The Development of Screening Methods to Identify Drugs to Limit ER Stress Using Wild-type and Mutant Serotonin Transporter

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The function of the serotonin transporter (SERT) is regulated by its membrane trafficking. Previously, we showed that the C-terminus-deleted mutant of SERT (SERTΔCT) exhibited an aberrant membrane trafficking and subsequent retention at the endoplasmic reticulum (ER). In addition, we found that proteasome inhibitor-induced ER stress resulted in the impairment of SERT membrane trafficking and retention of SERT at the ER, an impairment very similar to that of SERTΔCT. Based on the result that the chemical chaperone 4-phnylbutulic acid (4-PBA), which relieves ER stress, accelerated the membrane trafficking and upregulated SERT activity, we hypothesized that drugs that facilitate the membrane trafficking of SERT would have potential therapeutic effects on an ER stress-related disease. In this study, we aimed to develop simple screening methods for such drugs using SERT. We first validated the serotonin uptake assay using fluorescent substrates. This simple and reliable assay method was useful for screening for drugs that affected the wild-type SERT but not SERTΔCT. In addition, we verified an assay focusing on the formation of SERTΔCT aggregates. The drugs 4-PBA and SKF-10047 facilitated the trafficking of SERT to the membrane and reduced SERTΔCT aggregates, indicating that the drugs with such characters could be potential candidates for ER stress relief. For both assays, we clarified the usefulness of a high-content screening microscope. These results could pave the way for high-throughput screening for such drugs.

Key words: serotonin transporter, ER stress, membrane trafficking, chemical chaperone, Sigma-1 receptor

I.   Introduction

The serotonin transporter (SERT) terminates serotonergic neural transmission by the reuptake of serotonin into preterminals in the central nervous system [1, 10]. The SERT is a well-known target of antidepressants and is involved in the pathogenic mechanisms of mood disorder, anxiety disorder and autism [3, 7, 11, 14, 15, 18, 19, 23].

The serotonin uptake activity of SERT-expressing cells is regulated by the membrane trafficking of SERT [22, 24, 26, 27]. The SERT is translated and initially glycosylated at the endoplasmic reticulum (ER) and is transferred to the Golgi apparatus, where it is maturely glycosylated. Thereafter, SERT is transferred to the plasma membrane, where it serves its function. To elucidate the functional regulation of SERT, it is important to clarify the molecular mechanism by which SERT membrane trafficking is regulated.

In our previous study, we investigated the role of the SERT C-terminus in its membrane trafficking and glycosylation [13]. For this purpose, we characterized a C-terminus-deleted mutant of SERT (designated as SERTΔCT) by its uptake activity, localization and glycosy-

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lation. The results revealed that the membrane trafficking and glycosylation of SERTΔCT were disturbed and that its expression in the plasma membrane was robustly reduced, suggesting that the SERT C-terminus has a critical role in SERT membrane trafficking and glycosylation [13].

Endoplasmic reticulum stress (ER stress) is implicated in the pathogenesis of various neural diseases, such as neurodegenerative diseases and ischemic brain disease [2, 16, 17]. To elucidate the pathophysiological significance of SERT membrane trafficking in neural disease, we investigated how the SERT function was affected in the case of ER stress induced by proteasome inhibitors [13]. Our previous study demonstrated that ER stress impaired the membrane trafficking of SERT from the ER to the Golgi apparatus, thereby reducing the SERT activity of cells [13]. This result indicated that features of wild-type SERT with ER stress were very similar to that of SERTΔCT. In other words, SERTΔCT mimics the wild-type SERT exposed to ER stress. In addition, we elucidated that the chemical chaperone 4-phnylbutulic acid (4-PBA), which relieves ER stress, facilitated the membrane trafficking of SERT, thereby upregulating the cellular SERT activity [6].

Based on these previous findings, we hypothesized that the screening of drugs that accelerate the membrane trafficking of SERT would be useful for searching for therapeutic drugs that can improve ER stress-related disease. It is also speculated that drugs that modulate the SERT membrane trafficking would have beneficial effects on SERT-related neuropsychiatric diseases. In this study, we aimed to develop simple and valuable methods using SERT that can be applied to the high-throughput screening of such drugs.

II.   Materials and Methods

Materials

Glass-bottom culture dishes were purchased from MatTek Corporation (Ashland, OR, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin mixture were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), Biowest (Rue de la Caille, France) and Nacalai Tesque (Kyoto, Japan), respectively. An anti-DYKDDDDK tag mouse monoclonal antibody was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). An Alexa488-conjugated anti-mouse IgG antibody was purchased from Molecular Probe (Eugene, OR, USA). NucRed® Live 647 ReadyProbes® Reagent was purchased from Thermo Fischer Scientific (Tokyo, Japan); 4-phenylbutylate (4-PBA) and SKF-10047 were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Tocris Bioscience (Bristol, UK), respectively; and [³H] 5-HT (370 GBq/mmol) was purchased from PerkinElmer (Waltham, MA, USA). The Neurotransmitter Transport Assay Kit was purchased from Molecular Device Corporation (Sunnyvale, CA, USA). All other chemicals were of analytical grade.

Cell culture and transfection

A plasmid that can express FLAG-tagged rat SERT in mammalian cells was constructed as described previously [12, 13, 25]. Briefly, a rat SERT cDNA fragment with *Mun*I sites on both the 5' and the 3' ends was obtained by PCR using the full-length rat SERT cDNA as a template. PCR products were subcloned into the pTB701FL expression plasmid, which can add FLAG amino acid residues to the N-terminus of the target protein. The plasmid is designated here as pFLAG-SERT. A plasmid that can express a mutated form of SERT that lacks the C-terminus was made as described previously [13]. This plasmid is designated here as pFLAG-SERTΔCT.

COS-7 cells were cultured in DMEM supplemented with heat-inactivated FBS (10%), penicillin (100 unit/ml) and streptomycin (100 μg/ml). Culturing was performed in a humidified atmosphere containing 5% CO_2 at 37°C. For electroporation, pFLAG-SERT or pFLAG-SERTΔCT was transfected into COS-7 cells using an electroporator NEPA21 (NEPA GENE, Chiba, Japan) according to the protocol recommended by the supplier. Briefly, plasmids of 10 μg were transfected into 2×10^6 COS-7 cells and transfected cells were seeded into appropriate culture dishes.

[³H] 5-HT-Uptake Assay

Approximately 6×10^6 transfected COS-7 cells were equally divided into 24-well plates. As previously described, [³H] 5-HT uptake assays were carried out 48 hr after transfection [12, 13, 20, 21, 25]. In brief, the culture medium was removed and replaced with Krebs-Ringer-HEPES (KRH) buffer, which contained the following (in mM): NaCl, 120; KCl, 4.7; $CaCl_2$, 2.2; HEPES, 25; $MgSO_4$, 1.2; KH_2PO_4, 1.2; and glucose, 10; pH 7.4. After a 15-min pre-incubation period at 37°C, the cells were incubated for an additional 15 min in the presence of [³H] 5-HT. To prevent the degradation of 5-HT, 100 μM pargyline and 100 μM L-ascorbic acid were added to the KRH buffer. The uptake of [³H] 5-HT was stopped by washing three times with cold KRH buffer containing 10 μM fluvoxamine, and the cells were then lysed with RIPA buffer (10 mM Tris-HCl, 1% NP-40, 0.1% SDS, 0.1% sodium deoxycholate, 150 mM NaCl and 1 mM EDTA pH 7.4). The cell extracts were diluted in a scintillation cocktail (Clear-Sol II, Nakalai Tesque, Kyoto, Japan), and their radioactivity was measured with a liquid scintillation counter (LSC-5100, Aloka, Mitaka, Japan). The results were considered to represent the total 5-HT cellular uptake. [³H] 5-HT uptake in the presence of 10 μM fluvoxamine was also measured and was considered to represent the non-specific 5-HT cellular uptake. The specific 5-HT uptake was obtained by subtracting the non-specific 5-HT uptake from the total uptake amount. The 5-HT uptake assay was conducted at a concentration of 100 nM 5-HT. To calculate the uptake per mg of protein in the cells, the concentration of protein in the cell extract was measured using a BCA protein assay kit according to the manufacturer's directions (Pierce Biotech.,

Rockford, IL, USA). The SERT uptake activity was defined as the amount of [³H] 5-HT uptake per mg of protein.

The drugs (4-PBA and SKF-10047) were applied to culture medium 24 hr after the transfection, and cells were cultivated for another 24 hr until the uptake assay was performed.

Measurement of serotonin uptake using a fluorescent substrate

For this purpose, we used a Neurotransmitter Transporter Uptake Assay kit from Molecular Devices Corporation. This fluorescence-based assay can detect dopamine, noradrenaline and serotonin transporter activity in expressing cells. Experimental procedures were performed according to the manufacturer's recommended protocol (https://www.moleculardevices.com/products/assay-kits/neurotransmitter-transporter-uptake-assay-kit).

The wild-type SERT- or SERTΔCT-transfected cells were seeded into 96-well plates (µ-plate, ibid, Martinsried, Germany) at a density of 8000 cells per well. The drug application procedure was the same as that used for the [³H] 5-HT uptake assay. The fluorescence of the substrate incorporated into cells was measured using a high-content screening microscope, Opera Phenix (PerkinElmer, Waltham, MA, USA). For the detection of the cell nucleus, cells were stained with NucRed® Live 647 ReadyProbes® Reagent just before the uptake assay was performed.

The uptake assay started with the application of the fluorescent substrate in the extracellular buffer in each well. Five minutes after the application, the fluorescence of the cells was measured using the Opera Phenix microscope every 5 min for 45 min. The fluorescence of stained nuclei and incorporated substrates was excited by 640 and 488 nm laser-induced fluorescence, respectively. The images obtained were analyzed using Harmony® software associated with the Opera Phenix microscope.

Immunocytochemistry and counting aggregates

The 1×10^5 FLAG-SERTΔCT-transfected cells were seeded on glass-bottom culture dishes for the manual counting of aggregates. For analysis using the Opera Phenix microscope, the 8×10^3 cells were seeded into each well of a 96-well plate. The drugs were applied using the same protocol for the serotonin uptake assay.

Immunocytochemistry was carried out for FLAG-SERTΔCT-expressing COS-7 cells as previously described [12, 13, 20, 21, 25]. In brief, the cells were washed in PBS and fixed with 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 30 min at RT, washed twice with PBS, and then permeabilized with 500 µl PBS supplemented with 0.3% Triton X-100 and 5% normal goat serum (NGS) for 15 min. Then, the cells were washed twice in PBS with 0.03% Triton-X (PBS-T) and incubated for <1 hr at RT with the desired primary antibodies and for <1 hr at RT with the secondary antibodies in PBS-T and 1% NGS. The primary and secondary anti-

bodies used were the anti-DYKDDDDK tag mouse monoclonal antibody (diluted 1:1000) and the anti-mouse IgG conjugated with Alexa Fluor 488 (1:500), respectively.

For the manual counting of the number of aggregates, the fluorescent signals of cells in glass-bottom culture dishes were observed by fluorescence microscopy (BZ900, Keyence, Osaka, Japan). Three independent experiments were carried out. In each experiment, we counted the number of aggregates of 10–20 cells, calculated the average number of aggregates per cell and compared these values between the control and drug-treated groups.

For automatically counting aggregates, the fluorescent signals in the 96-well plate were observed using the Opera Phenix microscope. The NucRed® Live 647 ReadyProbes® Reagent was used for the detection of the nucleus. The fluorescence of the nucleus or FLAG-SERTΔCT was excited by 640 or 488 nm laser-induced fluorescence, respectively. The cell shape, cytosol and intracellular aggregates were recognized by Ready Made Solution soft #15, associated with Harmony® software. The number of aggregates in approximately 50–500 FLAG-SERTΔCT-expressing cells was automatically counted by the Opera Phenix microscope, and the average number of aggregates per cell was calculated.

Statistical analysis

Data analysis was performed and statistics were obtained using Prism 4 software (GraphPad Software, San Diego, CA). Statistical significance was determined by Student's t-tests or one-way ANOVA followed by Dunnett's post-test. If the P value was less than 0.05 (P<0.05), the difference was considered significant.

III. Results

Effects of 4-phenylutyric acid and SKF-10047 on the serotonin uptake activity of SERT and SERTΔCT

First, we focused on the drugs that have or induce chaperone activity. In our previous study, 4-phenylutyric acid (4-PBA), a chemical chaperones, was shown to accelerate the membrane trafficking of SERT, thereby, increasing the SERT activity [6]. To confirm this result, we investigated the effects of 4-PBA on the serotonin uptake activity of wild-type SERT or SERTΔCT, which was transiently transfected in COS-7 cells. For this purpose, we performed the conventional serotonin uptake assay using [³H] serotonin. As shown in Figure 1A, the uptake activity of SERTΔCT was almost 10% of that of wild-type SERT. Treatment with 3 mM 4-PBA for 24 hr increased the SERT activity by 43% or 71% of control levels in wild-type SERT or SERTΔCT-transfected cells, respectively (Fig. 1B), confirming the previous results.

SKF-10047 is known to exert its chaperone activity by binding as an agonist to the Sigma-1 receptor [8]. Therefore, we investigated the effect of SKF-10047, an agonist of the Sigma-1 receptor (Sig-1R), on the serotonin uptake
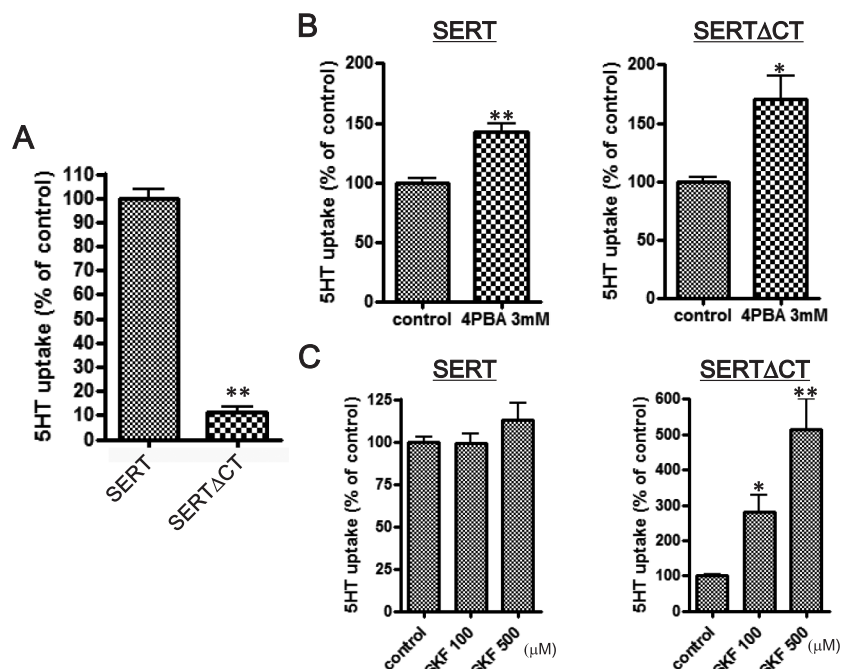
Fig. 1. Effects of 4-PBA and SKF-10047 on the uptake activity of SERT and SERTΔCT. The conventional serotonin uptake assay using [³H] serotonin was carried out for these experiments. **A**: Comparison of the serotonin uptake activity of wild-type SERT and SERTΔCT. The serotonin uptake activity of SERTΔCT was reduced to almost 1/10 of the level of that of wild-type SERT. The data represent the mean±S.E.M (**P<0.01 vs. non-treated control, n=4, Student's t-test). **B**: Effects of 3 mM 4-PBA on the serotonin uptake activity of SERT and SERTΔCT. Serotonin uptake activity was significantly augmented with 4-PBA more prominently in SERTΔCT than wild-type SERT. The data represent the mean±S.E.M (*P<0.05, **P<0.01 vs. non-treated control, n=4, Student's t-test). **C**: Effects of SKF-10047 on the serotonin uptake activity of SERT and SERTΔCT. SKF-10047 exhibited no significant effects on wild-type SERT, whereas SKF-10047 significantly increased the serotonin uptake activity of SERTΔCT in a dose-dependent manner. The data represent the mean±S.E.M (*P<0.05, **P<0.01 vs. non-treated control, n=8, one-way ANOVA with Dunnett's post-test).

activity of wild-type SERT or SERTΔCT. Treatment with SKF-10047 for 24 hr did not increase the activity of wild-type SERT but robustly increased the activity of SERTΔCT (Fig. 1C).

The rate of increase by 4-PBA and SKF-10047 was greater in SERTΔCT-expressing cells than in wild-type SERT-expressing cells. This result suggests that SERTΔCT is more sensitive to drugs with chaperone activity than wild-type SERT. The SERTΔCT would be a beneficial tool as a sensor for screening for drugs that accelerate the membrane trafficking of SERT.

Serotonin uptake assay using a fluorescent substrate for SERT

The conventional serotonin uptake assay using radio isotope-labeled substrate is not suitable for the high-performance screening of numerous drugs because this assay requires many complicated procedures, as described in the materials and methods. To overcome this limitation, we utilized a Neurotransmitter Transport Assay Kit, which was provided by Molecular Device [9]. The principles underlying this method are as follows: 1) the fluorescent substrate for SERT was applied to the SERT-expressing cells in an appropriate extracellular buffer. 2) This substrate was covered with masking dye in extracellular fluid. However, when the fluorescent substrate was incorporated into

cells via SERT, the masking dye detached from the fluorescent substrate. Therefore, only substrates incorporated into cells were detected as fluorescence. The serotonin uptake activity of SERT was reflected by the accumulation of fluorescence in cells. 3) This kit enables the real-time measuring of SERT uptake activity when used with a fluorescence imager such as the Opera Phenix microscope.

The wild-type SERT- or SERTΔCT-transfected cells seeded on 96-well plates were stained with NucRed® Live 647 for later detection of the nucleus. The observation using the Opera Phenix microscope began 5 min after the fluorescent substrates were applied to the cells. The fluorescence of the nucleus, which was detected by NucRed® Live 647, and cytosol, which was detected by the fluorescent substrate incorporated into cells via SERT, were measured every 5 min for 45 min. Figure 2 shows a representative fluorescence image of wells when SERT-transfected cells were seeded. The staining of nuclei showed that the number of cells detected in each well were nearly identical (Fig. 2A). In SERT-transfected cells, the fluorescence of incorporated substrates was apparently increased in control and 4-PBA treated wells at 45 min after the application compared with that at 5 min (Fig. 2B). To determine the non-specific uptake of substrates, the fluorescence was measured in the presence of 10 μM fluvoxamine, an inhibitor of SERT. No obvious change of
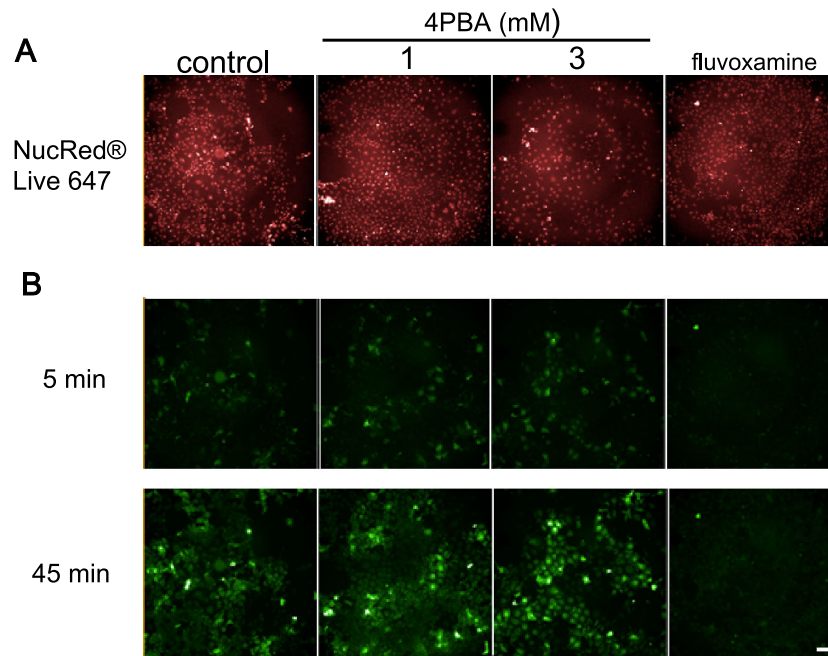
Fig. 2. Representative fluorescent images of SERT-expressing cells in each well. **A**: Nuclear staining by NucRed® Live 647. The fluorescence was obtained by excitation with a 640-nm laser. **B**: The images of fluorescent substrates incorporated into cells. The fluorescence was obtained by excitation with a 488-nm laser. Bar=100 μm.

fluorescence was observed in the fluvoxamine-treated well (Fig. 2B).

The fluorescence intensity of each cell was quantified using the Opera Phenix microscope. The fluorescence intensity of cells treated with target drugs represented the total cellular uptake of the substrate. The specific substrate uptake was obtained by subtracting the non-specific substrate uptake from the total substrate cellular uptake. Figure 3 shows the time course of the fluorescence intensity, which corresponded to the specific substrate uptake of SERT-expressing cells. The substrate uptake was gradually increased up to 45 min. The treatment with 3 mM 4-PBA significantly augmented the substrate uptake of SERT-expressing cells (Fig. 3A). By contrast, the treatment with 500 μM SKF-10047 tended to increase the substrate uptake of SERT, but the change was not significant (Fig. 3B). These results were very similar to those obtained using conventional radio isotope-based methods.

Next, we investigated the uptake of the fluorescent substrate in SERTΔCT-expressing cells. In contrast to the fluorescence change in SERT-expressing cells, there was no obvious change in the fluorescence in SERTΔCT-expressing cells 45 min after the application of the fluorescent substrate (Fig. 4). The fluorescence levels of SERTΔCT-expressing cells seemed to be comparable to those in the fluvoxamine-treated cells. The time courses of the fluorescence intensity are shown in Figure 5. In the 4-PBA-treated group, neither obvious specific uptake of fluorescence substrate nor significant effects of 4-PBA on SERTΔCT uptake activity were observed (Fig. 5A). In the
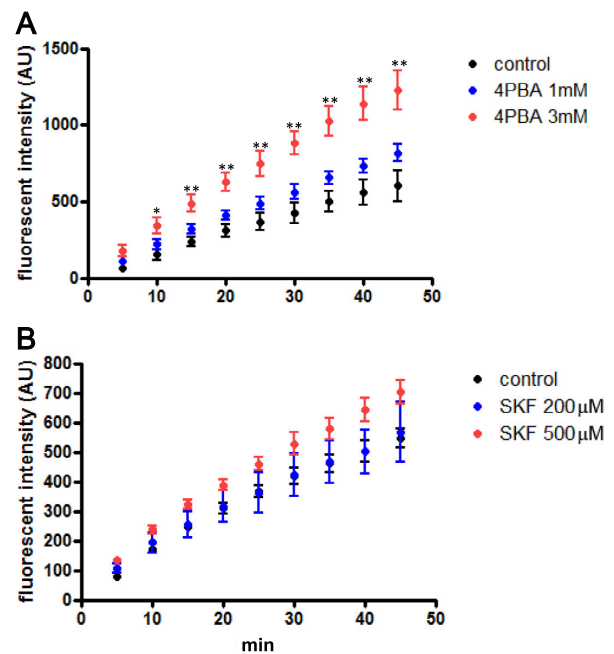
Fig. 3. Time course of fluorescent intensity in SERT-expressing cells. The three independent experiments were performed. In each experiment, the fluorescent intensities of approximately 400–2000 cells were averaged. Each point represents the mean±S.E.M of three experiments. (*P<0.05, **P<0.01 vs. non-treated control, n=3, one-way ANOVA with Dunnett's post-test). **A**: Time course of fluorescent intensity in SERT-expressing cells treated with 4PBA. The fluorescent intensity was gradually increased. Substrate uptake was significantly increased by 4-PBA at 3 mM compared with the control. **B**: Time course of fluorescent intensity in SERT-expressing cells treated with SKF-10047. SKF-10047 at 500 μM tended to increase substrate uptake.

4PBA (mM)

control   1   3   fluvoxamine

SERT

SERTΔCT
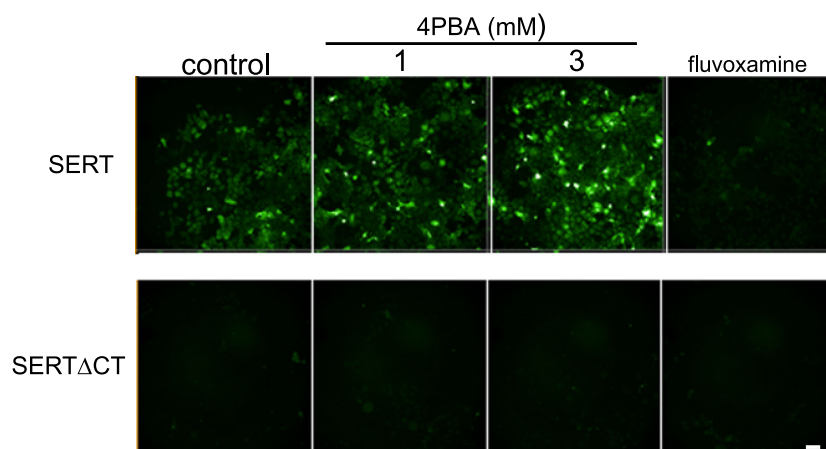
Fig. 4.   Representative fluorescent images of wild-type SERT- or SERTΔCT-expressing cells in each well. The images of fluorescent substrates incorporated into cells are shown and were obtained 45 min after the application of fluorescent substrates. Bar=100 μm.

SKF-10047-treated group, obvious specific uptake via SERTΔCT was not observed in the controls; however, although very faint, an increase in fluorescent intensity was observed in the SKF-10047-treated cells (Fig. 5B). These results for SERTΔCT obtained using the fluorescent substrate-based method was very different from those obtained using the radioisotope-based one.

Evaluation of drug effects using aggregation of SERTΔCT

As mentioned above and shown in a supplemental figure, SERTΔCT was mainly localized to the ER and was scarcely expressed at the plasma membrane. The representative immunostaining of SERTΔCT showed the aggregate formation at the ER in the perinuclear and cytosol compartments (Fig. 6A). Furthermore, the number of aggregates was reduced by the treatment with 4-PBA and SKF-10047 (Fig. 6A). These results led to the idea that aggregation of SERTΔCT was available for the evaluation of drugs that ameliorate ER stress.

First, we manually counted the number of aggregates for quantitative analysis. As shown in Figure 6B, the treatment with 1 and 3 mM 4-PBA significantly reduced the number of SERTΔCT aggregates. Additionally, 200 μM SKF-10047 reduced the number of SERTΔCT aggregates. These results were comparable to the incremental effects of 4-PBA and SKF-10047 on the uptake activity of SERTΔCT.

To automatically count the number of aggregates and pave the way to the high-performance screening of drugs, we again utilized the high-content screening microscope, Opera Phenix. As shown in Figure 7, the Opera Phenix microscope could recognize the cells and intracellular aggregates; therefore, this machine could automatically count the number of aggregates per cell. The quantitative analysis using the Opera Phenix microscope revealed that 4-PBA significantly reduced the number of SERTΔCT aggregates in a dose-dependent manner (Fig. 7B, left). The treatment with SKF-10047 also significantly reduced the
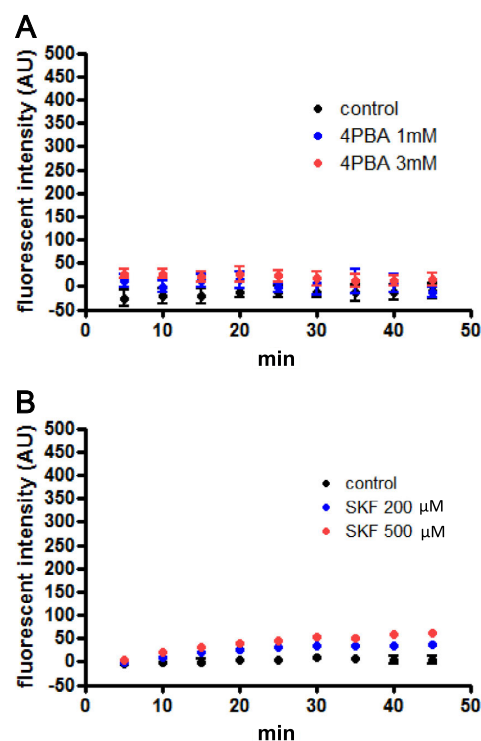

Fig. 5.   Time course of the fluorescent intensity in SERTΔCT-expressing cells. The three independent experiments were performed. In each experiment, the fluorescent intensities of approximately 400–2000 cells were averaged. Each point represents the mean±S.E.M of three experiments (n=3). **A**: Time course of fluorescent intensity in SERTΔCT-expressing cells treated with 4PBA. No apparent increase in fluorescent intensity was observed in SERTΔCT-expressing cells compared with the SERT-expressing cells. **B**: Time course of fluorescent intensity in SERTΔCT-expressing cells treated with SKF-10047. A very faint increase in fluorescent intensity was observed in SERTΔCT-expressing cells treated with SKF-10047. However, no apparent increase in fluorescent intensity was observed in the controls.

number of aggregates; however, dose-dependent effects were not observed (Fig. 7B, right). These results indicated that the high-content screening microscope was very bene-
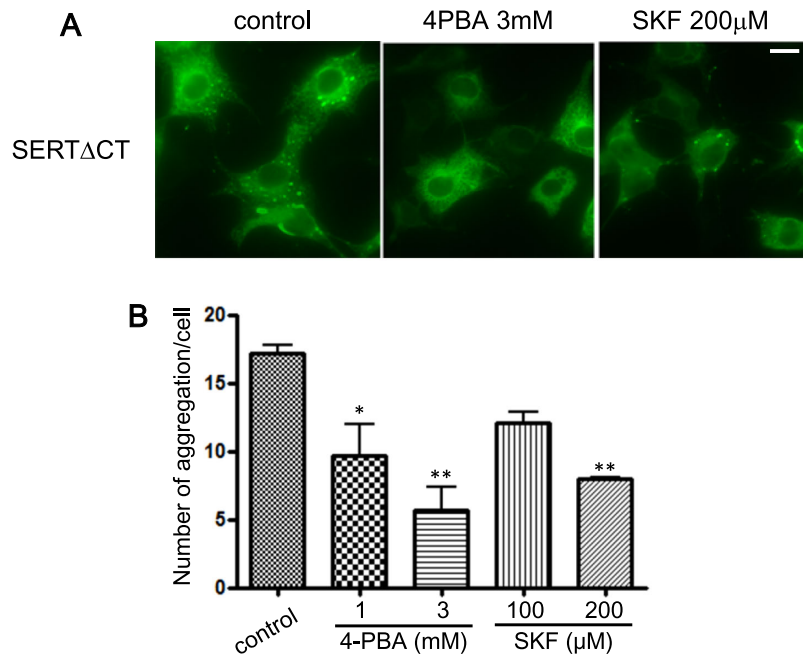
Fig. 6. Evaluation of drug effects using aggregation of SERTΔCT. **A**: Representative immunohistochemical images of SERTΔCT. Bar=10 μm. **B**: Effects of 4-PBA and SKF-10047 on aggregate formation of SERTΔCT. The three independent experiments were performed. In each experiment, the number of aggregates was manually counted from approximately 10–20 cells of each group and averaged. Both 4-PBA and SKF-10047 significantly reduced the number of SERTΔCT aggregates in a dose-dependent manner. The data represent the mean±S.E.M of three experiments. (*P<0.05, **P<0.01 vs. non-treated control, n=3, one-way ANOVA with Dunnett's post-test).
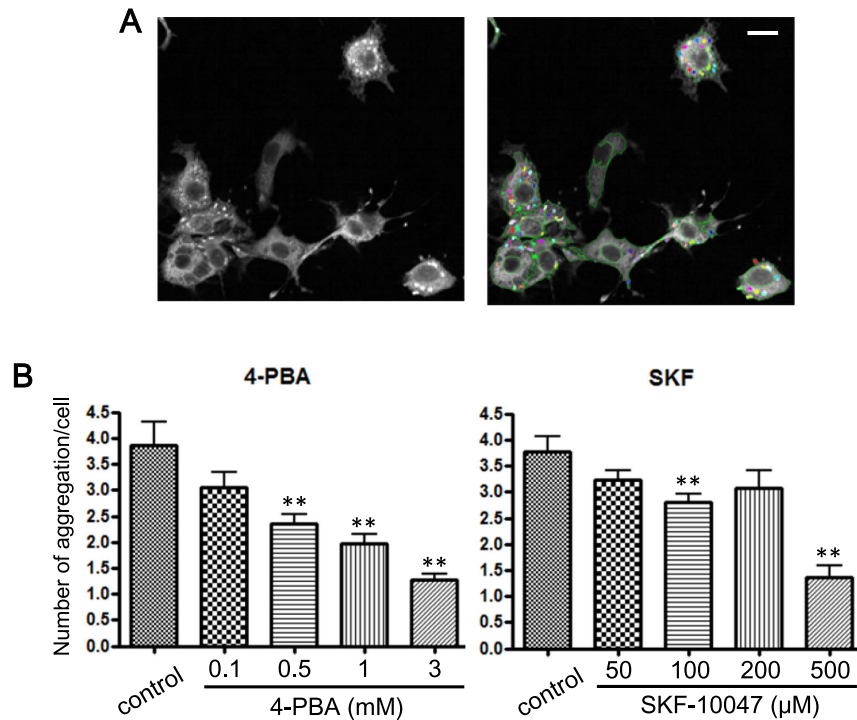
Fig. 7. Automatic count of SERTΔCT aggregates using the Opera Phenix microscope. **A**: Representative images of SERTΔCT aggregates. The left image shows the ordinal image of the cells. The right image shows that the Opera Phenix microscope recognized the cell shape and intracellular aggregates, which are indicated as colored line and dots, respectively. **B**: Effects of 4-PBA and SKF-10047 on SERTΔCT aggregates evaluated using the Opera Phenix microscope. The number of SERTΔCT aggregates in approximately 50–500 cells of each group was automatically counted using the Opera Phenix microscope. The number of SERTΔCT aggregates was significantly reduced by 4-PBA in a dose-dependent manner (left). SKF-10047 at the concentrations of 100 and 500 μM significantly reduced the number of SERTΔCT aggregates. (**P<0.01 vs. non-treated control, n=48–585, one-way ANOVA with Dunnett's post-test).

ficial for automatically counting aggregates of SERT∆CT, suggesting that this method would pave the way to a high-throughput screening of drugs that can attenuate ER stress.

IV. Discussion

The SERT∆CT would be used as a sensitive and beneficial sensor protein in case of screening the drugs that facilitates trafficking of membrane proteins, thereby ameliorates the ER stress

To elucidate the role of the SERT C-terminus in its membrane trafficking and glycosylation, we previously investigated the character of SERT∆CT, the C-terminus-deleted mutant of SERT, in light of its uptake activity, localization and glycosylation [13]. The results are summarized as follows: 1) The uptake activity of SERT∆CT was reduced to 1/10 of the level of that of wild-type SERT. 2) The maturely glycosylated SERT∆CT was reduced, whereas the immaturely glycosylated form was increased compared with the wild-type SERT. 3) The SERT∆CT was mainly localized at the ER and was not observed in plasma membrane, whereas wild-type SERT was expressed at both the ER and plasma membrane [13]. These results revealed that the disturbance of SERT∆CT membrane trafficking and glycosylation reduced the expression of functional SERT in the plasma membrane, thereby showing the critical role of the SERT C-terminus in SERT membrane trafficking and glycosylation. Indeed, it is known that the SERTC-terminus region has an export signal sequence that is important for transporting the SERT from the ER to the Golgi [4, 5].

We also previously investigated how ER stress affects the membrane trafficking of SERT to elucidate the patho-physiological significance of the SERT functional regulation via its membrane trafficking [13]. ER stress, induced by proteasome inhibitors, resulted in the impairment of SERT membrane trafficking and subsequent retention of SERT at the ER, the state of which was very similar to that of SERT∆CT [13]. These findings led to the idea that drugs that facilitate membrane trafficking of SERT, and especially SERT∆CT, would alleviate ER stress.

We first focused on a chemical chaperone and a sigma-1A receptor agonist as candidate drugs, which either have chaperon-like activity itself or enhance the chaperone activity, respectively. Our previous study already revealed that a chemical chaperone 4-PBA upregulated the SERT activity via accelerating its membrane trafficking [6], and our present results confirmed it. In this study, we also found that SKF-10047, a sigma-1A receptor agonist, upregulated the SERT activity. In our preliminary study, western blotting analysis confirmed that SKF-10047 also facilitated the membrane trafficking of SERT. These results indicate that 4-PBA and SKF-10047 are candidate drugs that validate the drug screening methods.

Importantly, the incremental effects of 4-PBA and SKF-10047 on serotonin uptake activity were more promi-

nent in the SERT∆CT than in the wild-type SERT. It is plausible that the ER stress relief drugs could have more apparent effects in the stressed state than normal state. These ideas suggest that the SERT∆CT would be a beneficial tool as a sensor protein for screening for drugs for this purpose.

Validation of the serotonin uptake assay using fluorescent substrates

To establish a simple and reliable assay system for screening various drugs with high performance, we tried to develop another method for a serotonin uptake assay other than the conventional radio isotope-based assay methods. For this purpose, we used the Neurotransmitter Transport Assay Kit provided by Molecular Device Corporation [9]. This assay kit enables the measurement of serotonin uptake activity by simple application of fluorescent substrates to wild-type SERT- or SERT∆CT-expressing cells. As shown in Figure 2, the fluorescence time-dependently increased in accordance with the accumulation of substrates in SERT-expressing cells, although the fluorescent intensity did not reach a plateau within the observed period in this study. By contrast, the fluorescent increase was not observed in fluvoxamine-treated cells. These results suggest that fluorescence accumulation occurred via SERT. The effects of 4-PBA and SKF-10047 on the SERT activity were very similar to those obtained using the radio isotope-based assay, indicating that this method is reliable and suitable for screening for drugs that affect the SERT uptake activity.

By contrast, the accumulation of fluorescence was scarcely observed in SERT∆CT-expressing cells. With SKF-10047 treatment, there was a very faint increase in fluorescence observed (Fig. 5B) possibly because the effect of SKF-10047 on SERT∆CT was robust. Based on the results provided from the manufacturer, the Km value of fluorescent substrate for the human SERT is 0.96 µM (https://www.moleculardevices.com/products/assay-kits/neurotransmitter-transporter-uptake-assay-kit), which is comparable to that of radioisotope-labeled serotonin for rat SERT, 0.32 µM [1]. These results suggest that the sensitivity for the detection of incorporated serotonin is higher in the radio isotope-based methods than in the methods using fluorescent substrates. It is unfortunately concluded that the serotonin uptake assay using fluorescent substrates is not suitable for screening drugs that affect the activity of SERT∆CT.

The aggregation of SERT∆CT would be a beneficial index for screening for drugs that accelerate membrane trafficking and attenuate ER stress

Based on the immunohistochemical observation, we found that the retained SERT∆CT tended to form aggregates. These aggregates were observed mainly in the peri-nuclear region and sparsely in the cytosol, where the ER was suggested to be localized (Fig. 6). The reduction of aggregates by a drug would become direct evidence that this drug possibly ameliorates ER stress. Therefore, we

focused on the appearance of SERTΔCT aggregates when 4-PBA or SKF-10047 was applied. The results of manually counting the aggregates showed that both 4-PBA and SKF-10047 significantly reduced the number of SERTΔCT aggregates in a dose-dependent manner.

Next, we tried to automatically count the SERTΔCT aggregates using the Opera Phenix microscope. The analysis software, which was originally prepared for lipid droplet analysis (Ready Made Solution #15), was suitable for this purpose. The results of automatically counting SERTΔCT aggregates were very similar to those using manual counting. With the automatic count, the number of analyzed cells was increased by approximately 10 times compared with the manual count, indicating that more reliable results could be obtained. Furthermore, the automatic count was finished within a few minutes. These results suggest that evaluation of SERTΔCT aggregates using high-content screening microscopes such as Opera Phenix would be a powerful method that can pave the way to the high-throughput screening of drugs that attenuate ER stress.

Conclusion

The analysis of fluorescent substrates was suitable for high-performance screening of drugs that affect the activity of wild-type SERT but was not applicable for screening SERTΔCT-affecting drugs. By contrast, the analysis of SERTΔCT aggregates was suitable for high-performance screening of drugs that provide ER stress relief. In both analyses, the high-content screening microscope was found to be a beneficial and powerful tool that can pave the way to the high-throughput screening of drugs with ER stress relief effects.

V.   Acknowledgments

VII.   References

 1. Blakely, R. D., Berson, H. E., Fremeau, R. T. Jr., Caron, M. G., Peek, M. M., Prince, H. K. and Bradley, C. C. (1991) Cloning and expression of a functional serotonin transporter from rat brain. *Nature* 354; 66–70.
 2. Bossy-Wetzel, E., Schwarzenbacher, R. and Lipton, S. A. (2004) Molecular pathways to neurodegeneration. *Nat. Med.* 10 Suppl; S2–9.
 3. Caspi, A., Sugden, K., Moffitt, T. E., Taylor, A., Craig, I. W., Harrington, H., McClay, J., Mill, J., Martin, J., Braithwaite, A. and Poulton, R. (2003) Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science* 301; 386–389.
 4. El-Kasaby, A., Just, H., Malle, E., Stolt-Bergner, P. C., Sitte, H. H., Freissmuth, M. and Kudlacek, O. (2010) Mutations in the carboxyl-terminal SEC24 binding motif of the serotonin transporter impair folding of the transporter. *J. Biol. Chem.* 285; 39201–39210.
 5. El-Kasaby, A., Koban, F., Sitte, H. H., Freissmuth, M. and Sucic, S. (2014) A cytosolic relay of heat shock proteins HSP70-1A and HSP90beta monitors the folding trajectory of the serotonin transporter. *J. Biol. Chem.* 289; 28987–29000.
 6. Fujiwara, M., Yamamoto, H., Miyagi, T., Seki, T., Tanaka, S., Hide, I. and Sakai, N. (2013) Effects of the chemical chaperone 4-phenylbutylate on the function of the serotonin transporter (SERT) expressed in COS-7 cells. *J. Pharmacol. Sci.* 122; 71–83.
 7. Han, D. D. and Gu, H. H. (2006) Comparison of the monoamine transporters from human and mouse in their sensitivities to psychostimulant drugs. *BMC Pharmacol.* 6; 6.
 8. Hayashi, T. and Su, T. P. (2007) Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca(2+) signaling and cell survival. *Cell* 131; 596–610.
 9. Jorgensen, S., Nielsen, E. O., Peters, D. and Dyhring, T. (2008) Validation of a fluorescence-based high-throughput assay for the measurement of neurotransmitter transporter uptake activity. *J. Neurosci. Methods* 169; 168–176.
10. Kuhar, M. J., Roth, R. H. and Aghajanian, G. K. (1972) Synaptosomes from forebrains of rats with midbrain raphe lesions: selective reduction of serotonin uptake. *J. Pharmacol. Exp. Ther.* 181; 36–45.
11. Lesch, K. P., Bengel, D., Heils, A., Sabol, S. Z., Greenberg, B. D., Petri, S., Benjamin, J., Muller, C. R., Hamer, D. H. and Murphy, D. L. (1996) Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. *Science* 274; 1527–1531.
12. Mochizuki, H., Amano, T., Seki, T., Matsubayashi, H., Mitsuhata, C., Morita, K., Kitayama, S., Dohi, T., Mishima, H. K. and Sakai, N. (2005) Role of C-terminal region in the functional regulation of rat serotonin transporter (SERT). *Neurochem. Int.* 46; 93–105.
13. Nobukuni, M., Mochizuki, H., Okada, S., Kameyama, N., Tanaka, A., Yamamoto, H., Amano, T., Seki, T. and Sakai, N. (2009) The C-terminal region of serotonin transporter is important for its trafficking and glycosylation. *J. Pharmacol. Sci.* 111; 392–404.
14. Olivier, B., Soudijn, W. and van Wijngaarden, I. (2000) Serotonin, dopamine and norepinephrine transporters in the central nervous system and their inhibitors. *Prog. Drug Res.* 54; 59–119.
15. Ozaki, N., Goldman, D., Kaye, W. H., Plotnicov, K., Greenberg, B. D., Lappalainen, J., Rudnick, G. and Murphy, D. L. (2003) Serotonin transporter missense mutation associated with a complex neuropsychiatric phenotype. *Mol. Psychiatry* 8; 933–936.
16. Paschen, W. and Mengesdorf, T. (2005) Cellular abnormalities linked to endoplasmic reticulum dysfunction in cerebrovascular disease—therapeutic potential. *Pharmacol. Ther.* 108; 362–375.
17. Paschen, W. and Mengesdorf, T. (2005) Endoplasmic reticulum stress response and neurodegeneration. *Cell Calcium* 38; 409–415.
18. Prasad, H. C., Steiner, J. A., Sutcliffe, J. S. and Blakely, R. D. (2009) Enhanced activity of human serotonin transporter variants

associated with autism. *Philos. Trans. R Soc. Lond. B Biol. Sci.* 364; 163–173.

19. Rudnick, G. and Wall, S. C. (1991) Binding of the cocaine analog 2 beta-[3H] carboxymethoxy-3 beta-(4-fluorophenyl)tropane to the serotonin transporter. *Mol. Pharmacol.* 40; 421–426.

20. Sakai, N., Sasaki, K., Nakashita, M., Honda, S., Ikegaki, N. and Saito, N. (1997) Modulation of serotonin transporter activity by a PKC activator and an inhibitor of type 1 and 2A serine/threonine phosphatases. *J. Neurochem.* 68; 2618–2624.

21. Sakai, N., Kodama, N., Ohmori, S., Sasaki, K. and Saito, N. (2000) Involvement of the actin cytoskeleton in the regulation of serotonin transporter (SET) activity: possible mechanism underlying SET regulation by protein kinase C. *Neurochem. Int.* 36; 567–579.

22. Samuvel, D. J., Jayanthi, L. D., Bhat, N. R. and Ramamoorthy, S. (2005) A role for p38 mitogen-activated protein kinase in the regulation of the serotonin transporter: evidence for distinct cellular mechanisms involved in transporter surface expression. *J. Neurosci.* 25; 29–41.

23. Sutcliffe, J. S., Delahanty, R. J., Prasad, H. C., McCauley, J. L., Han, Q., Jiang, L., Li, C., Folstein, S. E. and Blakely, R. D. (2005) Allelic heterogeneity at the serotonin transporter locus (SLC6A4) confers susceptibility to autism and rigid-compulsive behaviors. *Am. J. Hum. Genet.* 77; 265–279.

24. Tate, C. G. and Blakely, R. D. (1994) The effect of N-linked glycosylation on activity of the Na(+)- and Cl(−)-dependent serotonin transporter expressed using recombinant baculovirus in insect cells. *J. Biol. Chem.* 269; 26303–26310.

25. Yammamoto, H., Tanaka, S., Tanaka, A., Hide, I., Seki, T. and Sakai, N. (2013) Long-term exposure of RN46A cells expressing serotonin transporter (SERT) to a cAMP analog up-regulates SERT activity and is accompanied by neural differentiation of the cells. *J. Pharmacol. Sci.* 121; 25–38.

26. Zhu, C. B., Hewlett, W. A., Feoktistov, I., Biaggioni, I. and Blakely, R. D. (2004) Adenosine receptor, protein kinase G, and p38 mitogen-activated protein kinase-dependent up-regulation of serotonin transporters involves both transporter trafficking and activation. *Mol. Pharmacol.* 65; 1462–1474.

27. Zhu, C. B., Carneiro, A. M., Dostmann, W. R., Hewlett, W. A. and Blakely, R. D. (2005) p38 MAPK activation elevates serotonin transport activity via a trafficking-independent, protein phosphatase 2A-dependent process. *J. Biol. Chem.* 280; 15649–15658.