whereas OCT2 and OCT3 were expressed at very low levels in both cell lines, especially in RBL-2H3 Sc98 cells. The results of immunofluorescence (Fig. 4) suggested that the expression of OCT3 in primary rat peritoneal mast cells was much lower than that in wild-type RBL-2H3 cells. Similar expression patterns of the transporters observed by western

blotting were confirmed in wild-type RBL-2H3 and RBL-2H3 Sc98 cells. Based on these expression patterns, OCT1 and PMAT could be leading candidates response for extracellular histamine uptake in mast cells.

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4. Discussion

The release of mediators from mast cells depends on several molecular pathways such as classical exocytotic processes, *de novo* mediator production, vesicle trafficking and recycling, and exosomal and endosomal pathways. The intracellular resources for histamine release from mast cells include pre-packaged histamine, newly synthesized histamine, and taken up extracellular histamine. The mechanism and contribution of histamine uptake are not well understood, probably because the high level of endogenous histamine present in mast cells usually hampers its uptake. The present study dissected the mechanism of the histamine uptake system and identified possible histamine transporters located on the plasma and vesicle membranes, which may contribute to vesicle recycling and *de novo* mediator production, and therefore modulate the long term exocytotic ability of mast cells.

RBL-2H3 Sc98 cells, a recently identified mutant cell line of RBL-2H3 cells, have a very low histamine content owing to their lack of HDC activity. These cells have become a useful tool to study the mechanism of histamine uptake from extracellular spaces and uptake velocity because of their lack of prepackaged and newly synthesized histamine. The only histamine resource maintained in these cells is acquired by reuptake of extracellular histamine. RBL-2H3 Sc98 cells store histamine taken up from

the microenvironment in exocytotic vesicles via VMAT-2. VMAT-2 is essential for the transport and accumulation of monoamines in storage granules (Travis et al., 2000).

VMAT-2 recognizes histamine as a substrate and transports it via a mechanism different from that of its other substrates such as catecholamines and indoleamines (Merickel and Edwards, 1995). In IgE-sensitized RBL-2H3 Sc98 cells, the taken up and stored histamine was released from the cells after stimulation with the DNP-BSA antigen. The exocytotic process occurred with concomitant release of β -hexosaminidase, another granule-contained mediator.

Although we showed that RBL-2H3 Sc98 cells took up histamine from the microenvironment, it was unclear which transporter mediated its passage across the plasma membrane. Other biogenic amines have selective transporters that belong to the neurotransmitter sodium symporter family, including SERT for serotonin, dopamine transporter for dopamine, and norepinephrine transporter for noradrenaline (Rudnick, 2006). However, a histamine-selective transporter in mast cells has not been reported.

Recently, uptake-2 transporters, including OCT1, OCT2, OCT3, and PMAT, have been shown to transport some biogenic amines such as histamine, serotonin, and catecholamines. The expression of these transporters in human, rat, and mouse tissues has already been reported along with their substrate specificities (Grundemann et al.,

1999; Jonker and Schinkel, 2004; Engel and Wang, 2005; Duan and Wang, 2010; Koepsell, 2013; Duan et al. 2015). To ascertain which protein could potentially act as a histamine transporter in mast cells, we analyzed the ability of drugs, which are known selective inhibitors of each transporter, to suppress histamine uptake in RBL-2H3 Sc98 cells.

Uptake-2 transporters have common biogenic amine substrates. We evaluated the uptake of serotonin, dopamine, adrenaline, and noradrenaline by RBL-2H3 Sc98 cells, and found that they did not take up dopamine, adrenaline, or noradrenaline (data not shown). These results suggest that neuronal transporters DAT and NET are not present in these cells. In contrast, serotonin was taken up into the cells, which might have been facilitated by SERT or uptake-2 transporters. However, when both histamine and serotonin were added to the system, the uptake of amines was suppressed, suggesting that histamine and serotonin may compete for the same transporter. Because suppression of histamine uptake was greater than that of serotonin, the transporter appears to have a higher affinity for serotonin than histamine. Previously, it was reported that histamine is transported via OCT2 and OCT3, while serotonin is transported by OCT1 and OCT2 (Grundemann et al., 1999; Jonker and Schinkel, 2004).

Schneider et al. (2005) showed that histamine is taken up into murine basophils by OCT3, and revealed D-22 as a potent selective inhibitor of OCT3 (Duan et al., 2015). Because basophils and mast cells have properties that are similar to those of cytoplasmic granule-containing cells, OCT3 is capable of mediating histamine transport into RBL-2H3 mast cells. Therefore, we examined whether D-22 inhibits histamine uptake in RBL-2H3 Sc98 cells. Using D-22 at a single concentration of 0.01 μ M and total contact time of 45 min, histamine uptake was suppressed by approximately 30%. Other reported experimental methods, such as radioisotope labeling and fluorescence imaging, have demonstrated that D-22 is an effective inhibitor after only a short incubation period of 5–15 min (Schomig et al. 1993; Gasser et al., 2006; Inyushin et al., 2010).

Other inhibitors of each selected transporter, namely desipramine, cimetidine, corticosterone, and lopinavir, were used to block OCT1, OCT2, OCT3, and PMAT, respectively (Duan et al., 2015). Among these inhibitors, lopinavir suppressed histamine uptake the most in RBL-2H3 Sc98 cells. Desipramine slightly inhibited histamine uptake, whereas cimetidine and corticosterone did not show any inhibitory activities. There is no evidence that indicates inhibitory activities of lopinavir and desipramine against VMAT2. Some reported substrates and inhibitors of VMAT2

include reserpine, tetrabenazine, 3-amino-2-phenylpropene, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, 1-methyl-4-phenylpyridinium, and their respective derivative compounds (Wimalasena, 2011) with essentially different structures from lopinavir and desipramine. Therefore, further studies are required to investigate the effects of lopinavir and desipramine on VMAT2. However, using the RBL-2H3 cell line for this purpose will be challenging because there are both plasma and vesicle membrane transporters. Using reserpinized RBL-2H3 cells, the results suggested that the changes in histamine transport by lopinavir and desipramine were due to their activities towards only the plasma membrane transporter in RBL-2H3 cells and not in combination with vesicle membrane transporters.

In this study, we also profiled the expression of the transporters in wild-type RBL-2H3 cells, RBL-2H3 Sc98 cells, and rat peritoneal mast cells. OCT1 and PMAT were expressed at high levels in both RBL-2H3 Sc98 and RBL-2H3 cells. However, OCT2 and OCT3 were weakly or least detected in RBL-2H3 Sc98 cells. The patterns of protein expression in RBL-2H3 Sc98 cells were in parallel with the results obtained in the inhibitor experiments. OCT1 mRNA has also been detected in murine basophils, but its role in histamine uptake was not considered because [^3H]-histamine was poorly transported into OCT1-transfected 293 cells (Schneider et al. 2005). Histamine uptake

was suppressed by a high concentration of the OCT1 inhibitor desipramine in RBL-2H3 Sc98 cells. Therefore, OCT1 might be involved in histamine uptake at a high concentration. However, because of the low level of OCT3 expression and lack of histamine uptake suppression by corticosterone in RBL-2H3 Sc98 cells, the involvement of OCT3 in histamine uptake may be negligible in RBL-2H3 cells. These findings suggest that PMAT and OCT1 might act as histamine transporters in RBL-2H3 cells.

In the present study, histamine uptake into mast cells was directly dissected using a histamine-depleted mutant mast cell line, RBL-2H3 Sc98. Uptake-2 transporters modulate histamine uptake in mast cells, and PMAT and OCT1 may be established as candidate transporters for this uptake by studying the effect of various inhibitors. Our results suggest that exogenous histamine regulates mast cell functions and outward histamine release might affect allergic reactions and the inflammatory response, which is mediated by mechanisms other than conventional exocytosis.

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Conflict of Interest

The authors declare that they have no conflict of interest in relation to the content of this article.

References

- Barsumian, E.L., Iversky, C., Marianne, G.P., Siraganian, R.P., 1981. IgE-induced histamine release from rat basophilic leukemia cell lines: isolation of releasing and releasing clones. *Eur. J. Immunol.* 11, 317-323.
- Botchway, S.W., Parker, A.W., Bisby, R.H., Crisostomo, A.G., 2008. Real-time cellular uptake of serotonin using fluorescence lifetime imaging with two-photon excitation. *Micros. Res. Tech.* 71, 267-273.
- Cabut, M., Haegermark, O., 1966. Uptake, storage and release of histamine by rat peritoneal mast cells in vitro. *Acta. Physiol. Scand.* 68, 206-214.
- Day, M., Green, J.P., 1962. The uptake biogenic amines by neoplastic mast cell in culture. *J. Physiol.* 164, 227-237.
- Duan, H., Hu, T., Foti, R.S., Pan, Y., Swaan, P.W., Wang, J., 2015. Potent and selective inhibition of plasma membrane monoamine transporter by HIV protease inhibitors. *Drug Metab Dispos* 43, 1773-1780.
- Duan, H., Wang, J., 2010. Selective transport of monoamine neurotransmitters by human plasma membrane monoamine transporter and organic cation transporter 3. *J. Pharmacol. Exp. Ther.* 335, 743-753.
- Engel, K., Wang, J., 2005. Interaction of organic cations with a newly identified

- plasma membrane monoamine transporter. *Mol. Pharmacol.* 68, 1397-1407.
- Gasser, P.J., Lowry, C.A., Orchinik, M., 2006. Corticosterone-sensitive monoamine transport in the rat dorsomedial hypothalamus: potential role for organic cation transporter 3 in stress-induced modulation of monoaminergic neurotransmission. *J. Neurosci.* 26(3), 8758-8766.
- Gilfillan, M.A., Beaven M.A., 2012. Regulation of mast cell responses in health and disease. *Crit Rev Immunol.* 31, 475-529.
- Grundemann, D., Liebich, G., Kiefer, N., Koster, S., Schomig, E., 1999. Selective substrate for non-neuronal monoamine transporters. *Mol Pharmacol.* 56, 1-10.
- Husztai, Z., 2003. Histamine uptake into non-neuronal brain cell. *Inflamm Res*, 1:S03-6
- Inagaki, N., Nakai, N., Kimata, M., Kawasaki, H., Nagai, H. 2001. Recovery of purification-associated reduction in antigen-induced histamine release from rat peritoneal mast cells. *Biol. Pharm. Bull.* 24, 829-834.
- Inyushin, M., Kucheryavykh, Y., Kucheryavykh, L., Sanabria, P., Jimenez-Rivera, C., Struganova, I., Eaton, M., Skatchkov, S., 2010. Membrane potential and pH-dependent accumulation of decynium-22 (1,1'-diethyl-2,2'-cyanine iodide) fluorescence through OCT transporter in astrocytes. *Bol. Asoc. Med. P. R.* 102, 5-12.

- Jonker, J.W., Schinkel, A.H., 2004. Pharmacological and physiological functions of the polyspecific organic cation transporter* OCT2, 2, and 3 (SLC22A1-3). *J. Pharmacol. Exp. Ther.* 308, 2-9.
- Kiyoi, T., Liu, S., Sahid, M.N.A., Shudou, M., Maeyama, K., Mogi, M., 2018, High-throughput screening system for dynamic monitoring of exocytotic vesicle trafficking in mast cells. *PLoS One* 8, e0198785.
- Koepsell, H., 2013. The SLC22 family transporters of organic cations, anions, and zwitterions. *Mol. Asp. Med.* 34, 413-435.
- Komazawa, H., Yamaguchi, H., Hidaka, K., Ogura, J., Kobayashi, M., Iseki, K., 2013. Renal uptake of substrates for organic anion transporters Oat1 and Oat3 and organic anion transporter Oct1 and Oct2 is altered in rats with adenine-induced chronic renal failure. *J. Phar. Sci.* 102(3), 1086-1094.
- Kulczycki, A. Jr., Isersky, C., Metzger, H., 1974. The interaction of IgE with rat basophilic leukemia cells I. Evidence for specific binding of IgE. *J. Exp. Med.* 139, 600-616.
- Liu, S., Watanabe, S., Shudou, M., Kuno, M., Miura, H., Maeyama K., 2014. Upregulation of store-operated Ca^{2+} entry in the naïve CD4^{+} T cells with aberrant cytokine releasing in active rheumatoid arthritis. *Immunol.Cell Biol.*

92, 752-760.

- Maeyama, K., Hohman, R.J., Metzger, H., Beaven, M.A., 1986. Quantitative relationships between aggregation of IgE receptors, generation of intracellular signals, and histamine secretion in rat basophilic leukemia (2H3) cells - Enhanced response with heavy water. *J. Biol. Chem.* 261, 2583-2592.
- Maeyama, K., Sasaki, M., Watanabe, T., 1991. Simultaneous determination of intracellular calcium concentration and histamine secretion in rat basophilic leukemia cells (RBL-2H3). *Anal. Biochem.* 194, 316-325.
- Maeyama, K., Taguchi, Y., Sasaski, M., Wada, H., Beaven, M.A., Watanabe, T., 1988. Induction of histidine decarboxylase of rat basophilic leukemia (2H3) cells stimulated by higher oligomeric IgE or phorbol myristate acetate. *Biochem. Biophys. Res. Comm.* 151, 1402-1407.
- Merickel, A., Edwards, R.H., 1995. Transport histamine by Vesicular Monoamine Transporter-2. *Neuropharmacol.* 34, 1543-1547.
- Perdan-Pirkmajer, K., Pirkmajer, S., Raztresen, A., Krzan, M., 2013. Regional characteristics of histamine uptake into neonatal rat astrocytes. *Neurochem Res.* 38, 1348-1359.
- Rudnick, G., 2006. Structure/function relationship in serotonin transporter: New

- insights from the structure of a bacterial transporter, in: Sitte, H., Freissmuth, M. (Eds.), Neurotransmitter Transporters. Springer-Verlag Berlin, Heidelberg, pp. 59-73.
- Sakurai, E., Sakurai, E., Orelund, L., Nishiyama, S., Kato, M., Watanabe, T., Yanai, K., 2006. Evidence for the presence of histamine uptake into the synaptosomes of rat brain. *Pharmacology*. 78, 72-80
- Schneider, E., Machavoine, F., Pleau J.M., Bertron, A.F., Thurmond, R.L., Ohtsu, H., Watanabe, T., Schinkel, A.H., Dy, M., 2005. Organic cation transporter 3 modulates murine basophil functions by controlling intracellular histamine levels. *J. Exp. Med.* 202, 387-393.
- Schomig E., Babin-Ebell, J., Russ, H., 1993. 1,1'-Diethyl-2,2'-cynine (decynium22) potently inhibits the renal transport of organic cations. *Naunyn-Schmiedeberg Arch Pharmacol.* 347, 379-383.
- Tanaka, S., Nemoto, K.I., Yamamura, E., Ichikawa, A., 1998. Intracellular localization of the 74- and 53-kDa forms of L-histidine decarboxylase in rat basophilic/mast cell line, RBL-2H3. *J. Biol. Chem.* 273, 8177-8182.
- Travis, E.R., Wang, Y.M., Michael, D.J., Caron, M.G., Wightman, R.M., 2000. Differential quantal release of histamine and 5-hydroxytryptamine from mast

cells of vesicular monoamine transporter 2 knockout mice. PNAS. 97, 162-167.

Tuomisto, J., Tuomisto, L., 1980. Effects of histamine and histamine antagonists on the uptake and release of catecholamines and 5-HT in brain synaptosomes. Med Biol, 58, 33-37.

Wimalasena, K., 2011. Vesicular monoamine transporter: Structure-function, pharmacology, and medicinal chemistry. Med Res Rev, 31, 483-519.

Yamatodani, A., Fukuda, H., Wada, H., Iwaeda, T., Watanabe, T., 1985.

High-performance liquid chromatographic determination of plasma and brain histamine without previous purification of biological samples: cation-exchange chromatography coupled with post-column derivatization fluorometry. J. Chromatogr. 344, 115-123.

Figure Legends

Fig. 1 Histamine uptake by rat basophilic leukemia (RBL)-2H3 Sc98 cells. (A)

Dose response of histamine uptake in RBL-2H3 Sc98 cells. The indicated concentrations of histamine were added exogenously, and cells were

incubated for 12 h. The total amount of histamine taken up showed a concentration-dependent response based on the amount of exogenous histamine added to the medium. Values are shown as the mean $\pm$ S.E.M.

(n=3–5). (B) Time course of histamine uptake. After addition of 100 μ M exogenous histamine, cells were incubated for the indicated times. Values are shown as the mean $\pm$ S.E.M. (n=3–5). Exocytosis in RBL-2H3 and

RBL-2H3 Sc98 cells after stimulation with IgE-DNP-BSA was monitored by histamine (C) and β -hexosaminidase (D) release. The release of mediators

was examined in RBL-2H3 and RBL-2H3 Sc98 cells with and without 12 h of preincubation with exogenous histamine. Net release is plotted against the

DNP-BSA concentration. Values are shown as the mean $\pm$ S.E.M. (n=5). The potential uptake ability of RBL-2H3 Sc98 cells was also assessed by

evaluation of serotonin (E) and histamine (F) release after coincubation with

serotonin and histamine for 12 h. Values are shown as the mean $\pm$ S.E.M

(n=3–5, * $P < 0.05$).

Fig. 2 Uptake-2 transporters modulate histamine uptake in mast cells. (A)

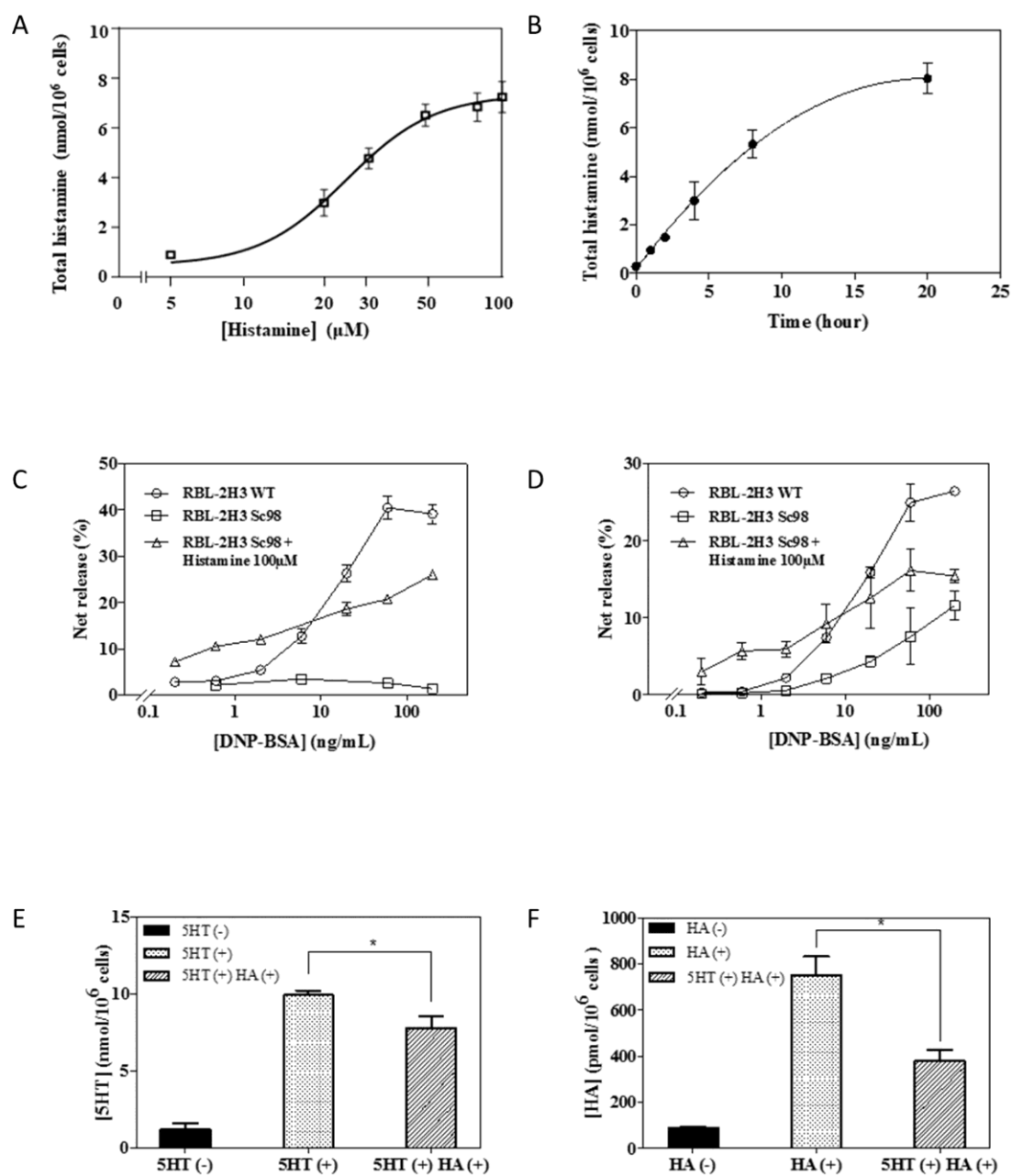
Cytotoxicity of D-22 in RBL-2H3 Sc98 cells was determined using WST-1 cell proliferation reagent. Cells were incubated for various times, 15 min, 30 min, 60 min, 2 h, and overnight (12 h), with different concentrations of D-22 (0.01, 0.1, 1, and 10 μ M). To evaluate histamine uptake by cells, histamine (100 μ M) was preincubated with cells for 12 h. (B) Blockade of histamine uptake by D-22. RBL-2H3 Sc98 cells were preincubated with D-22 for 15 min, and then the culture medium was replaced with fresh histamine (100 μ M)-containing medium. Uptake of histamine occurred for 12 h, and then histamine content was analyzed in the cells. Values are shown as the mean $\pm$ S.E.M. (n=5, * $P < 0.05$) (C) Dose response of histamine uptake inhibition by desipramine, cimetidine, corticosterone, and lopinavir. Before histamine uptake, RBL-2H3 Sc98 cells were preincubated with the regents at the indicated doses for 15 min. Histamine uptake occurred for 12 h with 100 μ M extracellular histamine. Values are shown as the mean $\pm$ S.E.M. (n=5). (D)

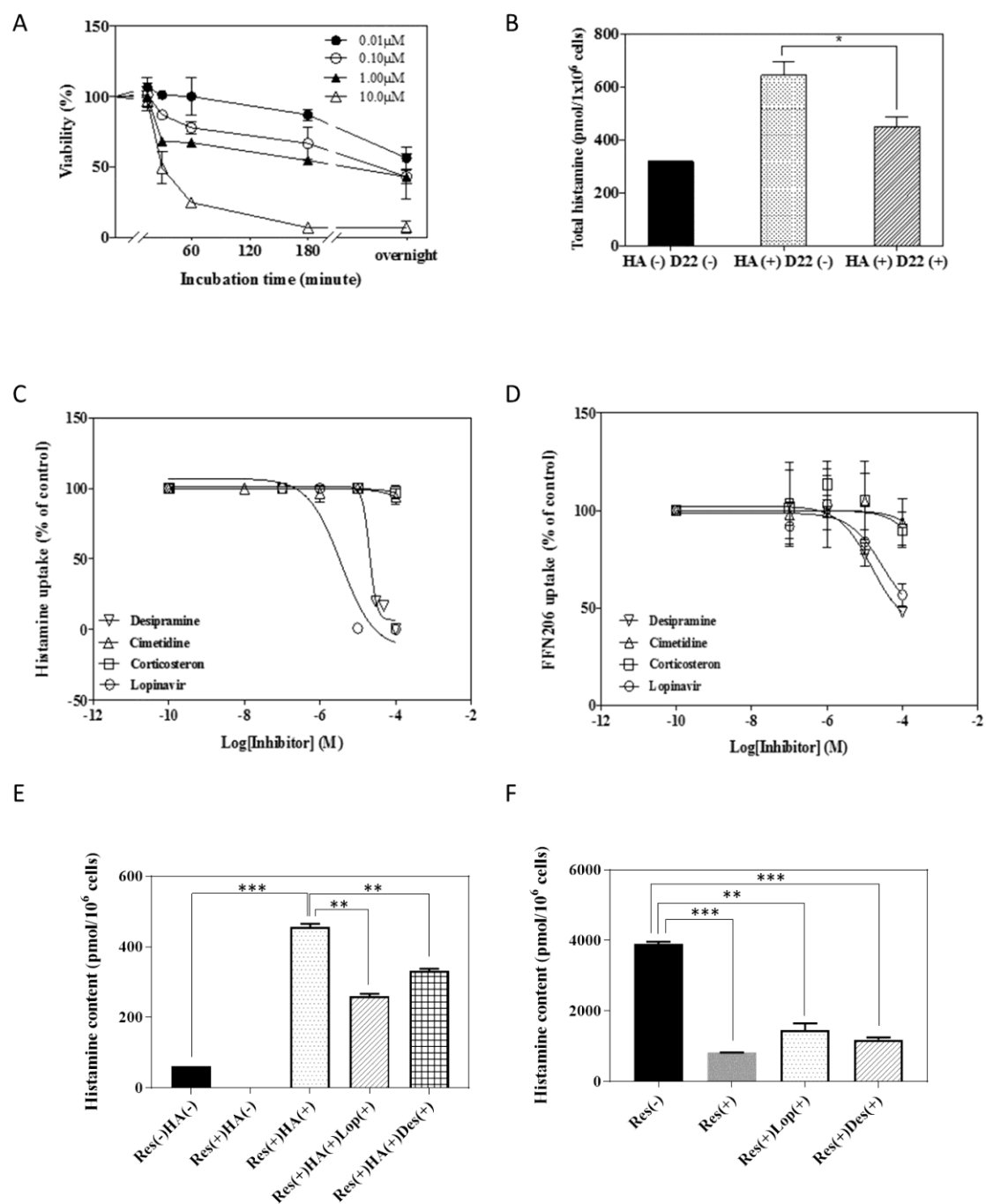
Dose response of FFN206 uptake inhibition by desipramine, cimetidine, corticosterone, and lopinavir. After 15 min of preincubation with the inhibitors, FFN206 was loaded into the cells for 2 h. Images were captured by a high throughput imaging analysis system, and the fluorescence intensity was evaluated. Values are shown as the mean $\pm$ S.E.M. (n=5). (E) Histamine uptake inhibition in reserpinized RBL-2H3 Sc98 cells by lopinavir and desipramine. For cell reserpinization, cells were preincubated with reserpine for 15 min before addition of any other inhibitor. Values are shown as the mean $\pm$ S.E.M. (n=5, ** P < 0.01; *** P < 0.001) (F) Histamine uptake inhibition in reserpinized wild-type RBL-2H3 cells by lopinavir and desipramine. Values are shown as the mean $\pm$ S.E.M (n=5, *** P < 0.001).

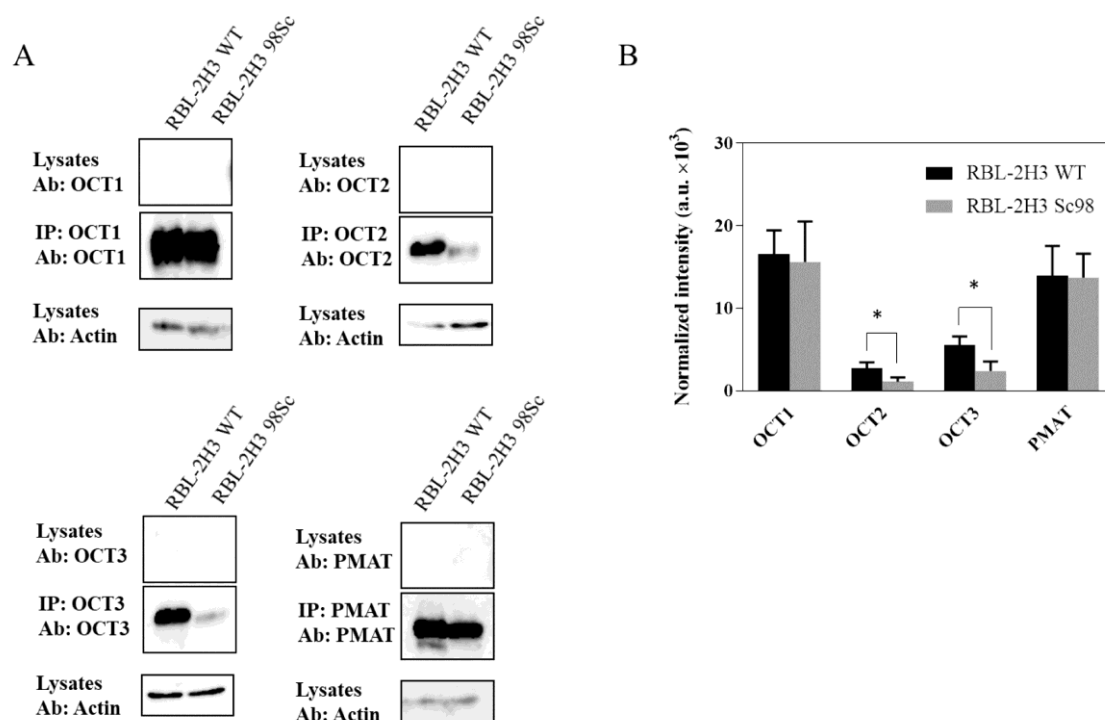
Fig. 3 Expression profiles of uptake-2 transporters in mast cells. The expression of OCT-1, OCT2, OCT3, and PMAT in RBL-2H3 and RBL-2H3 Sc98 cells was detected by western blotting after each transporter was precipitated from cell lysates using specific antibodies. (A) Representative blots of OCT-1, OCT2, OCT3, and PMAT in wild-type RBL-2H3 and RBL-2H3 Sc98 cells. Samples were derived from the same experiment, and gels/blots were

processed in parallel. IP, immunoprecipitated; Ab; antibody used for protein labeling. (B) Relative protein expression of each transporter in RBL-2H3 and RBL-2H3 Sc98 cells. Values are shown as the mean $\pm$ S.E.M (n=3, * P < 0.05).

Fig. 4 Immunofluorescence staining of uptake-2 transporters in mast cells. The expression of uptake-2 transporters, including (A) OCT1, (B) OCT2, (C) OCT3, and (D) PMAT, in primary rat peritoneal mast cells (RPMCs), wild-type RBL-2H3 cells (RBL-2H3 WT), and RBL-2H3 Sc98 cells was detected by immunofluorescence. The cells were stained for the indicated transporters (green) and with DAPI (blue). A representative overlay image ($\times 600$) is shown (scale bar: 20 μ m). (E) Average fluorescent intensity of each transporter in RPMCs, RBL-2H3 WT, and RBL-2H3 Sc98 cells. The average intensity was divided by the number of cells to determine the average intensity per cell. Values are shown as the mean $\pm$ S.E.M (n=25–37, * P < 0.05, ** P < 0.01).







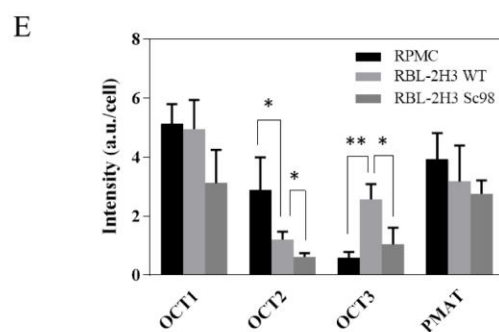
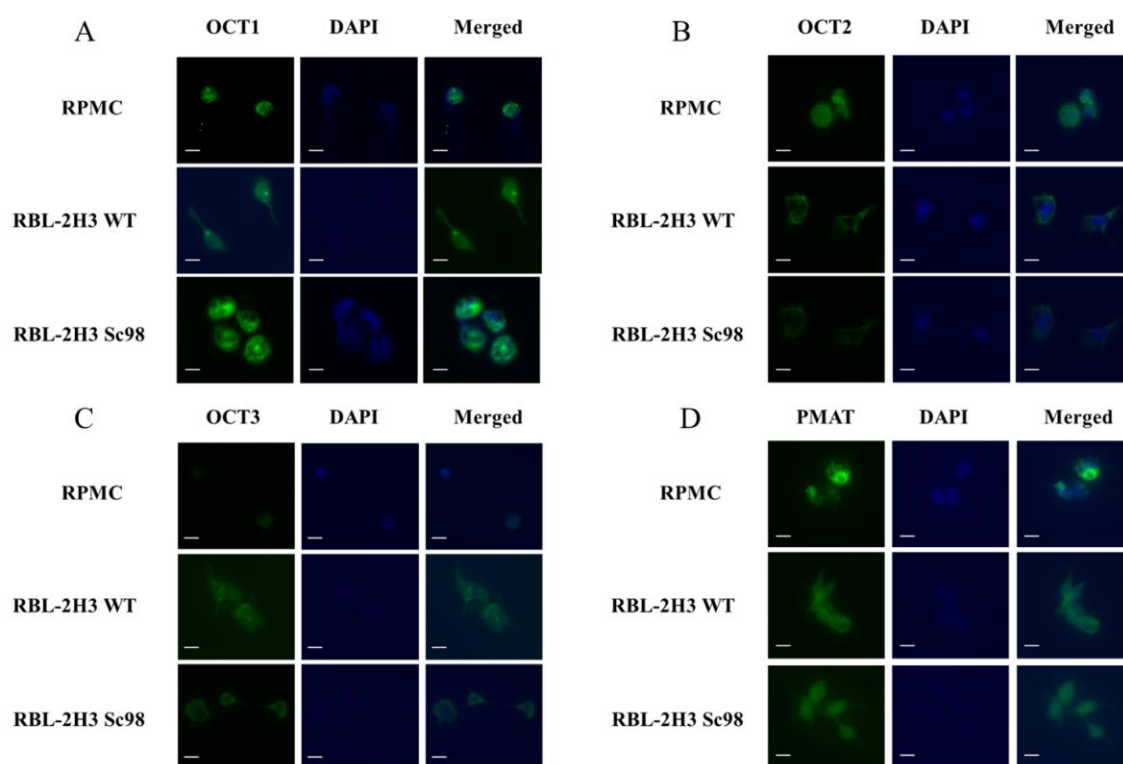


Table 1. Histamine content and histidine decarboxylase (HDC) activity of rat basophilic leukemia (RBL)-2H3 and RBL-2H3 Sc98 cells

	RBL-2H3	RBL-2H3 Sc98
Endogenous histamine (pmol/10 ⁶ cells)	20521.53 ± 452.63	89.18 ± 1.98
HDC activity (pmol·min ⁻¹ ·mg protein ⁻¹)	No stimulation	21.68 ± 0.47
	50nM PMA stimulation	73.32 ± 5.90*
	DNP 20ng/mL stimulation	39.00 ± 1.35 ^a
		N.D.

PMA, phorbol myristate acetate; N.D., not detected; ^a $P < 0.05$: significantly different from no stimulation group.