Accepted Manuscript

SKF-10047, a prototype Sigma-1 receptor agonist, augmented the membrane trafficking and uptake activity of the serotonin transporter and its C-terminus-deleted mutant via a Sigma-1 receptor-independent mechanism.

Masaya Asano, Serika Motoike, Chika Yokota, Naoto Usuki, Hikaru Yamamoto, Tomoaki Urabe, Kazusa Katarao, Izumi Hide, Shigeru Tanaka, Masashi Kawamoto, Masahiro Irifune, Norio Sakai

PII: S1347-8613(18)30206-8

DOI: https://doi.org/10.1016/j.jphs.2018.11.005

Reference: JPHS 562

To appear in: *Journal of Pharmacological Science*

Received Date: 24 July 2018

Revised Date: 11 November 2018

Accepted Date: 15 November 2018

Please cite this article as: Asano M, Motoike S, Yokota C, Usuki N, Yamamoto H, Urabe T, Katarao K, Hide I, Tanaka S, Kawamoto M, Irifune M, Sakai N, SKF-10047, a prototype Sigma-1 receptor agonist, augmented the membrane trafficking and uptake activity of the serotonin transporter and its C-terminus-deleted mutant via a Sigma-1 receptor-independent mechanism., *Journal of Pharmacological Science*, https://doi.org/10.1016/j.jphs.2018.11.005.

SKF-10047, a prototype Sigma-1 receptor agonist, augmented the membrane trafficking and uptake activity of the serotonin transporter and its C-terminus-deleted mutant via a Sigma-1 receptor-independent mechanism.

Masaya Asano[1], Serika Motoike[1, 2], Chika Yokota[1], Naoto Usuki[1], Hikaru Yamamoto[1, $], Tomoaki Urabe[1, 3], Kazusa Katarao[1], Izumi Hide[1], Shigeru Tanaka[1], Masashi Kawamoto[3], Masahiro Irifune[2], Norio Sakai[1, *]

1 Department of Molecular and Pharmacological Neuroscience

2 Department of Dental Anesthesiology

3 Department of Anesthesiology and Critical Care

Graduate School of Biomedical & Health Sciences, Hiroshima University

1-2-3 Kausmi, Minami-ku, Hiroshima 734-8551, Japan

* Address correspondence to: Norio Sakai

[1]Department of Molecular and Pharmacological Neuroscience

Graduate School of Biomedical & Health Sciences, Hiroshima University

1-2-3 Kausmi, Minami-ku, Hiroshima 734-8551, Japan

e-mail: nsakai@hiroshima-u.ac.jp

$ Present address:

Biosignal Research Center, Kobe University

1-1 Rokkodai-cho, Nada-ku, Kobe, 675-8501, Japan

Abstract

The serotonin transporter (SERT) is functionally regulated via membrane trafficking. Our previous studies have demonstrated that the SERT C-terminal deletion mutant (SERTΔCT) showed a robust decrease in its membrane trafficking and was retained in the endoplasmic reticulum (ER), suggesting that SERTΔCT is an unfolded protein that may cause ER stress. The Sigma-1 receptor (SigR1) has been reported to attenuate ER stress via its chaperone activity. In this study, we investigated the effects of SKF-10047, a prototype SigR1 agonist, on the membrane trafficking and uptake activity of SERT and SERTΔCT expressed in COS-7 cells. Twenty-four hours of SKF-10047 treatment (> 200μM) accelerated SERT membrane trafficking and robustly upregulated SERTΔCT activity. Interestingly, these effects of SKF-10047 on SERT functions were also found in cells in which SigR1 expression was knocked down by shRNA, suggesting that SKF-10047 exerted these effects on SERT via a mechanism independent of SigR1. A cDNA array study identified several candidate genes involved in the mechanism of action of SKF-10047. Among them, Syntaxin3, a member of the SNARE complex, was significantly upregulated by 48 h of SKF-10047 treatment. These results suggest that SKF-10047 is a candidate for ER stress relief.

Key words:
serotonin transporter; sigma-1 receptor; membrane trafficking; SKF-10047

Introduction

The serotonin transporter (SERT) terminates serotonergic neural transmission by reuptaking serotonin (5-HT) into presynaptic terminals in the central nervous system (1, 2). SERT is a well-known target of antidepressants and drugs of abuse (3-5). Therefore, SERT is thought to be involved in the pathogenic mechanisms underlying mood disorders, including depression and drug addiction (3, 6-8).

In addition, a SERT polymorphism in the promoter region is involved in vulnerability to stress and anxiety traits (9, 10). Previous reports revealed that missense mutations of the SERT gene were found in multiple families susceptible to autism (6-8). These findings emphasize that SERT is a key molecule that is widely related to various neuropsychiatric disorders, including autism, mood disorders, anxiety disorders, and other stress-related diseases. Therefore, the understanding of the SERT regulation mechanism may pave the way for the development of therapeutic strategies for these psychiatric diseases.

Membrane proteins, including SERT, are commonly glycosylated and folded in the endoplasmic reticulum (ER) after translation from mRNA and then transferred to the plasma membrane through the Golgi apparatus. SERT has two putative glycosylation sites in its second extracellular loop, which are first immaturely glycosylated by mannose (11). Then, SERT becomes a maturely glycosylated protein in the Golgi apparatus, followed by its trafficking to the plasma membrane (12-14). We examined the properties of the SERT C-terminal-deleted mutant (SERTΔCT) to clarify the role of the SERT C-terminus in the membrane trafficking and glycosylating modification of SERT (17). SERTΔCT was mostly retained in the ER and scarcely transferred to the plasma membrane, corresponding to a prominent decrease in serotonin uptake activity of SERTΔCT to 10% compared to that of wild-type SERT (8, 15-17). Western blotting analysis also revealed that the immaturely glycosylated form was increased, while the maturely glycosylated form was decreased in

cells expressing SERTΔCT (17).

Accumulating recent evidence has demonstrated that the SERT C-terminus contains signal peptides that promote the trafficking of SERT from the ER to the Golgi apparatus (15). To elucidate the physiological and clinical significance of SERT regulation via membrane trafficking and glycosylation, we focused on ER stress. We examined the effect of ER stress on SERT function. When ER stress is induced by a proteasome inhibitor, SERT membrane trafficking and uptake activity were decreased (17). On the other hand, 4-phenylbutyric acid (4-PBA), a chemical chaperone that functions as ER stress relief, promotes SERT membrane trafficking and uptake activity (18).

The Sigma 1 receptor (SigR1), which is mainly expressed in the ER, has diverse functions. Recent reports demonstrated that SigR-1A exerts neuroprotective effects by acting as a molecular chaperone (19-23). The chaperone activity of SigR-1A is thought to be exerted by the dissociation of GRP78/BiP (binding immunoglobulin protein), a binding molecular chaperone of SigR-1A at its C-terminus. SigR-1A agonists promote the chaperone activity of SigR-1A by detaching GRP78 / BiP from SigR-1A, while antagonists inhibit this process. (24)

Based on these findings, we investigated the effect of Sigma receptor ligands on SERT function to clarify the regulatory mechanism of SERT function via membrane trafficking. Here, we reported that SKF-10047, a prototype SigR1 agonist, significantly increased the uptake activity of SERTΔCT, the effects of which did not depend on the action of SigR1. This unique effect of SKF-10047 on the unfolded protein SERTΔCT may be useful for improving various pathophysiologies caused by ER stress.

Materials and Methods

Materials

An anti-DYKDDDDK tag (anti-FLAG) mouse monoclonal antibody was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). An anti-Sigma receptor goat polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). An anti-Syntaxin3 rabbit polyclonal antibody was purchased from Alomone labs. (Jerusalem, Israel). An anti-serotonin rabbit polyclonal antibody was purchased from Sigma-Aldrich (St Louis, MA, USA). An Alexa-conjugated anti-t IgG antibodies were purchased from Molecular Probe (Eugene, OR, USA). SKF-10047, NE-100 and PRE-084 were purchased from Tocris Bioscience (Bristol, UK). [^{3}H] 5-HT (370 GBq/mmol) was purchased from PerkinElmer (Waltham, MA, 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 (25). The plasmid is designated here as pFLAG-SERT. A plasmid that can express a C-terminus-deleted form of SERT was constructed as described previously (17). This plasmid is designated here as pFLAG-SERTΔCT.

COS-7 cells were obtained from the Riken Cell Bank (Tsukuba, Japan). AD293 cells, derived from the parental 293 cell line, were obtained from Agilent Technologies (Santa Clara, CA, USA). COS-7 and AD293 cells were cultured in DMEM supplemented with heat-inactivated FBS (10%), penicillin (100 units/ml) and streptomycin (100 μg/ml).

For electroporation, various plasmids were transfected into COS-7 or AD293 cells using an NEPA21 electroporator (NEPA GENE, Chiba, Japan) according to the protocol recommended by the

supplier. Briefly, 10 μg of plasmids were transfected into 2×10^6 COS-7 or AD293 cells, and transfected cells were seeded into appropriate culture dishes.

Generation of SigR1 knockdown cells

A plasmid containing target short hairpin sequences for SigR1 (SigR1 shRNA plasmid) was purchased from Origene (Rockville, MD, USA). The target shRNA sequence was GAGTATGTGCTGCTCTTCGGCACCGCCTT. To generate stable SigR1 knockdown AD293 cells, the SigR1 shRNA plasmid was transfected into cells by electroporation as described above. The transfected cells were diluted to approximately 100-500 cells/ml, seeded on the cultured dished and continuously cultured in the presence of puromycin (2 ng/ml). The cell colonies, which were grown from single cells, were picked 7-10 days after transfection. These cell colonies were continuously cultured in the presence of puromycin and were certified as stable SigR1 knockdown cells after the expression level of SigR1 was validated by western blotting.

To generate cells with transient knockdown of SigR1, the SigR1 shRNA plasmid was transfected into COS-7 cells concomitantly with pFLAG-SERT or pFLAG-SERTΔCT. Most of the analyses were performed two days after transfection.

Generation of Syntaxin3 knockdown or overexpressiong cells

For generating syntaxin 3(STX3) knockdown cells, double-stranded siRNAs corresponding to the sequence of human STX3 cDNA were purchased from Life technologies Japan (Tokyo, Japan). The target siRNA sequence was CCAAGCAGCTGACACAGGATGATGA and CCAACAACGTCCGGAACAAACTGAA, for #1 and #2 STX3-siRNA, respectively. The negative control siRNA was purchased from Ambion, Inc. (Austin, TX, USA). Each of 100 pmol STX3-siRNAor control siRNA was simultaneously transfected into COS-7 with pFLAG-SERTΔCT

by electroporation.

For generating STX3 overexpressing cells, a plasmid that can express Myc-DDK-tagged human STX3 isoform A (pMyc-DDK-STX3) was purchased from Origene. Five μg of pMyc-DDK-STX3 was also simultaneously transfected into COS-7 cells with pFLAG-SERTΔCT by electroporation.

5-HT-Uptake Assay

For the measurement of 5-HT uptake via wild-type SERT-expressing cells using a fluorescent substrate-based method, we used a Neurotransmitter Transporter Uptake Assay kit from Molecular Devices Corporation as previously described (26). In brief, the fluorescence of the substrate incorporated into cells was measured using a high-content screening microscope, Opera Phenix (PerkinElmer, Waltham, MA, USA). The fluorescence intensity per cell 45 min after the application of substrates was determined as the SERT uptake activity.

For the [^{3}H] 5-HT uptake assay of SERTΔCT-transfected COS-7 cells were carried out 48 h after transfection as previously described (17, 18, 26-28).

The drugs were applied to culture medium 24 h after the transfection, and the cells were incubated for another 24 h until the uptake assay was performed.

Western blotting analysis

Western blotting was carried out as previously described (17, 18, 26-28). In brief, the cell lysate sample (20 – 50μg protein) was electrophoretically separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) filters (Millipore). The PVDF filters were incubated with the anti-DYKDDDDK-tag mouse monoclonal antibody (diluted 1:1000), anti-Sigma receptor-goat polyclonal antibody (diluted 1:500) or anti-Syntaxin3-rabbit polyclonal antibody (diluted 1:500) for 16 h at 4°C. After three washing with PBS-T (0.01 M phosphate-buffered saline containing 0.03%

Triton X-100), the filters were incubated with horseradish peroxidase–conjugated anti-mouse or rabbit IgG antibody (diluted 1:10,000) for > 1 h at room temperature. The immunoreactive bands were visualized with a chemiluminescence detection kit (Chemi-Lumi One, Nacalai Tesque). The band densities were measured with a luminescent image analyzer (EZ-Capture MG; ATTO).

Immunocytochemistry

Immunocytochemistry was carried out for FLAG-SERT- or FLAG-SERTΔCT-expressing COS-7 cells as previously described (17, 18, 28). For immunostaining of FLAG-SERT, the primary and secondary antibodies used were the anti-DYKDDDDK tag mouse monoclonal antibody (WAKO, diluted 1:1000) and anti-mouse IgG conjugated with Alexa Fluor 546 (Molecular probe, 1:500), respectively. For immunostaining of serotonin, the SERT-expressed cells were treated with 1 μM serotonin for 15 min and then fixed with 4% paraformaldehyde. The primary and secondary antibodies used were the anti-serotonin rabbit polyclonal antibody (diluted 1:5000) and anti-rabbit IgG conjugated with Alexa Fluor 488 (Molecular probe, 1:500), respectively. Signals were observed by fluorescence microscopy (BIOREVO BZ-9000, KEYENCE, JAPAN).

Gene microarray analysis

We extracted the total RNA from the AD293 cell lines that were treated with /without 200 μM SKF-10047 for 24 h using an RNeasy mini kit (Qiagen, Hilden, Germany). The obtained RNAs were labeled, hybridized, washed, scanned and digitized by Toray Industries Inc., Tokyo, Japan.

Then, the detected signals for each gene were normalized by the global normalization method (the median of the detected signal intensity was adjusted to 25).

Measurement of chaperone activity using a-lactalbumin aggregates

BSA was denatured with 7.2 M guanidine hydrochloride at 25°C overnight in 210 μM phosphate buffer (pH 7.5). Aggregates of denatured BSA were formed by a 50-fold rapid dilution in phosphate buffer (14 mM KH2PO4, 86 mM K2HPO4, and 150 mM KCl (pH 7.5)) at 25°C and were further incubated for 3 h. Alpha-lactalbumin was reduced by incubating the protein in phosphate buffer containing 2.5 mM EDTA and 5 mM dithiothreitol for 30 min at 25°C. For the induction of reduced α-lactalbumin (r-LA) aggregation, r-LA was mixed with BSA aggregates in phosphate buffer. The mixture containing 140 μM r-LA and 4.2 μM BSA was incubated at 37°C. The aggregation formation of r-LA induced by BSA aggregates in the presence or absence of SKF-10047 was monitored by measuring turbidity at 488 nm using a spectrophotometer (Model 680 microplate reader, BIO-RAD Laboratories, Inc., USA) (29).

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.

Results

1. Effects of the SigR1 ligand SKF-10047 on the uptake activity of SERT

To elucidate the effects of SKF-10047 on the uptake activity of SERT, we transfected FLAG-SERT or FLAG-SERTΔCT cDNA into COS-7 cells. Twenty-four hours after the transfection, we treated the cells with SKF-10047 (0 -500 μM), and then, 24 h later, we performed the 5-HT uptake assay.

SKF-10047 at 500 μM significantly increased the uptake activity of wild-type SERT (Fig. 1A). Next, we examined the effects of SKF-10047 on the uptake activity of SERTΔCT. Our previous studies revealed that the activity of SERTΔCT was reduced to approximately one tenth of that of wild type (17, 18). As shown in Fig. 1B, SKF-10047 significantly and prominently increased the uptake activity of SERTΔCT in a dose-dependent manner.

The 5-HT uptake via wild-type SERT and SERTΔCT was immunohistochemically evaluated using an anti-5-HT antibody. In wild-type SERT-expressing cells, incorporated 5-HT was clearly observed as green fluorescence in the acquired images (Fig. 1C). This immunoreactivity was not observed in the presence of 10 μM fluvoxamine, a SERT inhibitor, indicating that the green fluorescence correspond to 5-HT, which was incorporated into cells via SERT (Fig. 1C).

SKF-10047 did not markedly affect the fluorescence intensity of incorporated serotonin in wild-type SERT-expressing cells. In contrast, in the SERTΔCT-expressing cells, SKF-10047 appeared to increase the amount of incorporated 5-HT in a dose-dependent manner.

2. Effects of SKF-10047 on the protein expression and glycosylation of SERT

To elucidate the effects of SKF-10047 on the protein expression and glycosylation of SERT, western blotting analysis was performed on SKF 10047-treated cells expressing FLAG-SERT or

FLAG-SERTΔCT.

Our previous study clarified that the SERT protein was recognized as multiple bands due to differences in glycosylated modification (17, 18). Based on our previous analyses using endoglycosidase, the band with a molecular size of approximately 80 kDa (arrowhead) corresponds to the functional and maturely glycosylated SERT that has gone through the Golgi apparatus, and the band with a molecular size of approximately 63 kDa (arrow) corresponds to the high-mannose-type immaturely glycosylated SERT in the ER (Fig. 2A). In addition, bands larger than 100 kDa were considered to be dimers of immaturely glycosylated SERT.

In the cells expressing wild-type SERT, the expression level of mature SERT was tended to be increased by SKF-10047, while that of immature SERT was significantly decreased (Supplemental Fig. 1A and 1B). The total expression level of mature and immature SERT was not influenced by the drug (Supplemental Fig. 1C). The expression ratio of mature SERT to immature SERT was evaluated as an index of membrane trafficking and glycosylated modification of SERT (Fig. 2A). SKF-10047 significantly increased the ratio in a dose-dependent manner, suggesting that SKF-10047 increases the expression of mature SERT and decreases that of immature SERT by promoting its membrane trafficking.

On the other hand, our previous studies revealed that maturely glycosylated SERTΔCT was scarcely observed, and most SERTΔCT was recognized as the immaturely glycosylated form (17, 18). In the cells expressing SERTΔCT, SKF-10047 significantly decreased the expression of immaturely glycosylated SERT in a dose-dependent manner (Fig. 2B).

3. Effects of SKF-10047 on the uptake activity of SERTΔCT in SigR1 knockdown cells

To elucidate whether the robust incremental effect of SKF-10047 on the uptake activity of SERTΔCT, as shown in Fig. 1, was mediated through SigR1, we generated SigR1 knockdown

AD293 cells and COS-7 cells using a shRNA plasmid targeting SigR1.

First, we validated the level of shRNA-induced SigR1 knockdown. The SigR1 shRNA reduced SigR1 expression by 53% and 56% in COS-7 cells and AD293 cells, respectively, compared to the expression in mock-transfected control cells (Fig. 3A). We compared the effects of SKF-10047 on the uptake activity of SERTΔCT between mock-transfected cells and SigR1 knockdown cells. In COS-7 cells, the increase in uptake activity of SERTΔCT caused by SKF-10047 (200 μM) was similar in SigR1 knockdown cells and mock-transfected cells (Fig. 3B). We performed the same studies using AD293 cells. The increase in uptake activity of SERTΔCT caused by SKF-10047 (0-500 μM) was not significantly different between SigR1 knockdown cells and mock-transfected cells (Fig. 3C). These results suggest that SKF-10047 did not exert its facilitatory effects on SERTΔCT function through SigR1.

We also examined whether the activity of wild-type SERT is affected by the knockdown of SigR1. We performed [^{3}H] 5-HT uptake assay instead of fluorescent substrate-based method for the reason as described figure legends (Supplemental Fig. 2). The knockdown of SigR1 did not affect the increasing tendency of uptake activity of wild-type SERT by SKF-10047 at 500μM (Supplemental Fig. 2).

To ascertain whether SKF-10047 acted directly on SERTΔCT as a chemical chaperone, we evaluated the chaperone activity of SKF-10047 *in vitro* using an α-lactalbumin aggregate assay. As shown in Supplemental Fig. 3, SKF-10047 did not exhibit any chaperone activity *in vitro*.

4. Effects of other SigR1 ligands on the uptake activity of SERTΔCT

Because SKF-10047, a prototype SigR1 agonist, possibly modified the function of SERT independent of its effect on SigR1, we investigated the effects of other sigma receptor ligands on the 5-HT uptake activity of SERTΔCT.

PRE-084, which has higher specificity for SigR1 than does SKF-10047 (19, 23, 30), did not affect the uptake activity of wild-type SERT or SERTΔCT at the concentration of 0 to 10 μM (Supplemental Fig. 4). Among the possible SigR1 ligands investigated in this study, pentazocine, NE-100, and haloperidol increased the activity of SERTΔCT (Supplemental Fig. 5A). However, the effect of these drugs on SERTΔCT uptake activity was not affected by SigR1 knockdown (Supplemental Fig. 5B).

5. cDNA array analysis to determine the underlying mechanism by which SKF-10047 affects the function of SERT and SERTΔCT

To clarify the underlying mechanism by which SKF-10047 affects SERT and SERTΔCT, we comprehensively examined the difference in mRNA expression between the control and cells treated with 200 μM SKF-10047 for 24 h using a cDNA array. The results are shown in Supplemental Table1.

We focused on Syntaxin3 (STX3) as the possible factor affecting the function of SERT via its membrane trafficking. STX3, a member of the SNARE (soluble NSF attachment protein receptor) complex, is known to be involved in endocytosis, thereby regulating the plasma membrane expression of proteins such as cystic fibrosis transmembrane regulator (CFTR) (31, 32). Therefore, we examined the effect of SKF-10047 on the protein expression of STX3. STX3 was recognized as two bands, corresponding to isoform A and B, main transcripts produced by alternative splicing (https://www.uniprot.org/uniprot/Q13277, Fig. 4) In agreement with the results of the cDNA array, 48 h of treatment with SKF-10047 significantly increased the protein levels of both isoforms of STX3 in a dose-dependent manner (Fig. 4).

6. Effects of SKF-10047 on the uptake activity of SERTΔCT in syntaxin3 (STX3) knockdown or

overexpressing cells

To elucidate the involvement of STX3 in the exertion of SKF-10047 effects on SERTΔCT, we investigated whether the incremental effects of SKF-10047 on SERTΔCT was affected by the knockdown or overexpression of STX3. We validated the level of STX3 by the siRNA-induced knockdown and STX3 overexpression by pMyc-DDK-STX3 transfection. The STX3-siRNA #1 and #2 reduced the expression of STX3 isoform A by 54% and 53% in COS-7 cells, compared to the expression in control siRNA-transfected cells (Fig. 5A, upper). Myc-DDK-tagged STX3 was recognized by immunoblotting for STX3, in addition to endogenous STX3 isoforms (Fig 5A, lower). The knockdown of STX3 did not exhibit any influence on the effects of SKF-10047 on the uptake activity of SERTΔCT (Fig. 5B). In contrast, SKF-10047 further increased the SERTΔCT uptake activity in STX3-overexpressing cells compared to that in mock-transfected cells (Fig. 5C). These results lead to the possible involvement of STX3 in the exertion of SKF-10047-induced incremental effects on SERTΔCT.

Discussion

The purpose of this study was to clarify the regulatory mechanism of SERT function by examining the influence of SigR1 ligands on SERT function. SERT uptake activity is thought to be regulated by membrane trafficking (17). SERT activity is decreased when the SERT protein is retained in the ER by the addition of mutations to the SERT C-terminus or by chemical treatment that impairs its membrane trafficking, such as treatment with brefeldin A or proteasome inhibitors (17, 18). In contrast, chemical chaperones, including 4-PBA, promote the membrane trafficking of SERT from the ER to the plasma membrane, thereby upregulating SERT function (18).

SigR1 is known to exist in the ER and exert chaperone activity when agonistic ligands bind to this receptor (19-23). Therefore, we predicted that SigR1 and its ligand affect the function of SERT. First, we examined the effects of SKF-10047, a prototype SigR1 agonist, on SERT membrane trafficking and uptake activity. As shown in Fig. 1, for both wild-type SERT and C-terminus-deleted mutant (SERTΔCT), SKF-10047 significantly increased SERT uptake activity. Interestingly, SKF-10047 prominently and robustly increased the uptake activity of SERTΔCT in a dose-dependent manner. These effects of SKF-10047 on SERT and SERTΔCT were in agreement with the results observed in our previous study (26). SERTΔCT was previously found to be retained in the ER as an unfolded protein (18, 26); therefore, these results revealed that SKF-10047 exerted more prominent effects on unfolded SERT than on wild-type SERT.

Next, we performed western blotting analysis to investigate the effects of SKF-10047 on protein expression and glycosylation. In cells expressing wild-type SERT, SKF-10047 decreased the expression of the immaturely glycosylated SERT and increased the expression of the maturely glycosylated SERT, suggesting that SKF-10047 could increase the uptake activity of wild-type SERT by accelerating the trafficking of immature glycosylated SERT in the ER to the plasma membrane.

In contrast, in cells expressing SERTΔCT, SKF-10047 clearly decreased the expression of the immaturely glycosylated SERT; however, whether the drug increased the expression of the maturely glycosylated SERT was not apparent because this form of SERT was scarcely observed in SERTΔCT -expressing cells. Nevertheless, SKF-10047 significantly increased the uptake activity of SERTΔCT. These results were coincident with our previous report showing that the chemical chaperone 4-PBA increased the uptake activity of SERTΔCT without the clear appearance of maturely glycosylated SERT (18). The reason why an increase in maturely glycosylated SERT was not observed instead of the observed decrease in immaturely glycosylated SERT is unknown. One possibility is that the expression of maturely glycosylated SERT may be lower than the level detectable by western blotting. Alternatively, SERTΔCT may remain in the immaturely glycosylated form when it is transported to the plasma membrane, and SERTΔCT may function as an incomplete transporter.

Based on a previous report demonstrating that SKF-10047 decreased the aggregate accumulation of SERTΔCT in the ER (26), SKF-10047 relieved SERTΔCT-induced ER stress by accelerating the degradation of immaturely glycosylated SERTΔCT through ERAD (endoplasmic reticulum-associated degradation). The involvement of ERAD may affect the membrane trafficking of SERTΔCT. At present, the detailed mechanism underlying the incremental effects of SKF-10047 on SERTΔCT function is not clear. Further experiments, including immunohistochemical studies with high resolution, are needed.

SigR1 was thought to exert its chaperone activity by the binding of SigR1 agonists to the receptor, the effects of which are in turn inhibited by SigR1 antagonists. To elucidate whether the incremental effect of SKF-10047 on the uptake activity of SERTΔCT was mediated through SigR1, we investigated the effect of this drug in SigR1 knockdown cells. However, the incremental effects of SKF-10047 on the uptake activity of SERTΔCT remained in SigR1 knockdown cells, suggesting that these effects of SKF-10047 were not through actions on SigR1. This idea was supported by the result

that PRE-084, which has higher specificity to SigR1 than does SKF-10047 (19, 23), did not affect the uptake activity of SERTΔCT. Based on these findings, we predicted that SKF-10047 increases the uptake activity of SERTΔCT by a SigR1-independent mechanism. Alternatively, SKF-10047 itself may have chemical chaperone activity, although not plausible based on the findings in Supplemental Fig. 3.

Therefore, to clarify the novel mechanism underlying the effects of SKF-10047 on SERTΔCT, we compared the difference in gene expression between SKF-10047-treated and control cells using a cDNA array. First, we chose the genes that showed sufficient endogenous expression with a global normalization value of more than 10. Next, we selected the genes that showed more than a two-fold change in expression in SKF-10047-treated cells compared to that in control cells (Supplemental Table 1). Among these, we focused on Syntaxin3 (STX3) as a gene that is related to membrane trafficking. STX3 is one of the SNARE proteins that induces exocytosis and facilitates the expression of membrane proteins, including transporters and receptors, to the plasma membrane (31, 32). Indeed, the plasma membrane expression of cystic fibrosis transmembrane regulator (CFTR), a membrane transporter, is known to be regulated by STX3. In addition, STX3 binds to SERT and regulates its function in CaCo-2 cells, an intestinal cell line (33). Although it is not clear whether SKF-10047 up-regulated the STX3 expression via SigR1-dependent mechanism or not at present, SKF-10047 increased the protein expression of STX3, as shown in Fig. 4. STX3 may be a candidate molecule that is involved in the novel mechanism of SKF-10047 on SERT.

To elucidate whether STX3 is involved in the exertion of SKF-10047 effects on SERTΔCT, we generated the STX3-knockdown or STX3-overexpressing cells, and investigated whether the knockdown and overexpression of STX3 affects the incremental effects of SKF-10047 on SERTΔCT uptake activity. As shown Fig.5, in STX3-overexpressing cells, not STX3-knockdown cells, SKF-10047 further increased the uptake activity of SERTΔCT, suggesting that STX3 is possibly and

involved in the SKF-10047 action on SERTΔCT via some mechanism. Further investigation is necessary to confirm the underlying mechanism by which STX3 could regulate the function of wild-type SERT or SERTΔCT.

Interestingly, SigR1 ligands other than SKF-1004, such as NE-100, pentazocine and haloperidol, increased the uptake activity of SERTΔCT, regardless of whether these ligands were agonists or antagonists. Furthermore, the effects of these drugs on SERTΔCT were retained in SigR1 knockdown cells. These findings lead to the possibility that SigR1 ligands other than just SKF-10047, which are thought to exert their effect through SigR1, regulate the function of membrane proteins through a novel mechanism. Among the ligands of SigR1, including SKF-10047, there might be a useful drug that can relieve ER stress.

In conclusion, SKF-10047 is a candidate drug for the relief of ER stress, which is caused by the accumulation of unfolded membrane proteins.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Sports and Culture and by grants from the Takeda Science Foundation, the Uehara Memorial Foundation and the Japanese Smoking Research Association. This work was performed using equipment at the Radiation Research Center for Frontier Science, Natural Science Center for Basic Research and Development, Hiroshima University.

Conflict of interest

Authors have no conflict of interest regarding this study.

References

1. Blakely RD, Berson HE, Fremeau RT, Jr., Caron MG, Peek MM, Prince HK, et al. Cloning and expression of a functional serotonin transporter from rat brain. Nature. 1991;354:66-70.
2. Kuhar MJ, Roth RH, Aghajanian GK. Synaptosomes from forebrains of rats with midbrain raphe lesions: selective reduction of serotonin uptake. The Journal of pharmacology and experimental therapeutics. 1972;181:36-45.
3. Han DD, Gu HH. Comparison of the monoamine transporters from human and mouse in their sensitivities to psychostimulant drugs. BMC Pharmacol. 2006;6:6.
4. Olivier B, Soudijn W, van Wijngaarden I. Serotonin, dopamine and norepinephrine transporters in the central nervous system and their inhibitors. Prog Drug Res. 2000;54:59-119.
5. Rudnick G, Wall SC. Binding of the cocaine analog 2 beta-[3H] carboxymethoxy-3 beta-(4-fluorophenyl)tropane to the serotonin transporter. Mol Pharmacol. 1991;40:421-6.
6. Ozaki N, Goldman D, Kaye WH, Plotnicov K, Greenberg BD, Lappalainen J, et al. Serotonin transporter missense mutation associated with a complex neuropsychiatric phenotype. Mol Psychiatry. 2003;8:933-6.
7. Prasad HC, Steiner JA, Sutcliffe JS, Blakely RD. Enhanced activity of human serotonin transporter variants associated with autism. Philos Trans R Soc Lond B Biol Sci. 2009;364:163-73.
8. Sutcliffe JS, Delahanty RJ, Prasad HC, McCauley JL, Han Q, Jiang L, et al. Allelic heterogeneity at the serotonin transporter locus (SLC6A4) confers susceptibility to autism and rigid-compulsive behaviors. Am J Hum Genet. 2005;77:265-79.
9. Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H, et al. Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science. 2003;301:386-9.
10. Lesch KP, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S, et al. Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. Science. 1996;274:1527-31.
11. Tate CG, Blakely RD. 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. 1994;269:26303-10.
12. Samuvel DJ, Jayanthi LD, Bhat NR, Ramamoorthy S. 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. 2005;25:29-41.
13. Zhu CB, Carneiro AM, Dostmann WR, Hewlett WA, Blakely RD. p38 MAPK activation

elevates serotonin transport activity via a trafficking-independent, protein phosphatase 2A-dependent process. J Biol Chem. 2005;280:15649-58.

14. Zhu CB, Hewlett WA, Feoktistov I, Biaggioni I, Blakely RD. 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. 2004;65:1462-74.
15. El-Kasaby A, Just H, Malle E, Stolt-Bergner PC, Sitte HH, Freissmuth M, et al. Mutations in the carboxyl-terminal SEC24 binding motif of the serotonin transporter impair folding of the transporter. J Biol Chem. 2010;285:39201-10.
16. Larsen MB, Fjorback AW, Wiborg O. The C-terminus is critical for the functional expression of the human serotonin transporter. Biochemistry. 2006;45:1331-7.
17. Nobukuni M, Mochizuki H, Okada S, Kameyama N, Tanaka A, Yamamoto H, et al. The C-terminal region of serotonin transporter is important for its trafficking and glycosylation. J Pharmacol Sci. 2009;111:392-404.
18. Fujiwara M, Yamamoto H, Miyagi T, Seki T, Tanaka S, Hide I, et al. Effects of the chemical chaperone 4-phenylbutylate on the function of the serotonin transporter (SERT) expressed in COS-7 cells. J Pharmacol Sci. 2013;122:71-83.
19. Cobos EJ, Entrena JM, Nieto FR, Cendan CM, Del Pozo E. Pharmacology and therapeutic potential of sigma(1) receptor ligands. Curr Neuropharmacol. 2008;6:344-66.
20. Hayashi T, Maurice T, Su TP. Ca(2+) signaling via sigma(1)-receptors: novel regulatory mechanism affecting intracellular Ca(2+) concentration. The Journal of pharmacology and experimental therapeutics. 2000;293:788-98.
21. Hayashi T, Su T. The sigma receptor: evolution of the concept in neuropsychopharmacology. Curr Neuropharmacol. 2005;3:267-80.
22. Hayashi T, Su TP. Regulating ankyrin dynamics: Roles of sigma-1 receptors. Proc Natl Acad Sci U S A. 2001;98:491-6.
23. Hayashi T, Su TP. Sigma-1 receptor ligands: potential in the treatment of neuropsychiatric disorders. CNS Drugs. 2004;18:269-84.
24. Hayashi T, Tsai SY, Mori T, Fujimoto M, Su TP. Targeting ligand-operated chaperone sigma-1 receptors in the treatment of neuropsychiatric disorders. Expert Opin Ther Targets. 2011;15:557-77.
25. Sakai N, Kodama N, Ohmori S, Sasaki K, Saito N. Involvement of the actin cytoskeleton in the regulation of serotonin transporter (SET) activity: possible mechanism underlying SET regulation by protein kinase C. Neurochem Int. 2000;36:567-79.
26. Katarao K, Murakawa S, Asano M, Usuki N, Yamamoto H, Shirafuji T, et al. The Development of Screening Methods to Identify Drugs to Limit ER Stress Using Wild-type

and Mutant Serotonin Transporter. Acta Histochem Cytochem. 2016;49:197-206.

27. Mochizuki H, Amano T, Seki T, Matsubayashi H, Mitsuhata C, Morita K, et al. Role of C-terminal region in the functional regulation of rat serotonin transporter (SERT). Neurochem Int. 2005;46:93-105.
28. Yammamoto H, Tanaka S, Tanaka A, Hide I, Seki T, Sakai N. 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. 2013;121:25-38.
29. Mimori S, Okuma Y, Kaneko M, Kawada K, Hosoi T, Ozawa K, et al. Protective effects of 4-phenylbutyrate derivatives on the neuronal cell death and endoplasmic reticulum stress. Biol Pharm Bull. 2012;35:84-90.
30. Su TP, Wu XZ, Cone EJ, Shukla K, Gund TM, Dodge AL, et al. Sigma compounds derived from phencyclidine: identification of PRE-084, a new, selective sigma ligand. The Journal of pharmacology and experimental therapeutics. 1991;259:543-50.
31. Collaco A, Marathe J, Kohnke H, Kravstov D, Ameen N. Syntaxin 3 is necessary for cAMP- and cGMP-regulated exocytosis of CFTR: implications for enterotoxigenic diarrhea. Am J Physiol Cell Physiol. 2010;299:C1450-60.
32. Tang BL, Gee HY, Lee MG. The cystic fibrosis transmembrane conductance regulator's expanding SNARE interactome. Traffic. 2011;12:364-71.
33. Nazir S, Kumar A, Chatterjee I, Anbazhagan AN, Gujral T, Priyamvada S, et al. Mechanisms of Intestinal Serotonin Transporter (SERT) Upregulation by TGF-beta1 Induced Non-Smad Pathways. PLoS One. 2015;10:e0120447.

Key words: serotonin transporter, sigma-1 receptor, membrane trafficking, SKF-10047

Figure legends

Fig. 1

Effects of SKF-10047 on the uptake activity of wild-type (WT) SERT or SERTΔCT.

A: Effects of SKF-10047 on the uptake activity of WT SERT.

In the cells expressing wild-type SERT, we measured the uptake of fluorescent SERT substrate per cell as the 5-HT uptake activity. We evaluated the uptake activity using the Neurotransmitter Transporter Uptake Assay kit as described in the Materials and Methods. SKF-10047 (500 μM) significantly increased the uptake activity of WT SERT (**P<0.05, n=9, one-way ANOVA with Dunnett's post-test).

B: Effects of SKF-10047 on the uptake activity of SERTΔCT.

We examined the effects of SKF-10047 on the uptake activity of SERTΔCT using a [^{3}H] 5-HT-uptake assay as described in Materials and Methods. SKF-10047 significantly and prominently increased the uptake activity of SERTΔCT in a dose-dependent manner (*P<0.05, n=4, one-way ANOVA with Dunnett's post-test).

C: Immunocytochemical analysis for evaluating 5-HT uptake via SERT.

After FLAG-SERT- or FLAG-SERTΔCT-expressing cells were treated with SKF-10047 for 24 h, the cells were treated with 1 μM serotonin for 15 min, and then fixed with 4% paraformaldehyde for later immunohistochemical procedures. Fixed cells were first reacted with the anti-FLAG antibody for SERT, followed by the anti-5-HT antibody for detecting the incorporated 5-HT. Cells were further visualized in red for SERT and green for 5-HT with Alexa 546-conjugated anti-mouse IgG or Alexa 488-conjugated anti-rabbit IgG, respectively.

In the cells expressing WT SERT, SKF-10047 did not markedly affect the fluorescence intensity of uptaken 5-HT (left panel). However, in the SERTΔCT-expressing cells, SKF-10047 appeared to

increase the fluorescence intensity of uptaken serotonin in a dose-dependent manner (right panel). The SSRI fluvoxamine completely blocked the uptake of serotonin both in WT SERT- and SERTΔCT-expressing cells.

Fig. 2

Effects of SKF-10047 on the protein expression and glycosylation of WT SERT or SERTΔCT.

A: Effects of SKF-10047 on the protein expression and glycosylation of WT SERT. SKF-10047 increased the expression of mature SERT and decreased the expression of immature SERT (left panel).

The ratio of mature SERT to immature SERT was significantly increased in a dose-dependent manner (*P<0.05, n=3, one-way ANOVA with Dunnett's post-test).

B: Effects of SKF-10047 on the protein expression and glycosylation of SERTΔCT.

SKF-10047 significantly decreased the expression of immaturely glycosylated SERT in a dose-dependent manner (*P<0.05, **P<0.01, n=3, one-way ANOVA with Dunnett's post-test).

Fig. 3

Effects of SKF-10047 on the uptake activity of SERTΔCT in SigR1 knockdown cells.

COS-7 cells were transiently transfected with SigR1 shRNA, and AD293 cells were stably transformed with the shRNA. We used cells transfected with a mock vector that did not contain shRNA as control cells.

A: Validation of SigR1 knockdown by SigR1 shRNA in COS-7 and AD293 cells.

The transfection of SigR1 shRNA reduced SigR1 expression by 53% and 56% in COS-7 cells and AD293 cells, respectively, compared to the expression in mock-transfected control cells.

B: Effects of SKF-10047 on the uptake activity of SERTΔCT in mock-transfected and SigR1

knockdown COS-7 cells.

The increase in SERT activity induced by SKF-10047 (200 μM) was similar in SigR1 knockdown cells and mock-transfected cells (n=4, ns, unpaired t-test).

C: Effects of SKF-10047 on the uptake activity of SERTΔCT in control and SigR1 knockdown AD293 cells. The increase in SERT activity induced by SKF-10047 (0-500 μM) was not significantly different between SigR1 knockdown cells and mock-transfected cells (n=4, ns, unpaired t-test).

Fig. 4

Effects of SKF-10047 on the protein expression of Syntaxin3 (STX3).

Syntaxin3 consists of some isoforms, produced by alternative splicing (https://www.uniprot.org/uniprot/Q13277). According to the predictive molecular size of isoforms, the isoform A and isoform B, main isoforms of STX3 were considered to be detected in COS-7 cells (left). Treatment of COS-7 cells with SKF-10047 for 48 h significantly increased the protein levels of both STX3 isoforms in a dose-dependent manner. Statistical analysis was performed for isoform B (*P<0.05, n=3, one-way ANOVA with Dunnett's post-test).

Fig. 5

Influence of knockdown or overexpression of Syntaxin3 on the incremental effects of SKF-10047 on the SERTΔCT uptake activity.

We examined the effects of SKF-10047 on the SERTΔCT uptake activity in STX3-konockdown or STX3-overexpressing COS-7cells, and compared the increasing rate in these cells to that in control cells.

A: Validation of STX3 knockdown by STX3 siRNA (upper) and overexpression by

pMyc-DDK-STX3 (lower) in COS-7 cells. The transfection of STX3 siRNA #1 and #2 reduced the expression of STX3 isoform A, a functional isoform, by 53% and 56%, respectively (upper). In pMyc-DDK-STX3-transfected cells, prominent expression of Myc-DDK-STX3 was seen in addition to endogenous STX3, isoform A and B.

B: Effects of SKF-10047 on the uptake activity of SERTΔCT in control siRNA-transfected COS-7 cells and STX3 siRNA #2-transfected COS-7 cells.

In both cells, 200μM SKF-10047 significantly increased the uptake activity of SERTΔCT (n=4, $P<0.001$, unpaired t-test). The increase in SERTΔCT uptake activity induced by 200μM SKF-10047 was similar in control siRNA-transfected cells and STX3 siRNA-transfected cells (n=4, ns, unpaired t-test).

C: Effects of SKF-10047 on the uptake activity of SERTΔCT in mock-transfected COS-7 cells and Myc-DDK-STX3-expressing COS-7 cells. In both cells, 200μM SKF-10047 significantly increased the uptake activity of SERTΔCT (n=4, $P<0.001$, unpaired t-test). The increase in SERTΔCT uptake activity induced by 200μM SKF-10047 in Myc-DDK-STX3-expressing cells was significantly higher than that in mock-transfected cells (n=4, $P<0.001$, unpaired t-test).

Supplemental Figure 1

A: Effects of SKF-10047 on the protein expression of maturely glycosylated wild-type SERT. SKF-10047 tended to increase the expression of mature SERT although the statistical significance was not obtained (n=4, ns, one-way ANOVA with Dunnett's post-test). .

B: Effects of SKF-10047 on the protein expression of immaturely glycosylated WT SERT. SKF-10047 dose-dependently decreased the expression of immature SERT with statistical significance (n=4, $P<0.05$, one-way ANOVA with Dunnett's post-test).

C: Effects of SKF-10047 on the total expression levels of WT SERT. Density of three bands as

shown in Fig. 2A was measured as the total expression level of WT SERT. The level was not affected by SKF-10047 (n=4, ns, one-way ANOVA with Dunnett's post-test).

Supplemental Figure 2

Effects of SKF-10047 on the uptake activity of wild-type SERT in SigR1 knockdown cells.

Instead of Neurotransmitter Transporter Uptake Assay kit, we used a [^{3}H] 5-HT-uptake assay for this experiment because a SigR1 shRNA plasmid simultaneously expresses Green fluorescent protein (GFP). Of note, our previous study demonstrated that SKF-10047 at 500μM tended to increase the uptake activity of WT SERT without statistical significance when we used a [^{3}H] 5-HT-uptake assay (26), which is incomparable to the result shown in Fig. 1A. In both mock-transfected and SigR1 knockdown cells, 500μM SKF-10047 slightly increased the WT SERT uptake activity, which is consistent with our previous study (26). There was no significant difference in the effects of SKF-10047on the SERT activity between mock-transfected and SigR1 knockdown cells (n=4, ns, unpaired t-test).

Supplemental Figure 3

The chaperone activity of SKF-10047 was evaluated using α-lactalbumin aggregates induced by denatured BSA. Aggregation was measured by the absorbance at 490 nm.

No significant change in absorbance was observed between the control and SKF-10047-treated groups, indicating that SKF-10047 did not exhibit chaperone activity.

Supplemental Figure 4

Effects of PRE-084 on the uptake activity of WT SERT and SERTΔCT.

PRE-084, which has higher specificity to SigR1 than does SKF-10047, did not affect the uptake

activity of either WT SERT or SERTΔCT at the concentration of 0 to 10 μM (n=4, one-way ANOVA with Dunnett's post-test).

Supplemental Figure5

Some SigR1 ligands increased the uptake activity of SERT, regardless of whether the ligand was an agonist or an antagonist.

A: Effects of several SigR1 ligands on the uptake activity of SERTΔCT. Among the SigR1 ligands tested, NE-100, an antagonist of SigR1, pentazocine, an agonist of SigR1, and haloperidol, an antagonist of SigR1, significantly increased the activity of SERTΔCT (**P<0.05, n=4, one-way ANOVA with Dunnett's post-test).

B: Effects of several SigR1 ligands on the uptake activity of SERTΔCT in mock-transfected and SigR1 knockdown COS-7 cells. The effect of these drugs on the activity of SERTΔCT was not affected by SigR1 knockdown (n=4, ns, unpaired t-test).

Supplemental Table 1

cDNA array results showed several genes that were upregulated by the treatment of AD293 cells with 200 μM SKF-10047 for 24 h.

We comprehensively examined the difference in mRNA expression between the control cells and the cells treated with 200 μM SKF-10047 for 24 h. The table shows the list of genes that have a global normalization value of more than 10 and an incremental ratio of more than 2. We focused on Syntaxin3 (STX3) as the possible molecule affecting the function of SERT via its membrane trafficking.

Fig.1

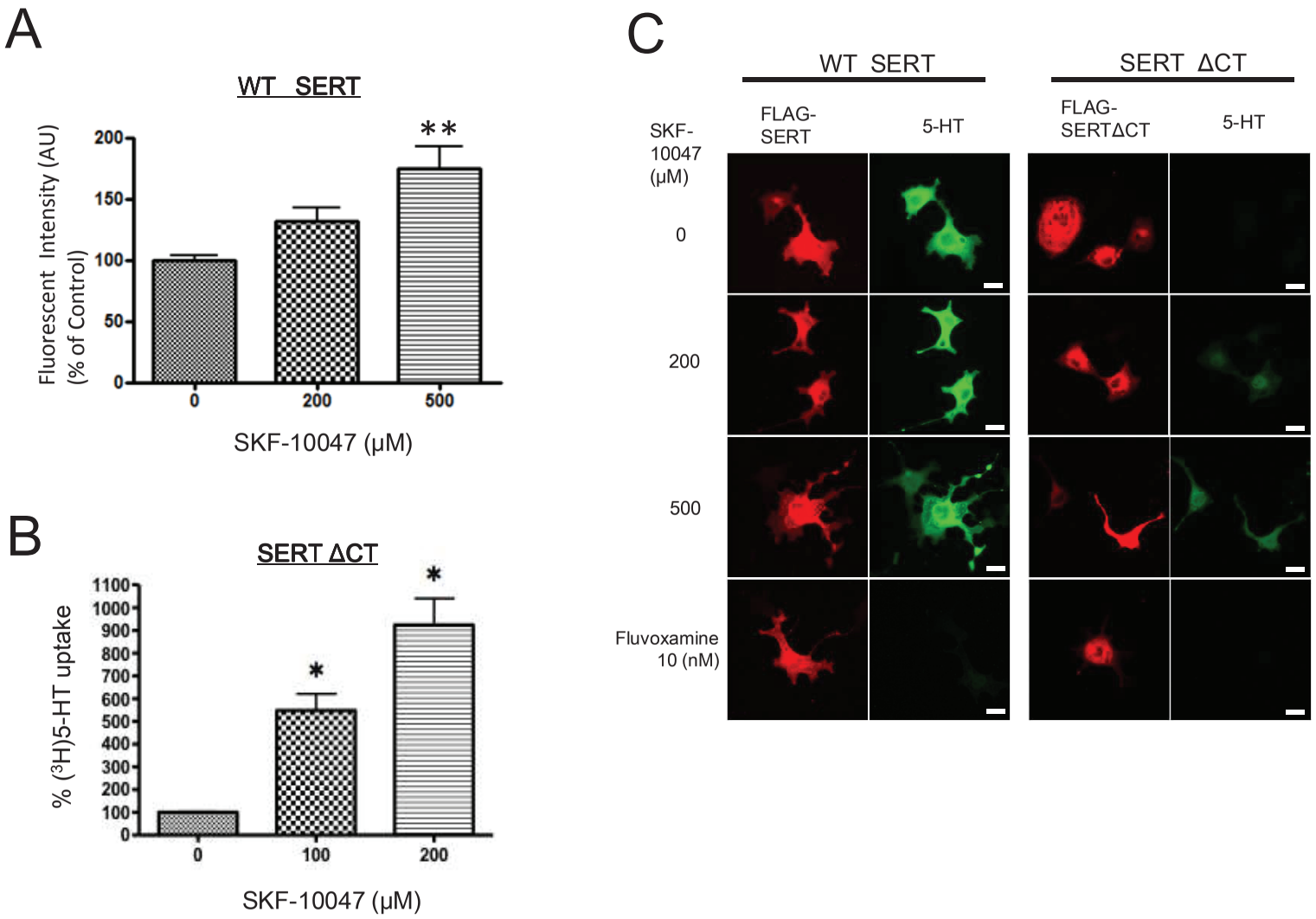

A
WT SERT
Fluorescent Intensity (AU)
(% of Control)
200
150
100
50
0
**
0
200
500
SKF-10047 (μM)
B
SERT ΔCT
% (3H)5-HT uptake
1100
1000
900
800
700
600
500
400
300
200
100
0
*
*
0
100
200
SKF-10047 (μM)
C
WT SERT
SERT ΔCT
FLAG-SERT
5-HT
FLAG-SERTΔCT
5-HT
SKF-10047 (μM)
0
200
500
Fluvoxamine 10 (nM)

Fig.2

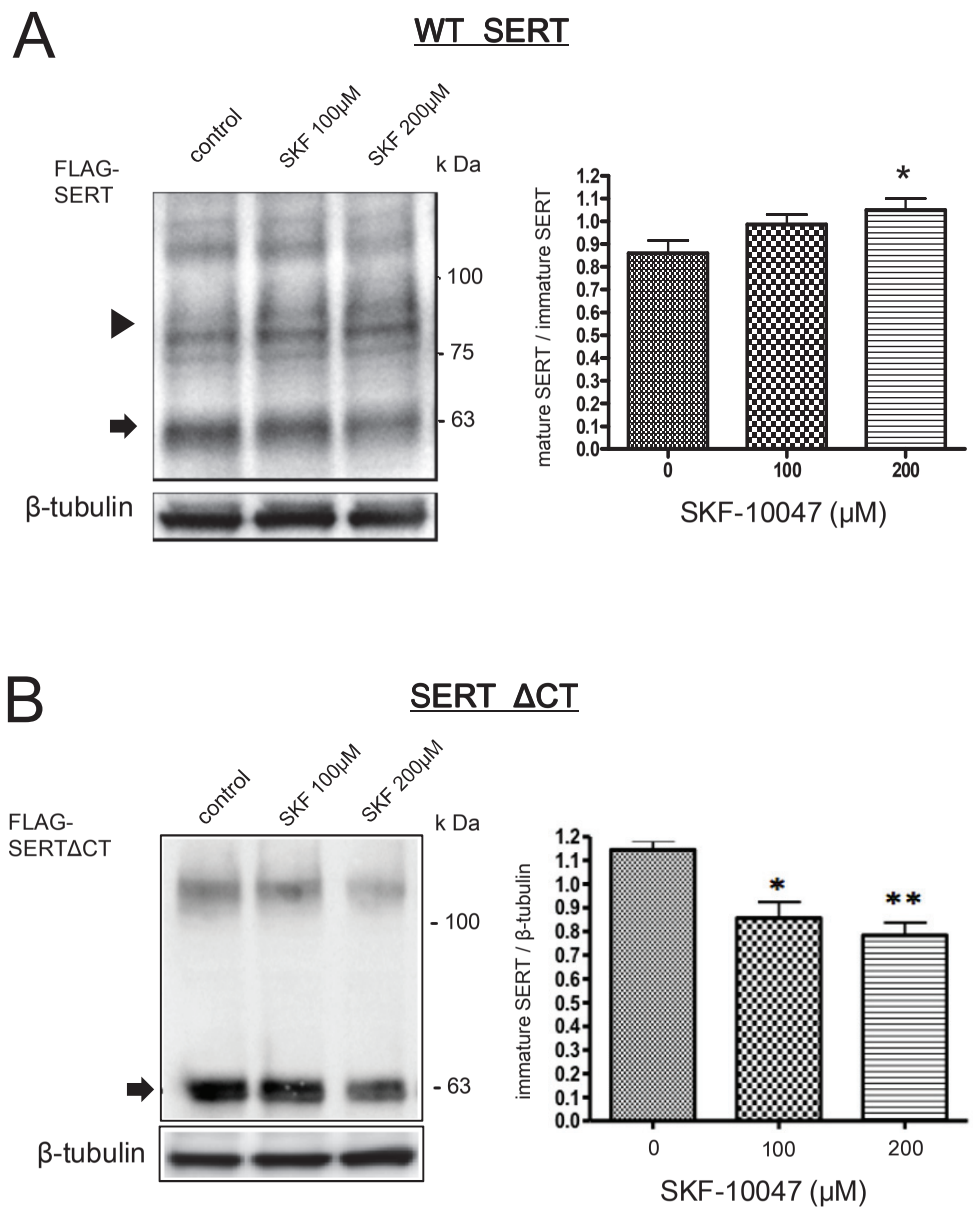

A
WT SERT
control
SKF 100μM
SKF 200μM
FLAG-
SERT
k Da
100
75
63
β-tubulin
mature SERT / immature SERT
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
0
100
200
SKF-10047 (μM)
B
SERT ΔCT
control
SKF 100μM
SKF 200μM
FLAG-
SERTΔCT
k Da
100
63
β-tubulin
immature SERT / β-tubulin
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
**
0
100
200
SKF-10047 (μM)

Fig.3

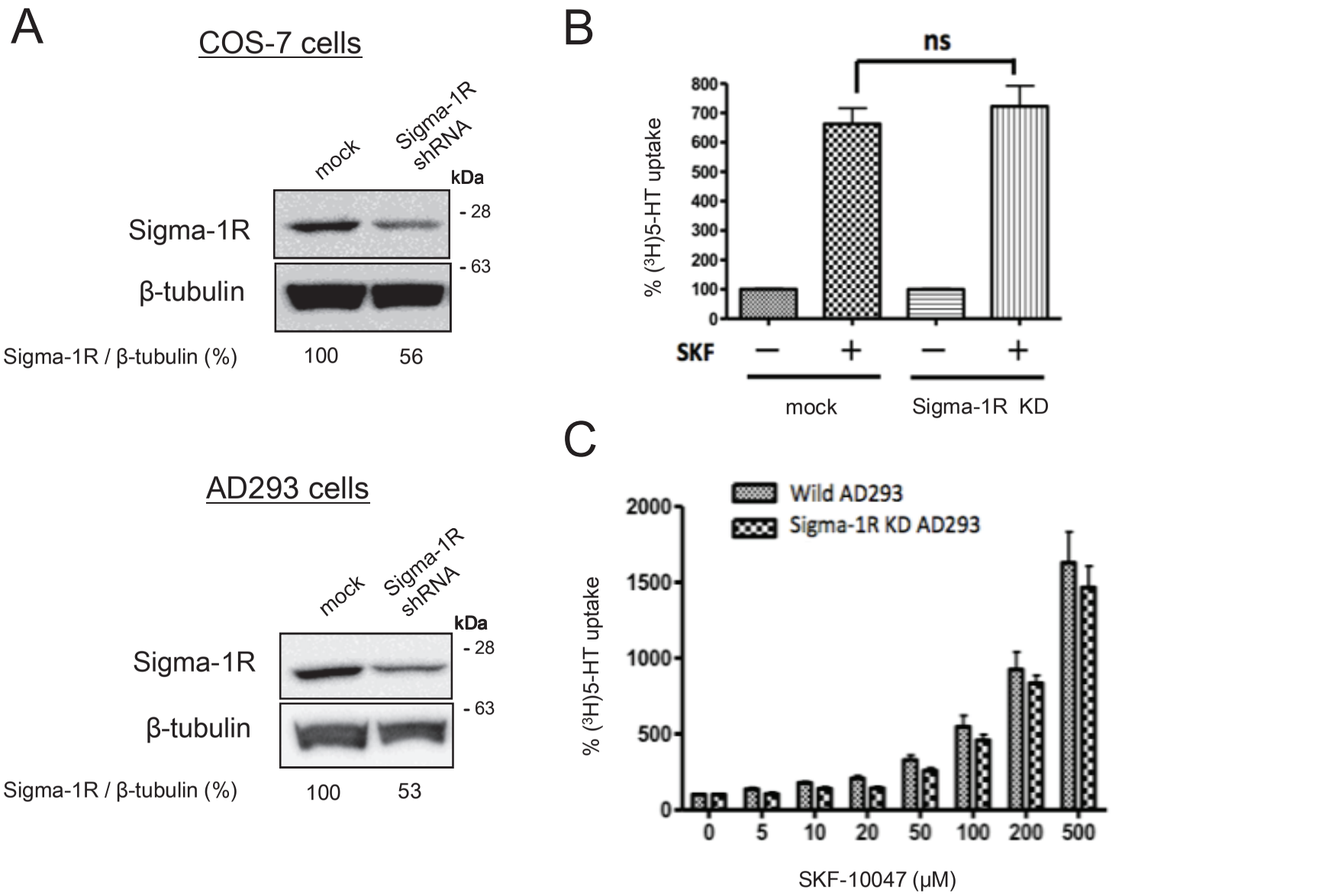

A
COS-7 cells
mock
Sigma-1R shRNA
kDa
Sigma-1R
β-tubulin
- 28
- 63
Sigma-1R / β-tubulin (%)
100
56
AD293 cells
mock
Sigma-1R shRNA
kDa
Sigma-1R
β-tubulin
- 28
- 63
Sigma-1R / β-tubulin (%)
100
53
B
ns
% (3H)5-HT uptake
800
700
600
500
400
300
200
100
0
SKF
−
+
−
+
mock
Sigma-1R KD
C
Wild AD293
Sigma-1R KD AD293
% (3H)5-HT uptake
2000
1500
1000
500
0
0
5
10
20
50
100
200
500
SKF-10047 (μM)

Fig.4

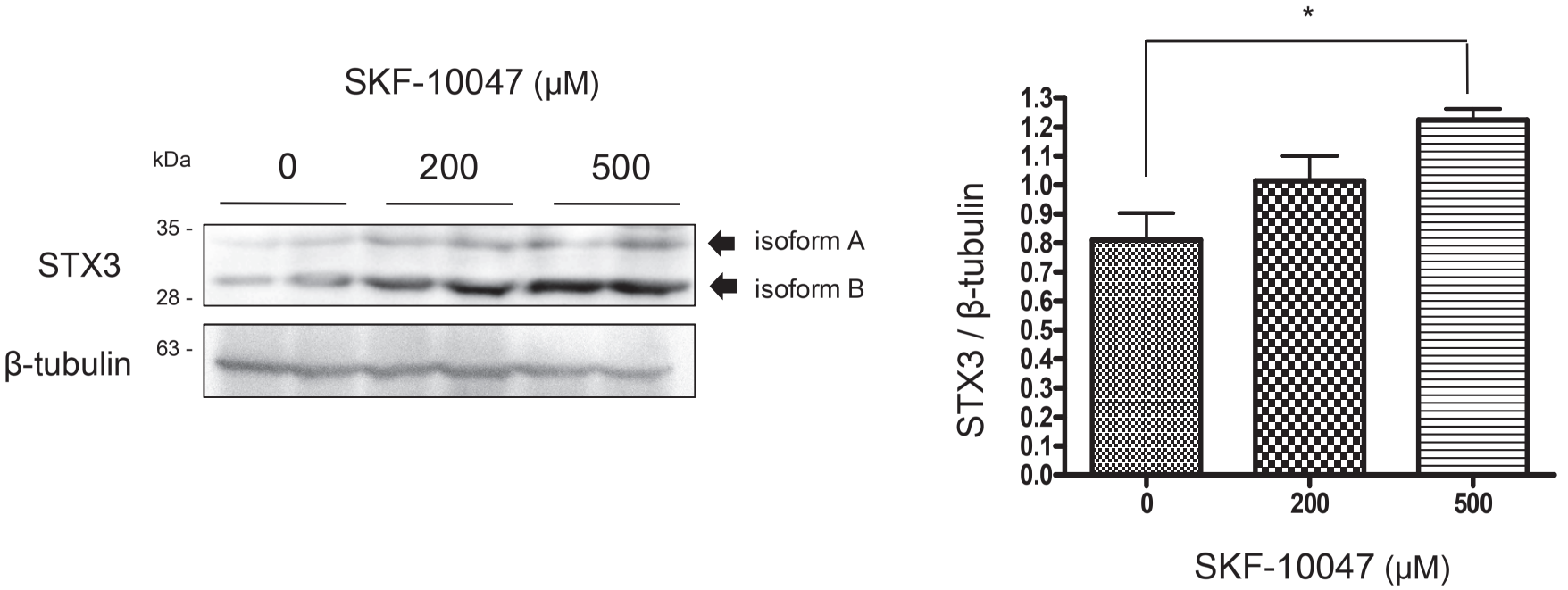
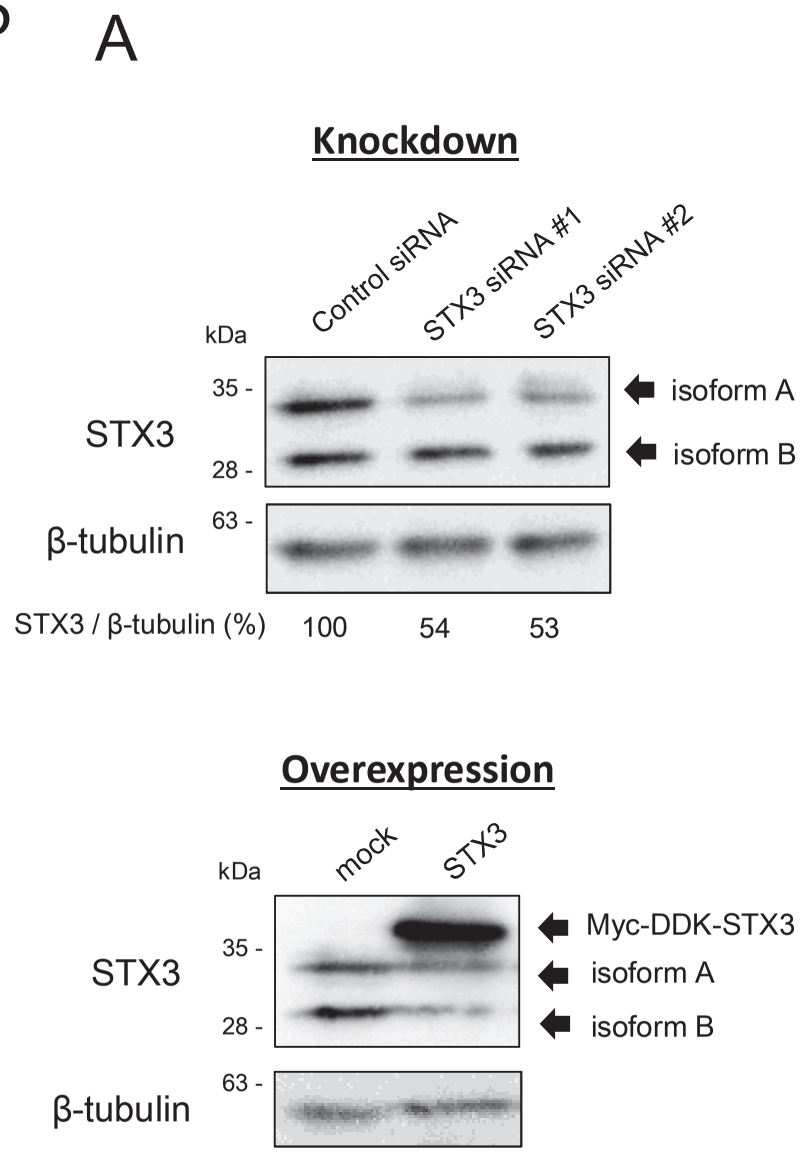
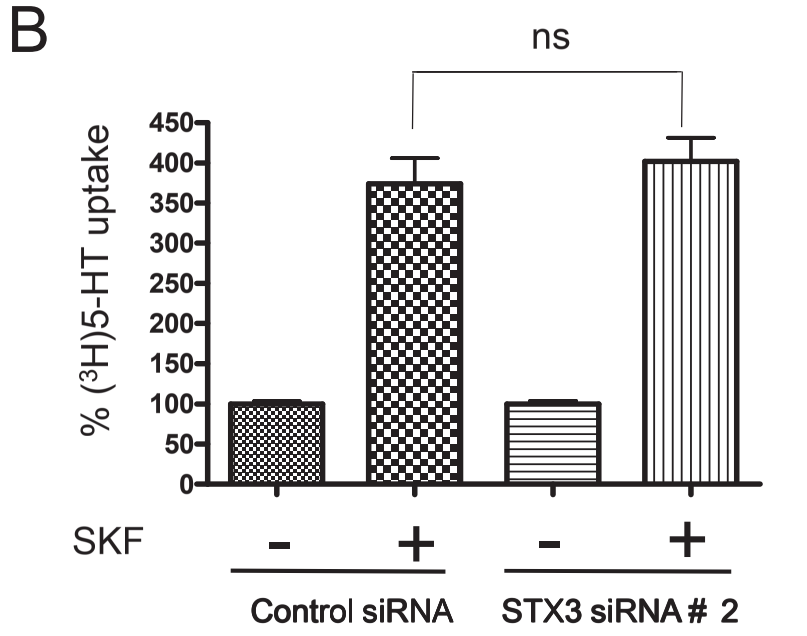
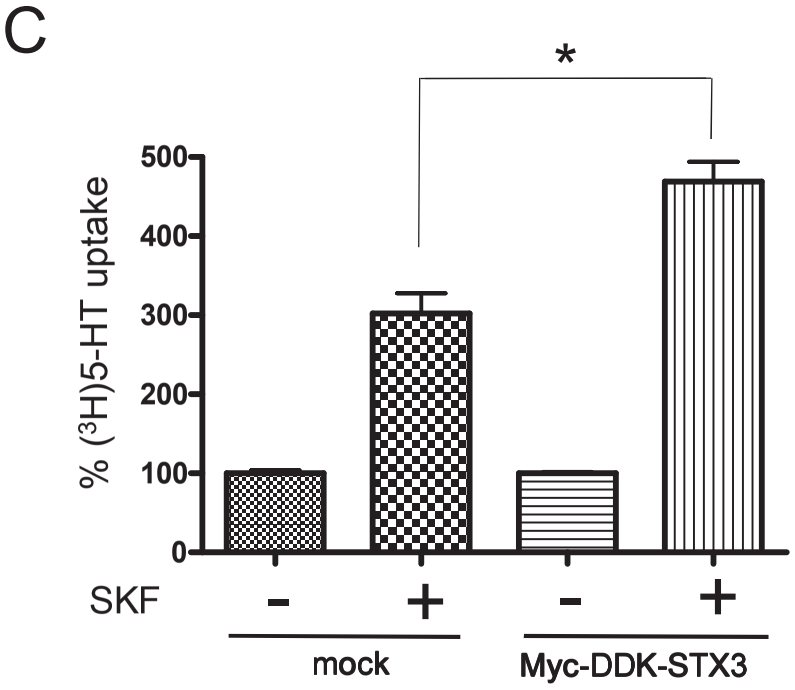

SKF-10047 (μM)
kDa
0
200
500
35 -
28 -
63 -
STX3
β-tubulin
isoform A
isoform B
*
STX3 / β-tubulin
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
200
500
SKF-10047 (μM)

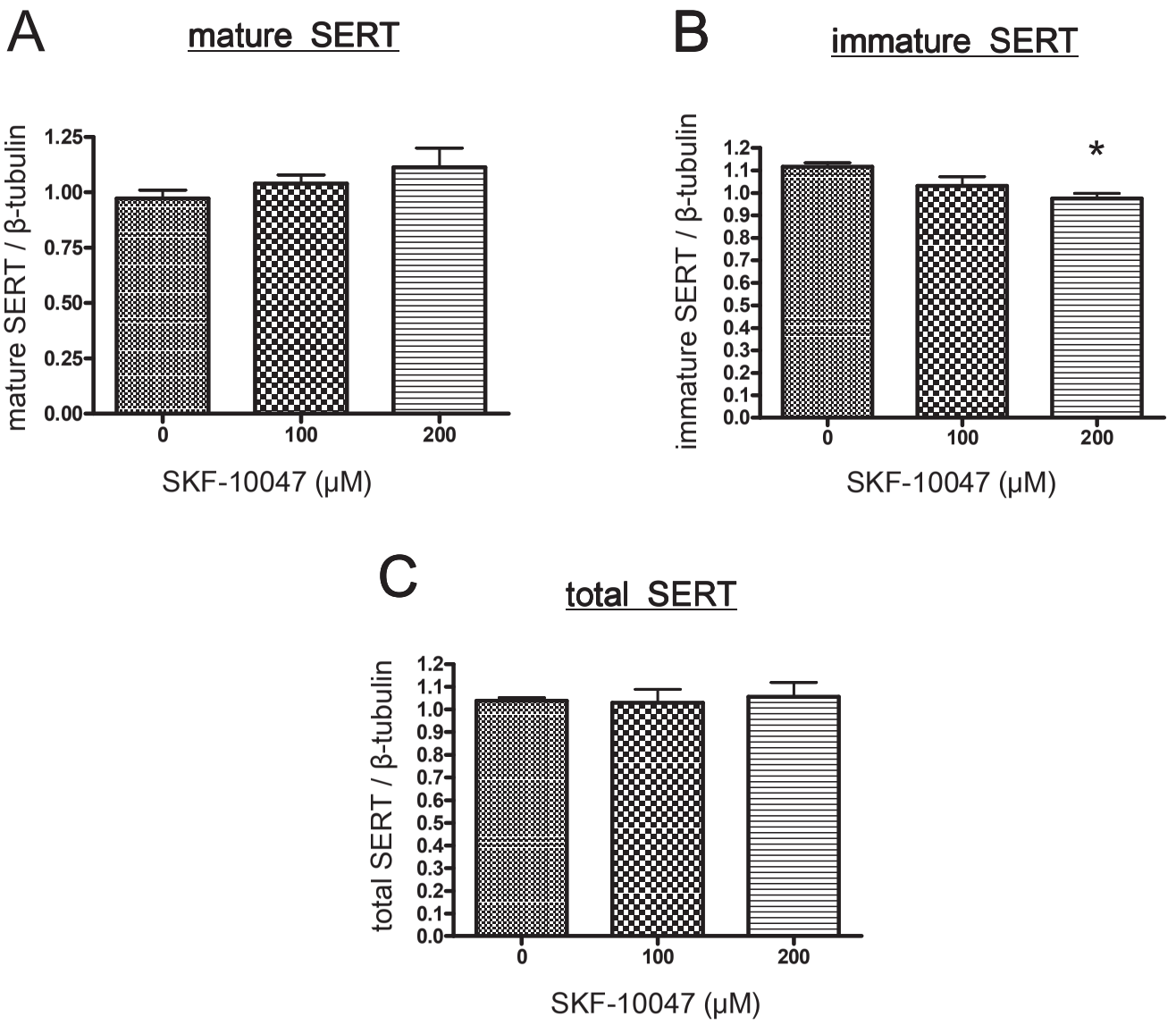
Fig.5


A
Knockdown
Control siRNA
STX3 siRNA #1
STX3 siRNA #2
kDa
35 -
28 -
STX3
isoform A
isoform B
63 -
β-tubulin
STX3 / β-tubulin (%) 100 54 53
Overexpression
mock
STX3
kDa
35 -
28 -
STX3
Myc-DDK-STX3
isoform A
isoform B
63 -
β-tubulin

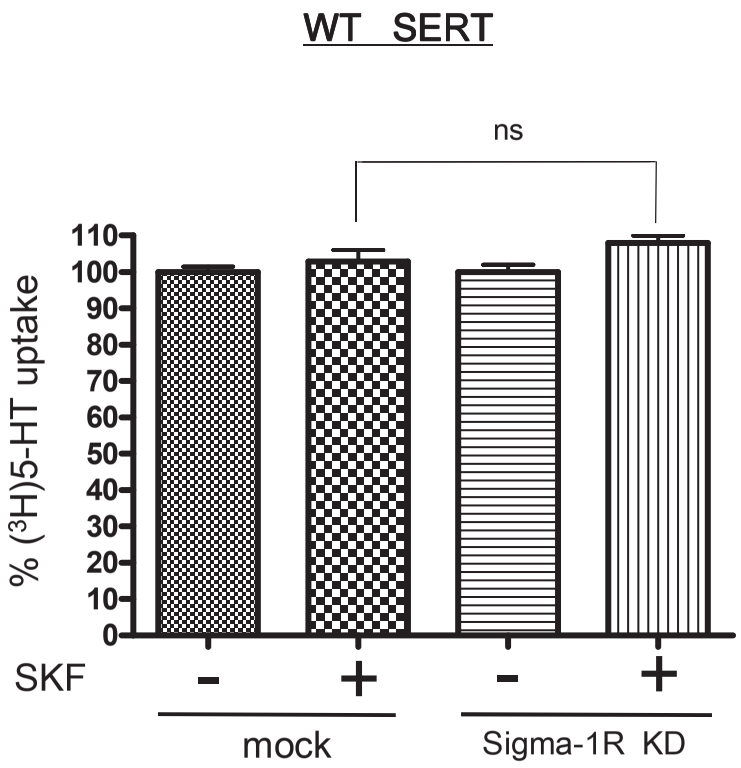

B
ns
% (3H)5-HT uptake
450
400
350
300
250
200
150
100
50
0
SKF
-
+
-
+
Control siRNA
STX3 siRNA # 2


C
*
% (3H)5-HT uptake
500
400
300
200
100
0
SKF
-
+
-
+
mock
Myc-DDK-STX3

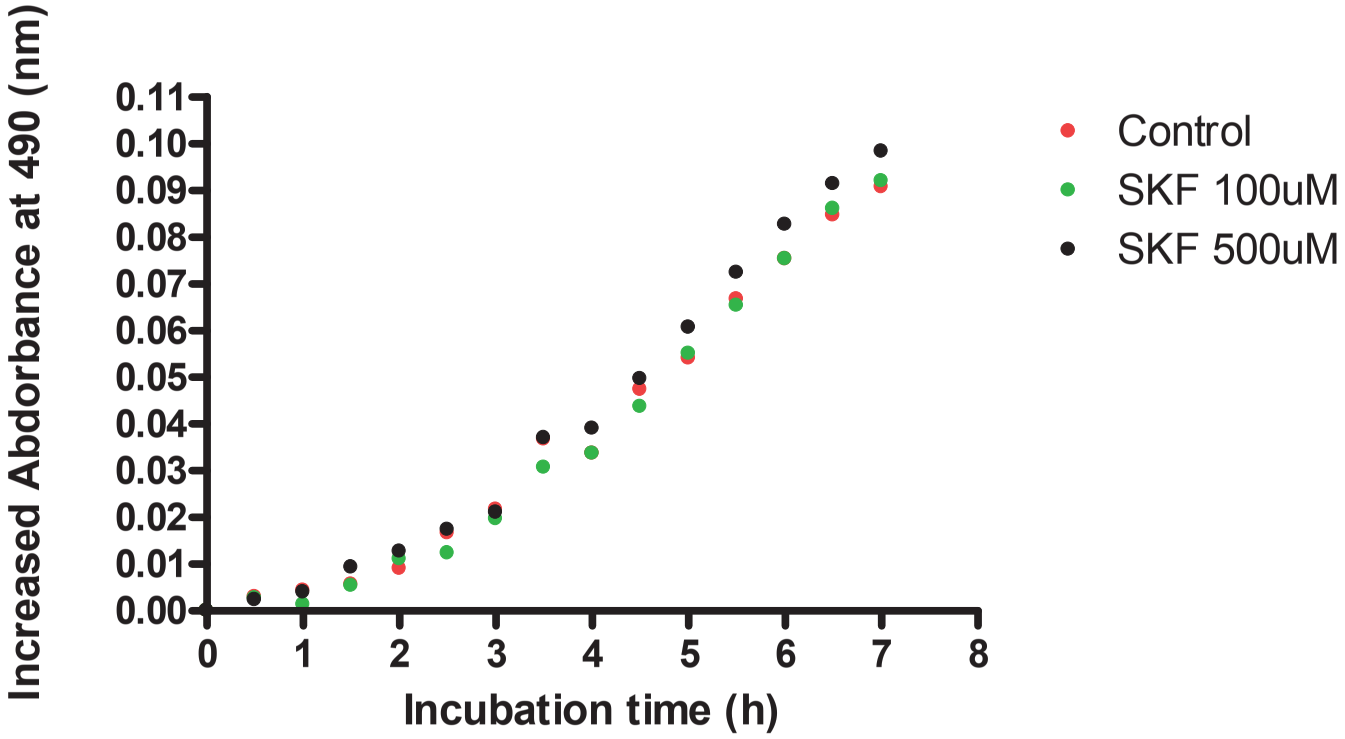
Supplemental Fig.1



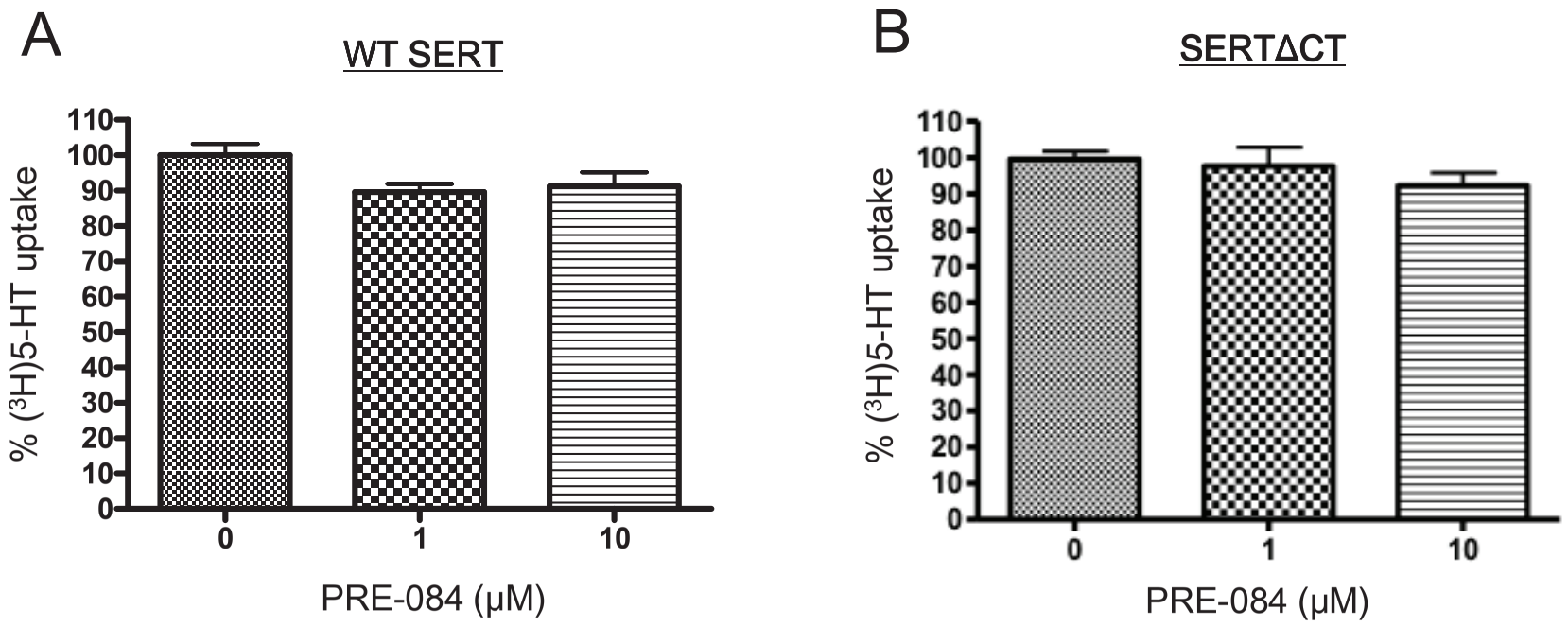
Supplemental Fig.2



Supplemental Fig.3

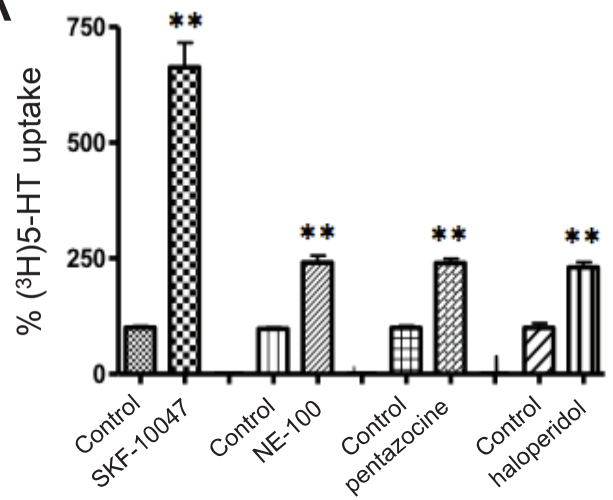


Supplemental Fig.4



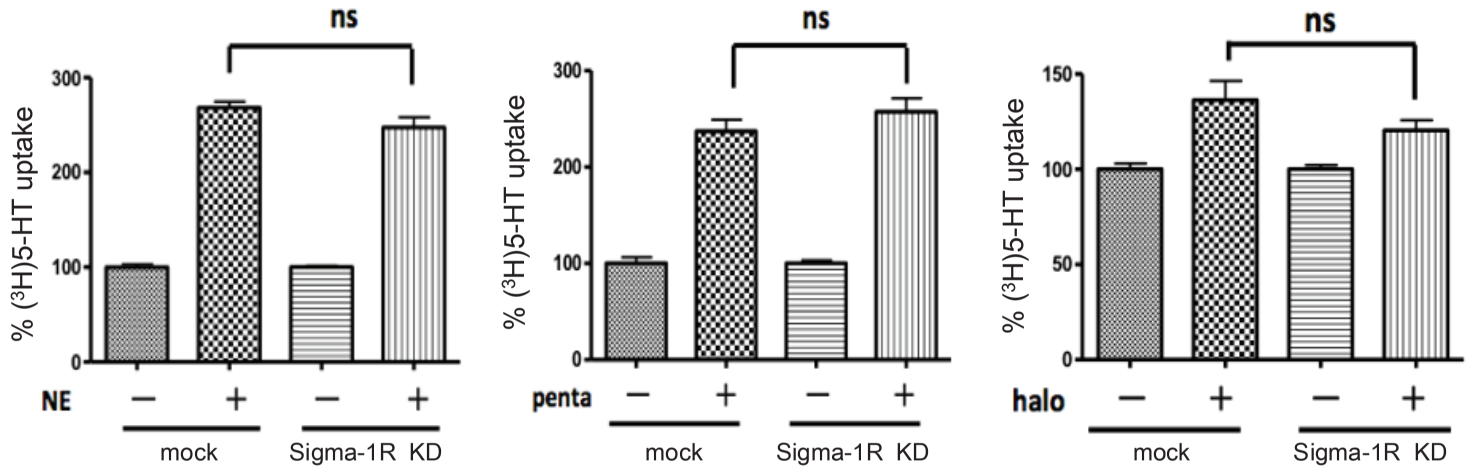
Supplemental Fig.5

A



Ligands	Concentration
SKF-10047	200μM
pentazocine	100μM
NE-100	10μM
haloperidol	10μM

B



Supplemental Table.1

up		global normalization		
symbol	**description**	**Control**	**SKF 200(μM)**	**ratio**
NUPR1	nuclear protein 1, transcriptional regulator	37	142	3.89
CXCL8	C-X-C motif chemokine ligand 8	107	274	2.56
DMRT1	doublesex and mab-3 related transcription factor 1	11	26	2.46
PSG2	pregnancy specific beta-1-glycoprotein 2	19	46	2.43
NXNL1	nucleoredoxin-like 1	15	35	2.37
SKIL	SKI-like proto-oncogene	21	49	2.34
PLEKHA4	pleckstrin homology domain containing A4	17	40	2.27
SLC20A1	solute carrier family 20 member 1	556	1232	2.22
BIRC3	baculoviral IAP repeat containing 3	28	62	2.21
TAC1	tachykinin precursor 1	18	39	2.17
HPCA	hippocalcin	15	33	2.15
TMEM231	transmembrane protein 231	14	31	2.14
ACHE	acetylcholinesterase (Yt blood group)	14	30	2.14
KIT	KIT proto-oncogene receptor tyrosine kinase	28	59	2.14
FBXO15	F-box protein 15	14	29	2.04
STX3	**syntaxin 3**	**285**	**579**	**2.03**
PVRIG	poliovirus receptor related immunoglobulin domain containing	11	21	2.02