

Oligomerization of the Human Serotonin Transporter and of the Rat GABA Transporter 1 Visualized by Fluorescence Resonance Energy Transfer Microscopy in Living Cells*

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Recent biochemical studies indicate that the serotonin transporter can form oligomers. We investigated whether the human serotonin transporter (hSERT) can be visualized as an oligomer in the plasma membrane of intact cells. For this purpose, we generated fusion proteins of hSERT and spectral variants of the green fluorescent protein (cyan and yellow fluorescent proteins, CFP and YFP, respectively). When expressed in human embryonic kidney 293 cells, the resulting fusion proteins (CFP-hSERT and YFP-hSERT) were efficiently inserted into the plasma membrane and were functionally indistinguishable from wild-type hSERT. Oligomers were visualized by fluorescence resonance energy transfer microscopy in living cells using two complementary methods, *i.e.* ratio imaging and donor photobleaching. Interestingly, oligomerization was not confined to hSERT; fluorescence resonance energy transfer was also observed between CFP- and YFP-labeled rat γ -aminobutyric acid transporter. The bulk of serotonin transporters was recovered as high molecular weight complexes upon gel filtration in detergent solution. In contrast, the monomers of CFP-hSERT and YFP-hSERT were essentially undetectable. This indicates that the homo-oligomeric form is the favored state of hSERT in living cells, which is not significantly affected by coin-cubation with transporter substrates or blockers. Based on our observations, we conclude that constitutive oligomer formation might be a general property of Na^+ / Cl^- -dependent neurotransmitter transporters.

It is widely accepted that tyrosine kinases and related receptors signal as dimers (1). Similarly, the oligomeric nature of voltage-dependent and ligand-gated ion channels is firmly established. In addition, over recent years it has been determined that other integral membrane proteins, which were originally thought to exist in monomeric form, actually form homo- and hetero-oligomers. For instance, this is true for several G protein-coupled receptors (2–5) and for a number of transporters, *e.g.* the erythrocyte glucose transporter-1 and the brain gluta-

mate transporter (6, 7). The structural organization of transporters is likely to determine their function. This consideration is particularly relevant in understanding the transporters that mediate the re-uptake of neurotransmitters from the synaptic cleft (8). These proteins depend on the presence of Na^+ and Cl^- and generate a current during transport, *i.e.* they may share properties similar to ion channels (9–12) that are known to be organized as oligomeric complexes. The human serotonin transporter (hSERT)¹ is a prototypic member of this family; its properties are of considerable clinical interest because the inhibitors are useful as antidepressants, and substrates that induce the reversal of transport (*e.g.* “ecstasy”) are abused (13). The complexity of the transport reaction is suggestive of a higher level of organization, and recent biochemical experiments on the SERT of different species indicate that the transporter can, in principle, form oligomeric structures (14–17).

However, it is at present unknown whether SERT exists as an oligomer in the membrane *in situ* and whether this is the preferred conformation. To address these issues, we applied a nondestructive method to visualize hSERT in living cells. This has become possible by the recent development of green fluorescent protein (GFP) variants that are suited to monitor close associations of fusion proteins by means of fluorescence resonance energy transfer (FRET), a quantum physical phenomenon that was first described by Förster (18).

The human SERT was tagged on its amino terminus with cyan fluorescent proteins (CFP) and yellow fluorescent proteins (YFP), respectively; these spectral variants of GFP have an appropriate spectral overlap of the donor (CFP) emission and the acceptor (YFP) excitation (19). Our observations show that hSERT exists as an oligomer in the membrane of living cells and that this is the preferred conformation. Moreover, this finding was not confined to the hSERT alone. A similar homoassociation exists with the rat GABA transporter 1 (rGAT1).

EXPERIMENTAL PROCEDURES

Materials—Tissue culture reagents were from Life Technologies, Inc. [³H]5-Hydroxytryptamine (HT) was from PerkinElmer Life Sciences. The following drugs were kindly donated: citalopram (Lundbeck A/S, København, Denmark), paroxetine (SmithKline Beecham, Worthing, United Kingdom), and cocaine (Dolda AG, Basel, Switzerland). Imipramine, *para*-chloroamphetamine, and serotonin were from Sigma. Meth-

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¹ The abbreviations used are: hSERT, human serotonin transporter; GFP, green fluorescent protein; FRET, fluorescence resonance energy transfer; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; GABA, γ -aminobutyric acid; rGAT1, rat GABA transporter 1; 5-HT, 5-hydroxytryptamine (= serotonin); hD₂R, human dopamine D₂-receptor; HEK-293, human embryonic kidney cells 293; FPLC, fast protein liquid chromatography.

ylenedioximethamphetamine ("ecstasy") was from Research Biochemical International (Natick, MA). All other chemicals were from commercial sources.

Plasmid Construction—hSERT cDNA was a generous gift of Dr. R. D. Blakely (Vanderbilt University, Nashville, TN). A *HindIII/XbaI* fragment (encompassing the coding region) was inserted into the plasmid pRC/CMV (Invitrogen), ligated into *HindIII/XbaI*-digested pEGFP-C1 (CLONTECH, Palo Alto, CA) to produce hSERT-pEGFP-C1, and transferred from this vector using *XhoI* to the plasmids pECFP-C1 and pEYFP-C1 (CLONTECH) to produce the plasmids hSERT-pECFP-C1 and hSERT-pEYFP-C1, respectively. GFP, CFP, or YFP is fused to the NH₂ terminus of hSERT and resides in the cytoplasm.

A *HindIII/StuI* fragment of the human dopamine D₂-receptor (hD₂R), encompassing the coding region and lacking eight COOH-terminal amino acids, was ligated into *HindIII/SmaI*-digested pYFP-N1 to produce the plasmid hD₂R-YFP; YFP is fused to the COOH terminus of hD₂R and situated in the cytoplasm of the cell.

As a positive control for FRET imaging, we constructed a fusion protein of CFP and YFP. The yellow variant, GFP10C, was a generous gift of Dr. Roger Tsien (University of California, San Diego, CA) and was subcloned into pEGFP-C1 COOH-terminal to the enhanced GFP, which was subsequently replaced by CFP (derived from pECFP-C1), thus resulting in a plasmid coding for a CFP-YFP tandem.

The cDNA encoding the rGAT1 was a generous gift of Dr. Patrick Schloss (ZI für Seelische Gesundheit, Mannheim, Germany). The coding region was excised using *BbrPI* and *KpnI*; blunt ends were generated at the *SalI* restriction site of the vector pEYFP-C1 (CLONTECH), which was digested afterward with *KpnI*. The rGAT1-cDNA was then inserted to result in the construct YFP-rGAT1. Subsequently, the rGAT1-cDNA was subcloned into the vector pECFP-C1 (CLONTECH) using the enzymes *SacI* and *KpnI* to generate the construct CFP-rGAT1.

Cell Culture and Transfection—Human embryonic kidney 293 (HEK-293) and HeLa cells were grown in minimal essential medium with Earle's salts and L-alanyl-L-glutamine (L-GlutaMAX I™, Life Technologies, Inc.), 10% fetal bovine serum, and 50 mg/liter gentamicin on 10-cm diameter cell culture dishes at 37 °C in an atmosphere of 5% CO₂, 95% air. One day before transfection, cells were replated to obtain subconfluent cultures either on glass coverslips (22 mm in diameter and placed into 6-well plates) or on 12-well plates (2 × 10⁵ cells/well plate) for uptake experiments. Transient transfections were performed with equal amounts of CFP and YFP plasmids using the CaPO₄ precipitation method or LipofectAMINE Plus™ as described (20).

Transport of [³H]5-Hydroxytryptamine—Uptake experiments were performed as described earlier (21). Cells transfected with either hSERT-wild type, CFP-hSERT, YFP-hSERT, or pECFP-C1, pEYFP-C1 vectors were incubated for 5 min at 22 °C in 0.5 ml of Krebs-Ringer-Hepes buffer (10 mM Hepes, 120 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 20 mM glucose, final pH 7.3) containing 2 μCi of [³H]5-HT (specific activity adjusted with unlabeled 5-HT). The amount of accumulated radioactivity was determined by liquid scintillation counting.

FRET Microscopy (Ratio Imaging and Donor Photobleaching FRET Microscopy)—Transfected HEK-293 or HeLa cells were investigated 1 day after transfection on a Nikon Diaphot TMD microscope using filter sets, which discriminate between CFP and YFP fluorescence (Omega Optical Inc., Brattleboro, VT) (CFP filter set: excitation, 440 nm; dichroic mirror, 455 nm; emission 480 nm; YFP filter set: excitation, 500 nm; dichroic mirror, 525 nm; emission, 535 nm), and a cooled CCD-camera (Kappa GmbH, Gleichen, Germany). For ratio imaging FRET microscopy, images were taken with the donor filter set (for CFP) and a FRET filter set (XF88, Omega Optical) with excitation of the donor (440 nm), a 455 nm dichroic mirror, and an emission filter for the acceptor (535 nm). Images were captured with both filter sets under identical conditions. This choice of settings was based on initial experiments with cells expressing CFP and YFP. These experiments were employed to verify that the imaging parameters did not result in spurious ratio images. Ratio images that are suited to detect a decrease in donor and an increase in acceptor fluorescence were calculated by dividing the acceptor filter image by the donor image (20, 22) using the NIH image software version 1.62. In principle, black (zero) ratio images are obtained even if an equal intensity of fluorescence is recorded with the donor and FRET filters. Nevertheless, to prevent the detection of false positive FRET images, the imaging conditions were adjusted to favor donor emission over acceptor emission. These settings introduce a bias against acceptor emission and, furthermore, underestimate FRET. We chose to sacrifice the sensitivity for detection of FRET for the sake of increasing the specificity of the signal.

Quantification of fluorescence ratios was achieved by imaging the

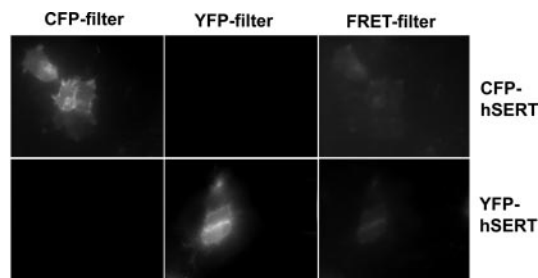


FIG. 1. Fluorescence microscopy. HEK-293 cells were transfected with plasmids encoding CFP-hSERT (top row) or YFP-hSERT (bottom row). The next day images (× 40 magnification) were taken using the CFP filter set (left column), the YFP filter set (middle column) and the FRET filter set (right column) as outlined under "Experimental Procedures." The images are representative of two different transfections with acquisition of five images with each different filter set.

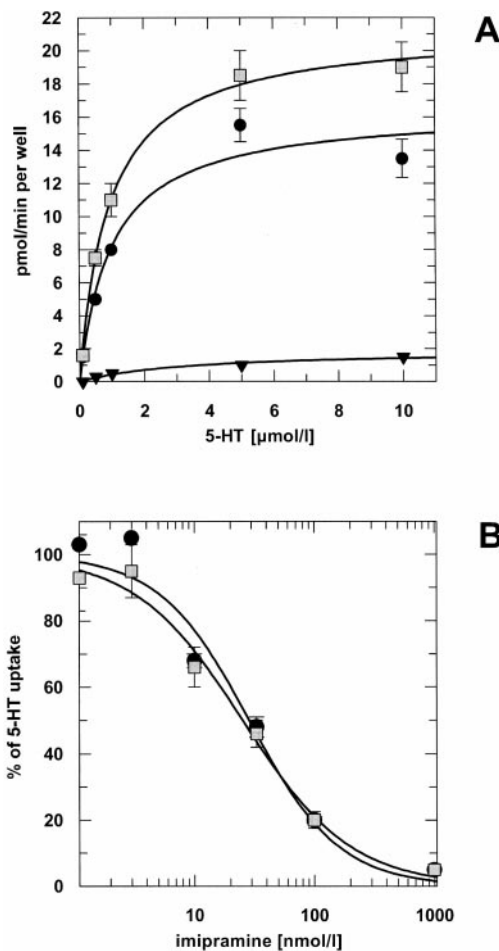


FIG. 2. Functional properties of YFP-hSERT and CFP-hSERT constructs. A, saturation analysis of 5-HT uptake. HEK-293 cells transfected with plasmids encoding CFP and YFP (▼), wild-type hSERT (●), or equal amounts of cDNA encoding CFP-hSERT and YFP-hSERT (gray square) were incubated with the indicated concentrations of [³H]5-HT for 5 min. B, inhibition of uptake by imipramine. HEK-293 cells transfected with plasmids encoding wild-type hSERT (●) or CFP-hSERT and YFP-hSERT (gray square) were incubated with increasing concentrations of imipramine to inhibit [³H]5-HT uptake.

transfected cells at lower magnification (× 10 objective) with donor and acceptor filter sets and by subsequently determining the integrated density values (ID = number of pixels × (mean intensity background)) for the calculation of the YFP:CFP fluorescence ratio at donor excitation.

Photobleaching FRET microscopy was done by continuous illumination with a 100-watt-mercury lamp and the CFP filter set with time series imaging for 1 min (with the acquisition of one image every 2 s), which was sufficient to bleach the donor to an extent of less than 20%.

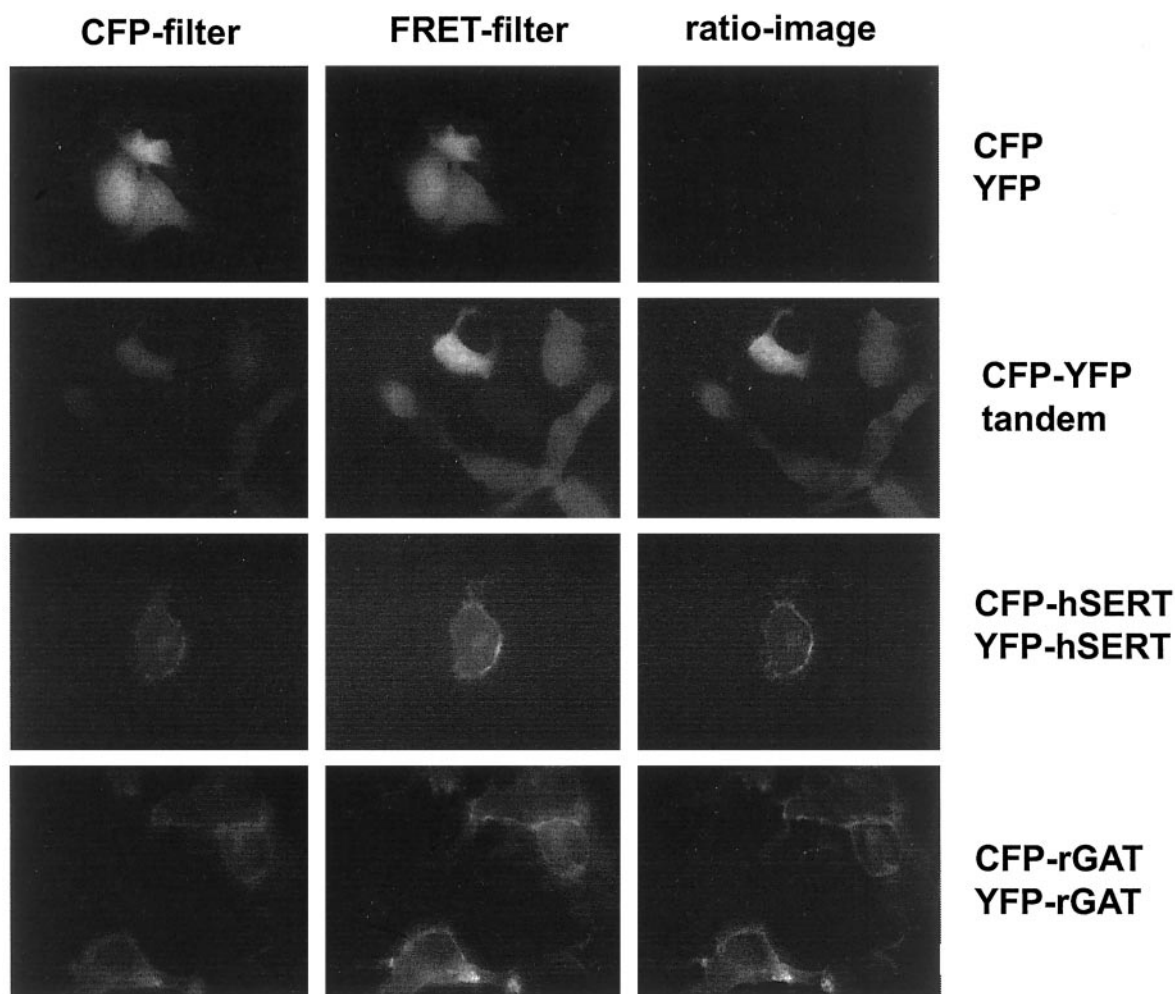


FIG. 3. **Ratio-imaging FRET microscopy.** HEK-293 cells were transfected with equal amounts of plasmids encoding CFP and YFP (top row, A–C), the CFP-YFP tandem (second row, D–F), equal amounts of vectors coding for CFP-hSERT and YFP-hSERT (third row, G–I), or CFP-rGAT and YFP-rGAT (bottom row, K–M). The next day, images ($\times 40$ magnification) were taken using the donor filter set (CFP-filter) (left column, A, D, G, K) and the FRET filter set (middle column, B, E, H, L) as outlined under “Experimental Procedures.” The ratio image (right column, C, F, I, M) represents the division of the FRET filter images by the donor filter images. The images are representative of 10 different transfections with the acquisition of 12 images with each different filter set.

Regions of interest were selected over the membrane, and fluorescence emission intensities were quantified using the NIH image software. The resulting decay curves were fitted to the equation for a single exponential decay approaching a constant value: fluorescence intensity = $A_0 * e^{-Kt} + \text{offset}$, where A_0 denotes the starting value, offset denotes the final fluorescence signal, and K is the decay constant. The time constant τ (fluorescence lifetime) is defined as $1/K$.

Fluorometry—Extracts were prepared from cells expressing CFP-hSERT and YFP-hSERT or CFP-hSERT and YFP by solubilizing the particulate fraction in phosphate-buffered saline containing 0.5% Nonidet P-40 and protease inhibitors (10 $\mu\text{g}/\text{ml}$ of aprotinin, 20 $\mu\text{g}/\text{ml}$ of phosphoramidon, 40 $\mu\text{g}/\text{ml}$ Pefabloc, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstatin, 1 mM EDTA from 1000 \times stock solutions). The insoluble material containing cell nuclei was removed by centrifugation at 14,000 $\times g$ for 15 min, and the supernatant was measured on a Jasco FP-920 spectrofluorometer. Emission wavelength scans were performed with excitation at 436 nm (for CFP and FRET measurements) or at 500 nm (for YFP). The bandwidth of excitation was 18 nm, and that of the emission was set to 10 nm. Emission scans with an excitation at 500 nm were performed to determine the amount of YFP without the influence of FRET. The minor contribution of YFP fluorescence to the emission curve at the CFP excitation wavelength (436 nm), which results from coexcitation of YFP at that wavelength (instead of sensitized fluorescence due to FRET), was subtracted from the emission scans, and the curves were normalized to equal CFP peak fluorescence at 476 nm. By that means, FRET can be detected by a shift of the emission scan at the acceptor wavelength showing the increase in acceptor fluorescence.

FPLC Analysis—Extracts were prepared from CFP-hSERT and YFP-hSERT expressing cells as described above and subjected to gel filtra-

tion on a Pharmacia FPLC system using a Superdex 200 matrix equilibrated with extraction buffer at a flow rate of 2 ml/min. The column (diameter = 16 mm, length = 2 m, volume = 402 ml) was calibrated with appropriate standards (Bio-Rad catalog no. 151-1901; 670, 158, 44, 17, and 1.35 kDa). Fluorescent proteins were detected during FPLC elution with a Jasco FP-920 spectrofluorometer in the kinetics mode using a 16- μl flow cell, excitation at 436 nm, and emission at 510 nm with an 18-nm bandwidth, respectively. Recording of the fluorescence signal was started briefly before the flow through of the void volume with read outs every 30 s for a total of 2 h.

Statistics—All results are expressed as means \pm S.E. values. The significance of differences among the means of various groups was determined by Student's *t* test for independent samples.

RESULTS AND DISCUSSION

The major aim of this study was to investigate the serotonin transporter in living cells by using GFP chimeras of this pharmacologically important transporter molecule. For that purpose, it is essential to verify the functional integrity of the fusion protein. This was achieved on several levels. Initially, we confirmed that the chimeric proteins comprising hSERT and different variants of GFP were inserted into the plasma membrane. Upon expression of CFP-hSERT and YFP-hSERT in HEK-293 cells (or in HeLa cells, data not shown), the bulk of the fluorescence was recorded over the plasma membrane (Fig. 1 and see Fig. 3G). The imaging of CFP-hSERT expressing cells with the YFP filter set showed no fluorescence signal. The same

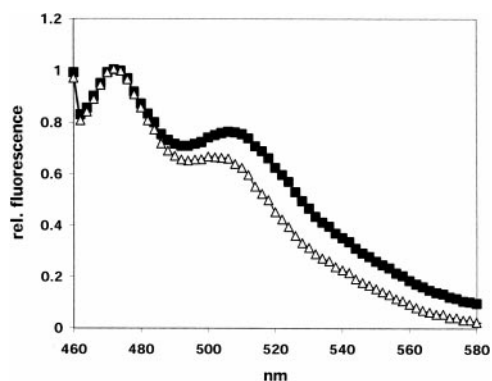


FIG. 4. **Scanning fluorometry of CFP- and YFP-hSERT-containing extracts.** HEK-293 cells were transfected either with equal amounts of CFP-hSERT and YFP-hSERT (■) or with CFP-hSERT in combination with YFP (△). One day after transfection, extracts were prepared and measured by spectrofluorometry as described under "Experimental Procedures." Acceptor fluorescence resulting from coexcitation of YFP at the CFP excitation wavelength was subtracted, and the emission scans were normalized to donor fluorescence.

was found with YFP-hSERT expressing cells and image acquisition with the CFP filter set, proving the specificity of the filters and the imaging conditions (Fig. 1). Uptake measurements showed that the fusion proteins of hSERT with GFP variants transported the natural substrate 5-HT with V_{max} and K_m values similar to those of wild-type hSERT (Fig. 2A; $V_{max} = 17 \pm 1$ versus 22 ± 2 pmol/min/well and $K_m = 0.99 \pm 0.23$ versus 0.94 ± 0.37 μ M for cells transfected with YFP-hSERT/CFP-hSERT and hSERT, respectively; $n = 3$). Cells transfected with CFP and YFP alone showed no specific uptake of 5-HT (Fig. 2A). Similarly, the inhibition of 5-HT influx by imipramine exhibited no significant difference in both cases ($IC_{50} = 16 \pm 2$ and 19 ± 3 nM, $n = 3$ for cells expressing YFP-hSERT/CFP-hSERT and hSERT, respectively, Fig. 2B). Thus, CFP- and YFP-tagged fusion proteins of hSERT are functional with characteristics that are very similar to the wild-type protein.

To visualize the oligomeric state of hSERT in living cells, we applied FRET microscopy using ratio imaging. The negative control consisted of cells that expressed CFP and YFP. In these cells, the donor image (Fig. 3A) showed a higher fluorescence signal than the FRET image (Fig. 3B) by using the imaging conditions as described under "Experimental Procedures." This observation results in a black ratio image (Fig. 3C). As a positive control, we used cells expressing the YFP-CFP tandem. Given the known Förster distance (~ 50 Å for CFP and YFP), the short tether in the CFP-YFP tandem, and its soluble nature, a strong intramolecular FRET was to be expected in the cytosol. The fluorescence intensity recorded with the FRET filter set (Fig. 3E) exceeded the intensity measured with the donor filter set (Fig. 3D), resulting in an intense cytosolic FRET signal in the ratio image (Fig. 3F).

If hSERT oligomerized in the plasma membrane, the intermolecular FRET ought to be seen in cells coexpressing YFP-hSERT and CFP-hSERT. These experiments were performed by the transient transfection of two different cell lines (HEK-293 and HeLa cells) with clear-cut evidence for FRET in each case. At the wavelength for donor excitation, fluorescence emission recorded with the CFP filter set (Fig. 3G) was obviously lower than the signal obtained with the FRET filter (Fig. 3H). This loss in donor emission is indicative of FRET. Accordingly, the ratio image revealed a signal confined to the cellular membrane (Fig. 3I). Cells expressing CFP-hSERT or YFP-hSERT alone did not exhibit any positive ratio image, thereby further supporting the specificity of this FRET imaging technique (Fig. 1). The clear positive ratio image of CFP-hSERT and YFP-

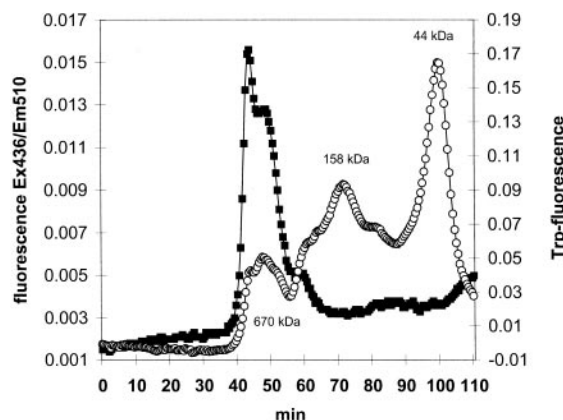


FIG. 5. **Gel filtration chromatography of CFP-hSERT and YFP-hSERT.** Detergent extracts were prepared from HEK-293 cells transfected with CFP-hSERT and YFP-hSERT. Extracts (■) were subjected to gel filtration chromatography on a FPLC system using Superdex 200 matrix calibrated with appropriate standards as indicated (○) (flow rate: 2 ml/min). Fluorescent proteins were detected during FPLC elution with a Jasco FP-920 spectrofluorometer (excitation = 436 nm, emission = 510 nm, bandwidth = 18 nm). Standard proteins were monitored by their inherent tryptophan fluorescence.

hSERT coexpressing cells can only be accounted for by intermolecular FRET due to oligomerization of the fluorescently tagged hSERT proteins. This oligomerization was detected not only in cells expressing CFP- and YFP-hSERT but also in cells expressing the CFP- and YFP-tagged rGAT1, which is a member of the same subfamily of Na^+/Cl^- -dependent neurotransmitter transporter proteins (sharing about 50% sequence homology) (Fig. 3, bottom line, $K-M$).

We also quantified FRET at a lower magnification in several independent experiments to obtain an average for a high number of transfected cells. The corresponding fluorescence ratio was significantly higher for images taken from cells expressing the YFP-CFP tandem (1.94 ± 0.37 , $n = 7$) and from cells coexpressing YFP-hSERT and CFP-hSERT (1.56 ± 0.13 , $n = 22$) or YFP-rGAT1 and CFP-rGAT1 (1.61 ± 0.18 , $n = 9$) than the ratio obtained for the negative control cells (*i.e.* coexpressing CFP and YFP, 0.54 ± 0.09 , $n = 13$).

The interaction between CFP- and YFP-tagged hSERT was further verified *in vitro* by means of emission scanning fluorometry. If CFP and YFP were in close proximity due to oligomerization of tagged hSERT molecules, this would result in FRET between CFP and YFP. Hence, an increase of the YFP fluorescence (sensitized acceptor fluorescence) ought to be observed at the excitation wavelength of CFP (*i.e.* the FRET donor), and this effect ought to be clearly detectable in emission wavelength scans. To test for sensitized acceptor fluorescence, we prepared extracts from cells expressing either CFP- and YFP-hSERT or CFP-hSERT with untagged YFP as control and recorded emission wavelength scans at the donor and acceptor excitation, respectively. Normalized emission scans of these extracts revealed a distinct sensitized acceptor fluorescence for the CFP-hSERT/YFP-hSERT sample as compared with control extracts (Fig. 4), which is clearly indicative of FRET, and this can arise only due to oligomerization of CFP- and YFP-hSERT.

The detection of intermolecular FRET unequivocally demonstrated the propensity of hSERT to form an oligomer in the cellular membrane of intact cells; however, it was not possible to estimate the relative proportion of transporters in the oligomeric or monomeric form by FRET. To verify that oligomers represented a sizable fraction of the total, CFP-hSERT and YFP-hSERT were solubilized from the plasma membranes in the presence of 0.5% Nonidet P-40 and subjected to gel filtration. The bulk of the transporter protein eluted in the high

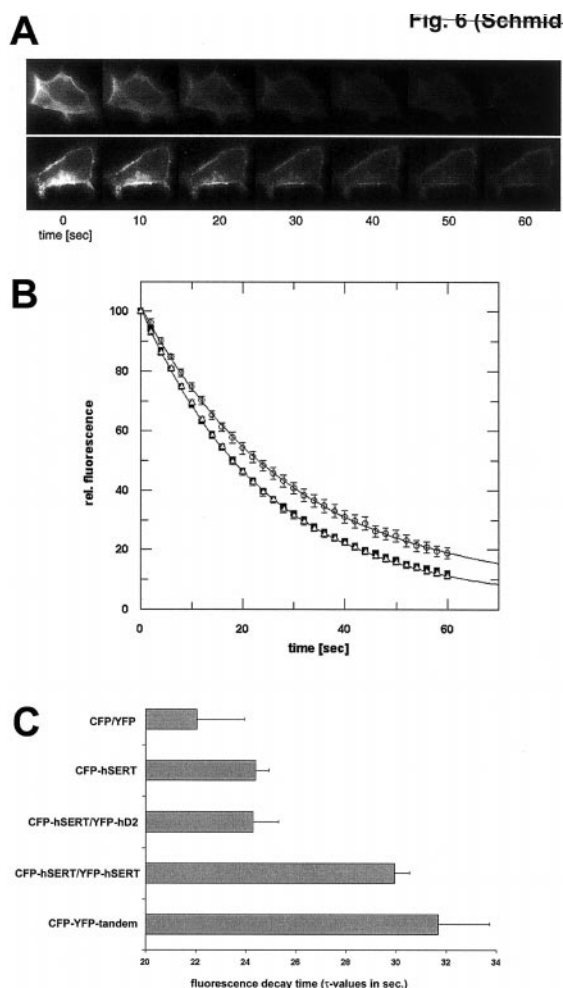


FIG. 6. Homo-oligomerization of hSERT as assessed by donor photobleaching FRET microscopy. HEK-2993 cells were transfected with various plasmids (CFP-hSERT or CFP-YFP tandem) or cotransfected with different combinations of constructs (CFP/YFP, CFP-hSERT/YFP-hSERT, or CFP-hSERT/hD₂R-YFP). The next day cells were exposed for 1 min to intense light using the donor filter set to selectively induce photobleaching of the donor (at $\times 60$ magnification, oil immersion). Fluorescence intensity was measured in the defined regions of the cellular membrane and fitted according to the equation given under “Experimental Procedures” to obtain a donor fluorescence decay curve and the time constant τ . **A**, representative images illustrating the decrease of the donor fluorescence of CFP-hSERT in the absence (*top*) and presence (*bottom*) of YFP-hSERT. **B**, fluorescence intensity was measured in the defined regions of the cellular membrane and fitted according to the equation given under “Experimental Procedures.” Donor fluorescence decay curves for CFP-hSERT alone (\blacksquare) ($n = 7$), CFP-hSERT cotransfected with YFP-hSERT (\circ) ($n = 31$), or hD₂R-YFP (\triangle) ($n = 6$) are shown. **C**, mean fluorescence decay τ values of cells transfected with the indicated combinations of expression plasmids (obtained from several independent experiments). For n value see under “Results and Discussion.”

molecular mass range with a peak at about 600–800 kDa (Fig. 5); two shoulders were also evident (at about 550 kDa and 320 kDa, respectively), which may represent distinct multimers (*e.g.* dimers and tetramers). A detailed spectroscopic analysis of the fluorescent peak using wavelength scans was not feasible because of the high dilution of tagged hSERT in the peak, which required detector settings that are not appropriate to determine FRET unequivocally. However, it seems unlikely that these high molecular mass complexes comprising CFP- or YFP-tagged hSERT are monomeric forms of the transporter molecule surrounded by detergent micelles because the size of Nonidet P-40 micelles is expected to be only 60–90 kDa based on the average number of detergent molecules in micelles

(100–150) and the molecular mass of 603 for the Nonidet P-40 monomer. Nevertheless, we cannot rule out that hSERT may associate with additional membrane proteins, and these may contribute to the formation of large molecular weight complexes. In fact, it is very likely that hSERT forms a complex with synaptic proteins because it is specifically targeted to synaptic specializations in neurons (and in differentiated PC12 cells).² Furthermore, we cannot formally exclude the possibility that nonfunctional hSERT aggregates are incorporated in the high molecular weight peak. However, we note that we did not detect a significant portion of hSERT monomers by employing native gel electrophoresis. In contrast, in the denatured form hSERT migrated with the relative molecular mass predicted for the monomer (data not shown). Thus, taken together, these data are consistent with the notion that hSERT is a constitutive oligomer.

It is worth pointing out that the existence of higher order complexes has also been postulated based on molecular sieve chromatography, cross-linking, and coimmunoprecipitation experiments (14, 15, 17). Our observations confirm and extend these findings by directly visualizing hSERT oligomers in the membranes of living cells.

To rule out the possibility that the FRET signal we detected between CFP- and YFP-tagged hSERT resulted from transient collision events within the membrane (and presumably nonspecific interaction), we aimed to investigate CFP-hSERT in combination with other transmembrane proteins fused to YFP. However, a different method has to be used for the detection of FRET in this case because the distinct nature of different transmembrane proteins might result in differences in expression levels or subcellular distribution. The prerequisite for the calculation of FRET ratio images is that both fluorophores exhibit the same intracellular distribution and that they are present in equal amounts. These requirements are met by employing essentially identical plasmids encoding homotypic proteins, such as CFP and YFP or CFP-hSERT and YFP-hSERT, which differ only by nine amino acids within the core region of the fluorophore but are otherwise identical. They may not be fulfilled if the proteins that are coexpressed differ substantially in amino acid composition. Thus, we employed the method of donor photobleaching FRET microscopy (2), which relies on the fact that the bleaching of a donor fluorophore is slower in the presence of a FRET acceptor because energy is transferred to the acceptor (and thus unavailable for bleaching the donor). The time constant of the fluorescence decay is independent of fluorophore concentration (as long as the acceptor is in excess) and is not affected by the differences in intracellular distribution (2).

If high levels of illumination were applied through the donor filter set, donor fluorescence emission rapidly declined (Fig. 6A), and this process was monoexponential (Fig. 6B). As expected, the time constant of fluorescence decay was significantly lower in cells coexpressing CFP and YFP than in cells expressing the CFP-YFP tandem, the time constant τ being 22.0 ± 1.9 s ($n = 10$) and 31.7 ± 2.1 s ($n = 14$), respectively ($p = 0.015$, see Fig. 6C). Similarly, the fluorescence decay of CFP-hSERT alone significantly was faster than CFP-hSERT in the presence of YFP-tagged hSERT (24.4 ± 0.5 s, $n = 7$, and 29.9 ± 0.6 s, $n = 31$, respectively; $p = 0.02$). The fact that YFP-hSERT slowed the decay in the fluorescence of CFP-hSERT was indicative of intermolecular FRET and thus represented an independent confirmation of the ratio imaging results. As a prototypical structurally unrelated transmembrane protein, we chose the human dopamine D₂ receptor fused to YFP (hD₂R-

² H. Just, M. Freissmuth, and H. H. Sitte, unpublished observation.

YFP). In cells expressing CFP-hSERT and hD₂R-YFP (in excess), a time constant τ of 24.3 ± 1.0 s ($n = 6$) was calculated, *i.e.* similar to τ of CFP-hSERT alone. This means that there is no significant interaction between hSERT and hD₂R in the cellular membrane. Hence, statistical transient collision events between structurally unrelated transmembrane proteins cannot account for the FRET signal observed between CFP- and YFP-hSERT. However, it was reported that the hD₂R is able to hetero-oligomerize with another membrane protein, the human somatostatin receptor (23), as detected by FRET microscopy as well.

An intriguing question was whether different agonistic or antagonistic drugs acting at hSERT would interfere with the oligomeric organization of the transporter as detected by intermolecular FRET. To answer this question, we applied donor-bleaching FRET microscopy of CFP- and YFP-hSERT expressing cells before and 3, 6, and 9 min after the addition of drugs (at a concentration of about $10 \times IC_{50}$). As antagonistic substances, we used citalopram, cocaine, imipramine, and paroxetine as transporter substrates 5-HT, methylenedioxymethamphetamine ("ecstasy"), and *para*-chloroamphetamine were applied. We could not detect any significant alteration of the τ values and thus no change of the intermolecular FRET, indicating that these compounds do not considerably modify the homo-oligomerization of CFP- and YFP-hSERT (data not shown).

In conclusion, we unambiguously found that the human serotonin transporter forms homo-oligomers within the cellular membrane of living cells. Moreover, a similar homo-oligomerization was found for the structurally related rat GABA transporter 1. It is therefore attractive to speculate that homo-oligomer formation is a property that is common to all members of the subfamily of the Na⁺/Cl⁻-dependent neurotransmitter transporters.

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REFERENCES

- Heldin, C. H. (1995) *Cell* **80**, 213–223
- Gadella, T. W., Jr., and Jovin, T. M. (1995) *J. Cell Biol.* **129**, 1543–1558
- Jordan, B. A., and Devi, L. A. (1999) *Nature* **399**, 697–700
- Overton, M. C., and Blumer, K. J. (2000) *Curr. Biol.* **10**, 341–344
- Rocheville, M., Lange, D. C., Kumar, U., Sasi, R., Patel, R. C., and Patel, Y. C. (2000) *J. Biol. Chem.* **275**, 7862–7869
- Pessino, A., Hebert, D. N., Woon, C. W., Harrison, S. A., Clancy, B. M., Buxton, J. M., Carruthers, A., and Czech, M. P. (1991) *J. Biol. Chem.* **266**, 20213–20217
- Haugeto, O., Ullensvang, K., Levy, L. M., Chaudhry, F. A., Honore, T., Nielsen, M., Lehre, K. P., and Danbolt, N. C. (1996) *J. Biol. Chem.* **271**, 27715–27722
- Rudnick, G. (1997) in *Neurotransmitter Transporters: Structure, Function, and Regulation* (Reith, M. E. A., ed) pp. 73–100, Humana Press, Inc., Totowa, NJ
- Bruns D., Engert F., and Lux H. D. (1993) *Neuron* **10**, 559–572
- Mager S., Min C., Henry D. J., Chavkin C., Hoffman B. J., Davidson N., and Lester H. A. (1994) *Neuron* **12**, 845–859
- Sitte, H. H., Huck, S., Reither, H., Boehm, S., Singer, E. A., and Pifl, C. (1998) *J. Neurochem.* **71**, 1289–1297
- Petersen, C. I., and DeFelice L. J. (1999) *Nat. Neurosci.* **2**, 605–610
- Schloss, P., and Williams, D. C. (1998) *J. Psychopharmacol.* **12**, 115–121
- Ramamoorthy, S., Leibach, F. H., Mahesh, V. B., Ganapathy, V. (1993) *Placenta* **14**, 449–461
- Jess, U., Betz, H., and Schloss, P. (1996) *FEBS Lett.* **394**, 44–46
- Chang, A. S., Starnes, D. M., and Chang, S. M. (1998) *Biochem. Biophys. Res. Commun.* **249**, 416–421
- Kilic, F., and Rudnick, G. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 3106–3111
- Förster, T. (1948) *Ann. d. Physik (Leipzig)* **2**, 55–75
- Pollok, B. A., and Heim, R. (1999) *Trends Cell Biol.* **9**, 57–60
- Schmid, J. A., Birbach, A., Hofer-Warbinek, R., Pengg, M., Burner, U., Furtmüller, P. G., Binder, B. R., and de Martin, R. (2000) *J. Biol. Chem.* **275**, 17035–17042
- Sitte, H. H., Scholze, P., Schloss, P., Pifl, C., and Singer, E. A. (2000) *J. Neurochem.* **74**, 1317–1324
- Periasamy, A., and Day, R. N. (1999) *Methods Cell Biol.* **58**, 293–314
- Rocheville, M., Lange, D. C., Kumar, U., Patel, S. C., Patel, R. C., and Patel, Y. C. (2000) *Science* **288**, 154–157