Antidepressant-induced internalization of the serotonin transporter in serotonergic neurons

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ABSTRACT **A deficiency of serotonergic signaling is thought to be involved in the etiology of depression. Thus, drugs blocking the reuptake of serotonin back into the neurons are widely used in treatment of this disease; however, their delayed effect in remission of patients suggests that the clinical response does not rely on simple serotonin uptake inhibition but may include further regulatory mechanisms. We have analyzed cellular serotonin transporter (SERT) expression on exposure to the selective serotonin reuptake inhibitor citalopram in serotonergic neurons expressing the native SERT allele in its natural surroundings. Biotinylation of SERT-expressing HEK293 cells, as well as confocal microscopy analysis in these cells and in serotonergic neurons, revealed that exposure to citalopram time dependently reduces the amount of cell surface-expressed SERT. Furthermore, in serotonergic neurons, longer exposure to citalopram not only caused an internalization of SERT proteins from the cell surface but also induced a redistribution of SERT from neurite extensions into the soma. This process was reversible on drug removal. Microarray analysis performed on citalopram-treated serotonergic neurons revealed that antidepressant treatment does not alter SERT mRNA expression, suggesting that SERT trafficking from and to the cell membrane is regulated on the posttranscriptional level.—Lau, T., Horschitz, S., Berger, S., Bartsch, D., Schloss, P. Antidepressant-induced internalization of the serotonin transporter in serotonergic neurons. *FASEB J.* 22, 1702–1714 (2008)**

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In the central nervous system, reuptake of serotonin (5-hydroxytryptamine, 5-HT) from the synaptic cleft is mediated by a specific serotonin transporter protein (SERT), which belongs to a family of related Na^+/Cl^--dependent transporter proteins, including the transporters for norepinephrine, dopamine, glycine, and γ-aminobutyric acid (GABA) (1). SERT plays a crucial role in serotonergic neurotransmission, as it controls the concentration of free active neurotransmitter in the synaptic cleft and represents a molecular target for clinically effective antidepressants, as well as for drugs of abuse (2–4). Among other findings, a reduced activity of serotonergic neurotransmission has been postulated in the pathogenesis of depression (5, 6), and several drugs elevating serotonergic activity are widely used for the pharmacotherapy of depression (7). Selective serotonin reuptake inhibitors (SSRIs) bind to SERT, thereby blocking 5-HT uptake and consequently enhancing synaptic 5-HT concentration. Although inhibition of uptake is achieved rapidly and efficiently, improvement of mood occurs only after more than 1 wk on antidepressant therapy (8–10). This delay suggests that it is not simple serotonin uptake inhibition *per se*, but rather additional regulatory mechanisms that may underlie the therapeutic effect. These mechanisms are thought to include 5-HT-mediated elevated expression of neurotrophic factors such as brain-derived neurotrophic factor (BDNF), which, in turn, enhance the sprouting of serotonergic axons (8, 11–13). Another mechanism is thought to rely on the regulation of the efficiency of the clearance of 5-HT from the extrasynaptic space by modulation of SERT protein expression on the cell surface. In this regard, it has been reported that long-term treatment with SSRIs leads to a significant reduction of SERT density in hippocampal and dorsal raphé slices in rats (14, 15). Comparably, the efficiency of norepinephrine uptake into PC12 cells and of GABA uptake in primary dissociated rat hippocampal cultures is down-regulated on exposure to the respective transporter antagonists (16, 17). Further experiments, using non-neuronal cell lines stably expressing the transporters for norepinephrine and serotonin, revealed that antagonist-induced down-regulation of transmitter uptake results from a reduction of the maximal transport rate, with no change in the apparent substrate affinity (18, 19). As shown with radioligand binding experiments or cell surface labeling, this seems to result from a reduction of transporter molecules on the cell surface. This goes along with *in vivo* findings that antidepressants reduce serotonin

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clearance from extracellular space to a greater extent by down-regulation of the serotonin transporter than by acute blockade of the transporter (14, 20).

Here, we have investigated the cell surface expression of SERT molecules on exposure to the antidepressant citalopram *in vitro*. We have used two different human embryonic kidney 293 (HEK293) cell lines, one stably expressing the recombinant human SERT (hSERT), the other one expressing hSERT tagged with a green fluorescent protein (GFP). In addition, we have analyzed murine stem cell-derived serotonergic neurons expressing the endogenous SERT (21). Cell surface biotinylation was performed to estimate SERT membrane expression in the absence or presence of citalopram treatment in hSERT-expressing HEK293 cells. In all three of the cell lines, we also visualized SERT cellular localization by confocal microscopy in the absence and presence of citalopram with an antibody, which recognizes an extracellular epitope of the protein. Thus, in the absence of detergents, SERT molecules located on the cell surface are detected, whereas in the presence of a detergent, both intracellular and cell surface-expressed transporter molecules are detected. Following SERT-GFP trafficking in HEK293 cells in the absence of detergents makes it possible to directly compare cell surface-localized SERT by antibody staining with cellular SERT distribution displayed by GFP fluorescence. Microarray analysis performed on citalopram-treated 5HT neurons and control neurons should reveal whether antidepressant treatment does alter SERT mRNA expression.

MATERIALS AND METHODS

Materials

Sulfo-*N*-hydroxysuccinimide (NHS) -biotin was from Perbio (Aalst, Belgium). The enhanced chemiluminescence (ECL) detection system was purchased from NEN (Wilmington, DE, USA). [^{3}H]-labeled citalopram (82 Ci/mmol) and [^{3}H]-labeled 5-HT (10.2 Ci/mmol) were purchased from Amersham (Freiberg, Germany) and NEN, respectively.

Cell surface biotinylation

Surface expression of SERT was monitored by biotinylation, as previously described (22), with some modifications. Cells were treated with sulfo-NHS-biotin for 1 h at room temperature. The unbound reagent was washed away with 100 mM glycine in PBS/Ca-Mg (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 9.6 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 7.3), and the signal was quenched by incubation in 100 mM glycine in PBS/Ca-Mg for 30 min at room temperature. Cells were washed with PBS/Ca-Mg and resuspended in lysis buffer [10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 0.1% sodium-dodecyl-sulfate (SDS); 1% Triton X-100; 1% sodium deoxycholate] supplemented with protease inhibitors (1 μg/ml leupeptin, 1 μM pepstatin, 1 mg/ml soybean trypsin inhibitors, 1 mM iodoacetamide, and 250 mM PMSF) for 1 h at 4°C with shaking. Lysates were centrifuged at 15,800 *g* for 30 min at 4°C, and the supernatant was loaded onto a 0.5 ml avidin column (prewashed with PBS/Ca-Mg). The column was washed with 250 μl PBS/Ca-Mg, sealed, and incubated for 1 h at room temperature. Afterward, it was washed successively with 4 ml PBS/Ca-Mg, 100 μl Laemmli buffer, 500 μl 100 mM glycine in PBS/Ca-Mg, and 100 mM glycine in PBS/Ca-Mg, pH 2.8. Blue fractions were collected and separated on 10% SDS-PAGE according to the method of Laemmli (23). Proteins were transferred for 2 h at 160 mA onto nitrocellulose, blocked with 5% milk powder in transfer Western blot buffer (TWB) (10 mM Tris-HCl, pH 8.0; 120 mM NaCl; 0.5% Tween-20), and immunoblotted with anti-SERT (1:4000) overnight at 4°C [anti-SERT C-20 antibody and horseradish peroxidase (HRP) -conjugated anti-goat secondary antibody were from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Detection was performed after incubation with an HRP-conjugated secondary antibody (1:10,000) using the ECL detection system, and quantification was performed using the image analysis system AIS/C (Imaging Research, St. Catherines, ON, Canada).

Cell culture

Generation of HEK293 cells stably expressing a hSERT-GFP fusion protein

HEK293 cells were transfected with the cDNA of hSERT-GFP (kindly provided by Dr. Harald Sitte, Institute of Pharmacology, Medical University of Vienna, Vienna, Austria) by calcium phosphate precipitation, as described by Schloss and Betz (24). After transfection, the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 95% humidified air with 5% CO_2. Selection of clonal lines permanently expressing SERT-GFP was performed as described earlier (25). Stably transfected cells (HEKhSERT-GFP cells) were selected with G418 (1 mg/ml) and maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and G418 (500 μg/ml).

Maintenance and differentiation of 1C11 cells

1C11 cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Serotonergic differentiation of 1C11 cells was induced by the addition of dibutyryl cAMP to 1 mM and cyclohexane carboxylic acid to 0.05% final concentrations (21).

Immunofluorescence and confocal analysis

Subconfluent cells were grown on coverslips and fixed with 4% paraformaldehyde for 15 min as described previously (26). Antibodies targeted against an extracellular loop of SERT were obtained from Advanced Targeting Systems (San Diego, CA, USA); the secondary antibodies were from Molecular Probes (Leiden, The Netherlands). Antibodies were incubated in PBS containing 0.2% fish skin gelatin in the presence or absence of 0.01% saponin. Coverslips were mounted in fluorescent mounting medium (DakoCytomation, Copenhagen, Denmark). Confocal images were acquired on a Leica TCS SP2 imaging system mounted on a DM IRE2 microscope using an ×63 oil planachromat lens (NA 1.3), an argon laser (458–514 nm), and a green neon laser (543 nm) (Leica Microsystems, Wetzlar, Germany). Images

were acquired with the following settings: the photo multiplier for GFP detection was set to an offset of 3.1, gain of 1.6, and beam expander of 6 V; the photomultiplier for Alexa545 detection was set to an offset of 0.2, gain of 1.6, and beam expander of 6 V. Both channels were acquired with a pinhole setting of 0.47 and run at low laser powers (22.88%). These imaging conditions allowed acquisition of very few or no overexposed or saturated images. To avoid crosstalk between two channels, double-labeled images were acquired subsequently, using an excitation beamsplitter (DD488/543; Leica).

Microarray analysis

For this experiment, four 10-cm petri dishes of 1C11 cells were differentiated to serotonergic cells, as described above. At day 7, two 10-cm petri dishes were treated with citalopram (500 nM) for 24 h. At day 8, all four plates were harvested, and RNA was extracted from the cells using the RNeasy lipid tissue minikit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. The quality of the isolated total RNA was monitored on an Agilent Bioanalyser 2100 using RNA 6000 NanoChips (Agilent, Santa Clara, CA, USA). The RIN values for the 4 RNA samples were all above 9.8. For the synthesis of biotinylated cRNA, 5 μg of total RNA of each sample was reverse transcribed using the one-cycle cDNA synthesis kit (Affymetrix, Santa Clara, CA, USA). cDNA was purified *via* the sample cleanup module (Affymetrix) and biotinylated *via in vitro* transcription using the IVT labeling kit (Affymetrix). Labeled cRNA was then purified *via* the sample cleanup module (Affymetrix). All steps with the Affymetrix kits were done according to the manufacturer's protocol. The yield of cRNA was between 70 μg and 140 μg. Each cRNA sample (n=4) was then fragmented and hybridized onto two GeneChip mouse expression arrays (430A and 430B; Affymetrix). The analysis of the gene expression data was done using GeneSpring 7.2 software (Silicon Genetics, Redwood City, CA, USA). Affymetrix-generated CEL files of the respective expression arrays were preprocessed by the GC-RMA method. Differentially regulated genes were accessed by the following steps. First, global normalization of expression data to the 50th percentile and normalization of expression levels per gene (where mean expression levels of nontreated group served as control sample) were performed by built-in algorithms. Next, all genes with a fold change greater than 1.6 between the two groups were extracted from data set.

RESULTS

Cell surface biotinylation of recombinant SERT in HEK293 cells

To study the regulation of cell surface expression of SERT in more detail, we performed surface biotinylation of HEK293 cells stably expressing the human SERT protein (HEKhSERT cells; ref. 25). To measure the effect of citalopram on SERT expression, we incubated HEKhSERT cells for 4 h with 500 nM citalopram or medium (control). After an intense washout of the antidepressant, the cells were treated as described in Materials and Methods. Whereas in control cells, nearly all SERT immunoreactivity was detected in the eluate fraction, that is, cell surface expressed, treatment of HEKhSERT cells with 500 nM citalopram resulted in a significant reduction of SERT molecules on the cell surface, with a concomitant increase in the cell interior, as shown in the washout fraction (**Fig. 1**). Densitometric quantification of the biotinylation signals revealed a reduction to 56.9 ± 6.3%, as compared to control cells (n=4; P<0.05; nonparametric Mann-Whitney U test).

Confocal microscopy of SERT in HEKhSERT cells

To address the question of whether the citalopram-induced reduction of SERT membrane expression is due to intracellular trafficking, we next followed SERT cellular distribution in HEKhSERT cells in the absence and presence of citalopram, using confocal microscopy. Here, we used an antibody that recognizes an extracellular epitope. Thus, in the absence of detergents, the antibody only labeled SERT molecules that are located on the cell surface. In the presence of a detergent, detected immunoreactivity reflected both cell surface-expressed and intracellular transporter molecules. As shown in **Fig. 2**, without exposure of HEKhSERT cells

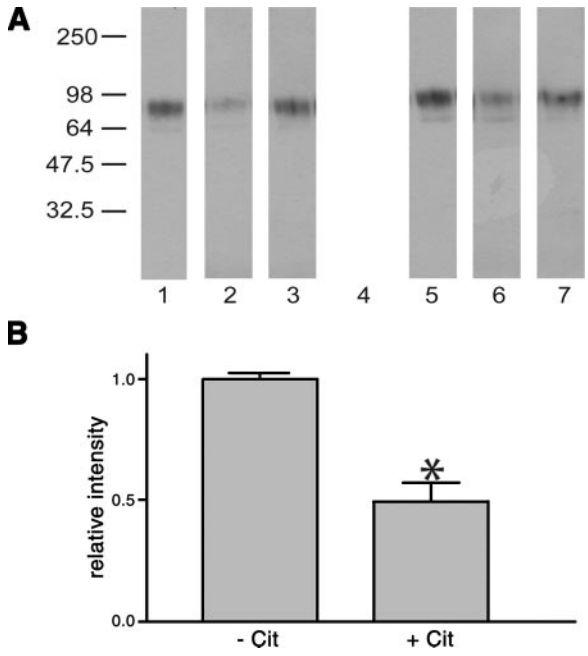

Figure 1. Effect of citalopram treatment on cell surface SERT density. HEKhSERT cells were treated for 4 h with 500 nM citalopram or vehicle (control). After the treatment, biotinylation was performed as described in Materials and Methods. After solubilization and purification over an avidin column, biotinylated proteins were subjected to SDS-PAGE, and immunoblotting was performed using an anti-SERT antibody. *A*) A representative SERT immunoblot (lane 1: molecular weight marker; lanes 2–4: untreated HEKSERT; lane 2: lysate; lane 3: washout from avidin column; lane 4: eluate from avidin column; lanes 5–7: citalopram-treated HEKhSERT; lane 5: lysate; lane 6: washout; and lane 7: eluate). *B*) Quantitative analysis of SERT immunosignals of 4 independent experiments. *P < 0.05; nonparametric Mann-Whitney U test.

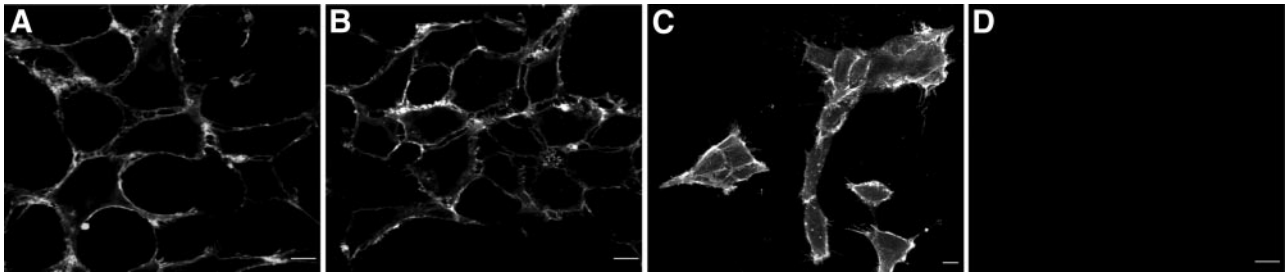

Figure 2. Effect of citalopram on the cellular localization of SERT in HEKhSERT cells. HEKhSERT cells were incubated with 500 nM citalopram or vehicle for 1 h. In control cells, immunostaining with a SERT antibody generated against an extracellular epitope of the transporter was observed in the presence (*A*) and absence (*B*) of the detergent saponin. On exposure to citalopram, SERT immunoreactivity was seen in the presence (*C*) but not the absence (*D*) of saponin. Scale bars = 20 μm.

to citalopram, SERT was detected by the antibody both in the absence and presence of the detergent saponin. After treatment of HEKhSERT cells with 500 nM citalopram for 1 h, SERT molecules were detected in the presence but not in the absence of saponin. This suggests that exposure to citalopram induces internalization of SERT molecules to intracellular compartments, where they are not degraded but still accessible to the antibody in the presence of saponin. It should be noted that the confocal image acquisition shown here was performed under optimized conditions, as described in Materials and Methods. Nonoptimized conditions with higher power settings still revealed noninternalized SERT proteins on the cell surface in Fig. 2*D*. However, at such settings SERT staining signals in Fig. 2*A–C* are over the detection ceiling.

This finding supports our previous observation that [^{3}H]5HT uptake in HEKhSERT cells is reduced after exposure to citalopram (18). In this study, [^{3}H]5HT transport rate had been shown to recover after removal of the drug. Consequently, we next investigated by confocal microscopy whether SERT is translocated back to the cell surface after removing citalopram. We performed time kinetics, in which we incubated the HEKhSERT cells for different times with citalopram and then analyzed cell surface staining in the absence of saponin. As shown in **Fig. 3**, SERT surface staining started to vanish after 10 min and disappeared after 2 h of citalopram exposure. In one set of experiments, after 2 h of citalopram exposure, one aliquot of cells was kept for another 2 h in citalopram-containing medium, while another aliquot was kept in citalopram-free medium. As seen in Fig. 3, after 2 h in the absence of citalopram, SERT immunoreactivity reappeared at the cell surface. The same result was obtained in the presence of the protein synthesis inhibitor cycloheximide (30 μg/ml), which indicates that the reappearance of transporter molecules at the cell surface was not due to newly synthesized SERT but rather resulted from trafficking from an intracellular pool still visible in the presence of saponin (see Fig. 2*C*), back to the cell membrane. In control experiments, treatment with 500 nM citalopram did not affect the cell surface expression of the human dopamine transporter (hDAT) stably expressed in HEK293 cells (data not shown).

Confocal microscopy of SERT-GFP in HEKhSERT-GFP cells

For further investigation, we isolated a clonal cell line stably expressing a GFP-tagged SERT. HEK293 cells were transfected with an hSERT-GFP cDNA, in which the GFP-coding region was fused in frame to the C-terminus of the hSERT, resulting in HEKhSERT-GFP cells. This cell line allowed us to visualize cell surface-expressed SERT (by antibody staining in the absence of saponin) simultaneously with intracellular localized transporter proteins (GFP fluorescence). As shown in **Fig. 4**, in the absence of citalopram, GFP fluorescence of hSERT-GFP (first column) was almost exclusively found in the plasma membrane paralleled by SERT antibody staining (second column); a merged image of the channels is also shown (third column). On exposure to citalopram, GFP fluorescence time dependently translocated more to intracellular compartments, and antibody staining disappeared from the cell surface. After 120 min, no antibody staining was seen in cells exposed to citalopram. After exchanging the citalopram-containing medium with citalopram-free medium (containing cycloheximide at 30 μg/ml), hSERT-GFP was translocated back to the cell membrane and thus again accessible to antibody staining without detergent.

Regulation of cell surface expression of SERT in serotonergic neurons

Most studies describing the regulation and subcellular trafficking of neurotransmitter transporter have been performed on heterologous expression systems expressing recombinant proteins. To elucidate whether citalopram affects the cellular localization of SERT in its natural surroundings in a way similar to that shown above for SERT in HEK293 cells, we studied the effect of exposure to citalopram on SERT expression in stem cell-derived serotonergic neurons. To this intent, we used murine F9-derived 1C11 cells (21). This cell line expresses the neuroprogenitor-specific marker nestin,

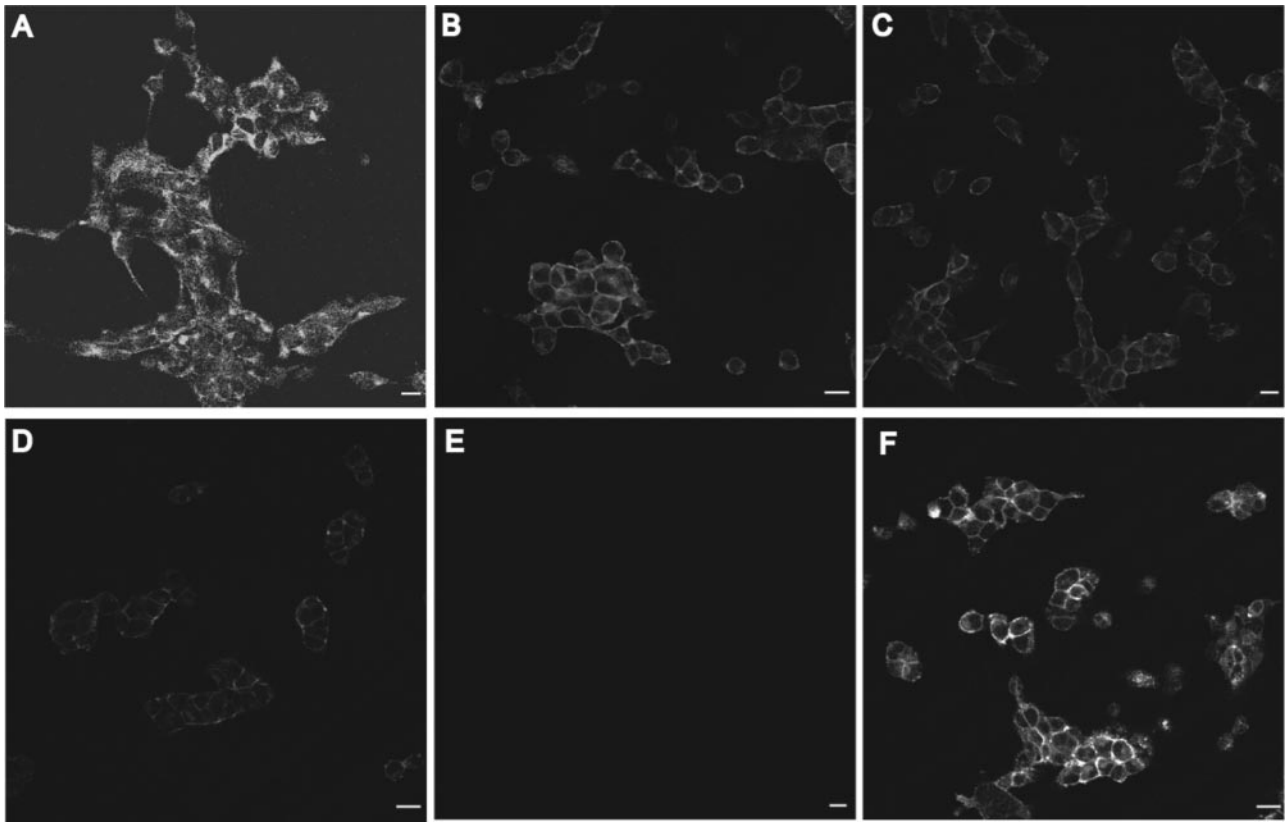

Figure 3. Reversibility of citalopram-induced SERT internalization into HEKhSERT cells. HEKhSERT cells were incubated for different time periods with 500 nM citalopram or vehicle: *A*) control; *B*) 15 min; *C*) 30 min; *D*) 1 h; *E*) 2 h. Thereafter, the medium was removed, the cells were gently washed twice with medium at 37°C, fixed, and immunostained as described in Materials and Methods. *F*) The cells were treated with citalopram for 2 h, the SSRI-containing medium was removed, and the cells were kept in fresh medium in the absence of citalopram but in the presence of the protein synthesis inhibitor cycloheximide for another 2 h. Scale bars = 50 μm.

and on appropriate differentiation, 1C11 cells have been shown to acquire a serotonergic phenotype. They develop neurite extensions and express proteins typical for serotonergic neurons, such as different 5HT receptors, SERT, and tryptophane hydroxylase (21). When labeling differentiated 1C11 ($1C11^{5HT}$) cells with the anti SERT antibody, we observed intense staining on cell bodies and along neurite extensions. Such staining was also seen with immunocytochemical detection of SERT in serotonergic fibers in brain and cell bodies in the raphé nuclei (27).

As shown in **Fig. 5**, in $1C11^{5HT}$ cells, SERT was detected by the antibody both in the absence and presence of saponin. Treatment of $1C11^{5HT}$ cells with 500 nM citalopram for 6 h induced internalization of SERT molecules to intracellular compartments, as indicated by the absence of SERT immunoreactivity in the absence of saponin. As can be seen in the presence of the detergent, citalopram not only caused an internalization of SERT molecules from the cell surface but also induced a retraction of SERT from the neurite extensions into the somata of the neurons. Notably, incubation of serotonergic neurons with 500 nM nisoxetine, a selective antagonist of the norepinephrine transporter, showed no effect on SERT surface expression; neither did incubation with serotonin itself up to 10 μM (data not shown).

To ensure that citalopram treatment does not exert any unspecific effects on neurite extensions, we examined SERT staining in the presence of saponin and simultaneously compared the cell morphology of cells exposed to citalopram to that of control cells by bright-field microscopy. As shown in **Fig. 6**, treatment with citalopram for 4 and 12 h did not affect the morphology and neurite outgrowths of differentiated $1C11^{5HT}$ neurons but clearly redistributed SERT immunoreactivity to the cell soma.

Next, we performed time kinetics, in which we incubated the $1C11^{5HT}$ cells for different times with citalopram and then monitored SERT cell surface staining in the absence or presence of saponin. As shown in **Fig. 7**, SERT expression on the cell surface was strongly diminished after 3 h of citalopram exposure and was completely undetectable after 12 h. After replacing the citalopram-containing medium by citalopram-free medium, antibody staining in the presence of saponin revealed that SERT was transported from the soma back into the neurite extensions but was only very weakly expressed on the cell surface (Fig. 7*I*, *J*). After another 3 h in citalopram-free medium, SERT reap-

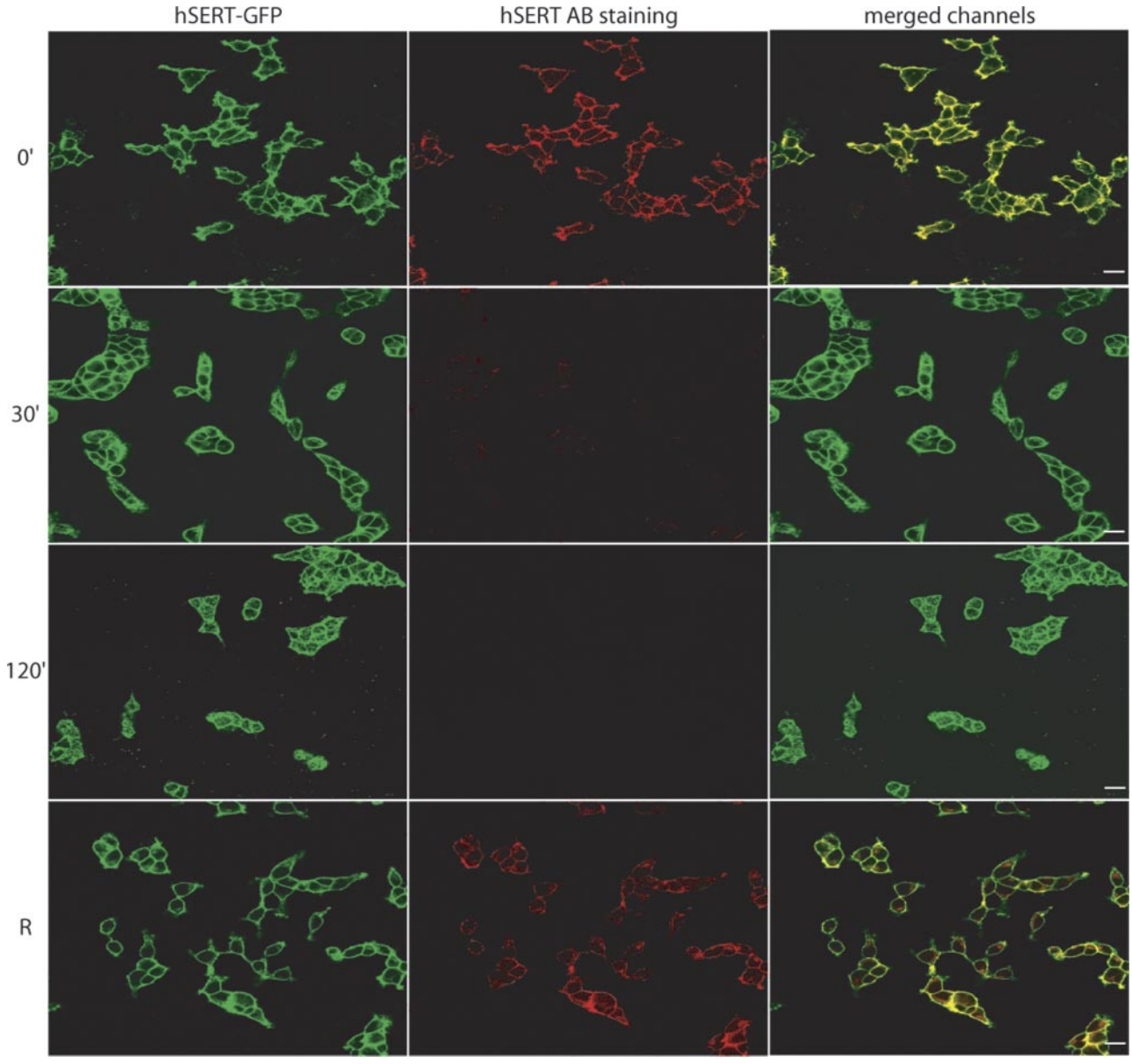

Figure 4. Time dependence of citalopram-induced internalization of hSERT-GFP. Column 1: GFP fluorescence; column 2: hSERT-GFP antibody staining in the absence of saponin; column 3: merged images of the two channels. The time points of citalopram treatment are indicated. The last row shows the reappearance of hSERT-GFP at the cell membrane 2 h after exchanging citalopram-containing medium for citalopram-free medium (containing cycloheximide 30 μg/ml). Scale bars = 50 μm.

peared on the cell surface of the neurons (Fig. 7*K*, *L*). This was also seen in the presence of the protein synthesis inhibitor cycloheximide (30 μg/ml), suggesting that the reappearance of transporter molecules at the cell surface was not due to newly synthesized SERT, but rather resulted from trafficking from an intracellular pool in the cell body.

To characterize citalopram-induced internalization of SERT in $1C11^{5HT}$ cells in more detail, we followed citalopram-induced SERT internalization together with cholera toxin subunit B (CTSB) conjugated to Alexa 555 dye (**Fig. 8**), a mouse antisyntaxin 1A antibody (**Fig. 9**), and a mouse anti-TGN38 antibody in the presence of saponin (**Fig. 10**). CTSB is a well-established tool to follow retrograde labeling in neurons (28, 29) and is also used as a marker for lipid rafts (30, 31), which segregate specific proteins and thus are important in protein trafficking. As shown in Fig. 8, the addition of CTSB (1 μg/ml) to the medium before drug application revealed a colocalization of SERT and CTSB in transport structures in the proximate neighborhood of the plasma membrane. Already after 30 min of drug treatment, neuritic SERT staining was slightly reduced, and after 1 h, only a small amount of SERT was still found on the cell extensions. SERT immunoreactivity was strongly accumulated in perinuclear regions, where

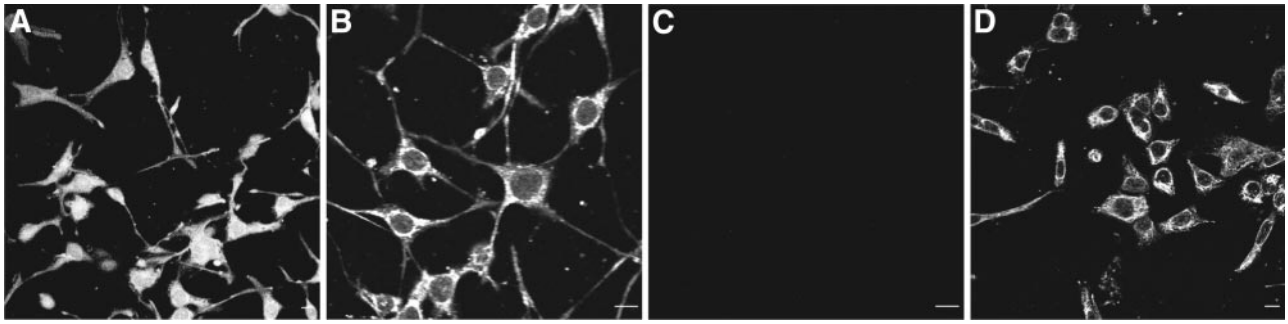

Figure 5. Effect of citalopram on the cellular localization of SERT in 1C11^{5HT}cells. 1C11^{5HT} cells were incubated with 500 nM citalopram or vehicle for 6 h. In control cells, immunostaining with an anti-SERT antibody was generated against an extracellular epitope of the transporter was observed in the absence (*A*) and presence (*B*) of the detergent saponin. On exposure to citalopram, SERT immunoreactivity was seen in the presence (*D*) but not the absence (*C*) of saponin. Note that in saponin-treated cells, exposure to citalopram restricted SERT immunoreactivity to the cell body. Scale bars = 20 μm.

it still colocalized with CTSB. In contrast, CTSB (present in the medium throughout drug exposure) was continuously incorporated in the neurites and retrogadely transported to the cell soma, thereby, always labeling the whole cell. This also confirmed the neuronal integrity during citalopram-induced retraction of SERT from neurites to the cell soma (also compare Fig. 5).

When following SERT internalization with an antibody against syntaxin 1A (a marker for recycling vesicles) for more than 12 h, no colocalization was observed (see Fig. 9), whereas costaining with an antibody against TGN38, revealed that after 12 h a large part of SERT had accumulated in the trans-Golgi-network (see Fig. 10).

Microarray analysis

Recently, it was reported that infusing short-interfering SERT-selective RNA (siRNA) into the ventricular system significantly reduced the mRNA concentration of SERT in raphé nuclei (32). Further, a specific and widespread down-regulation of SERT-binding sites was

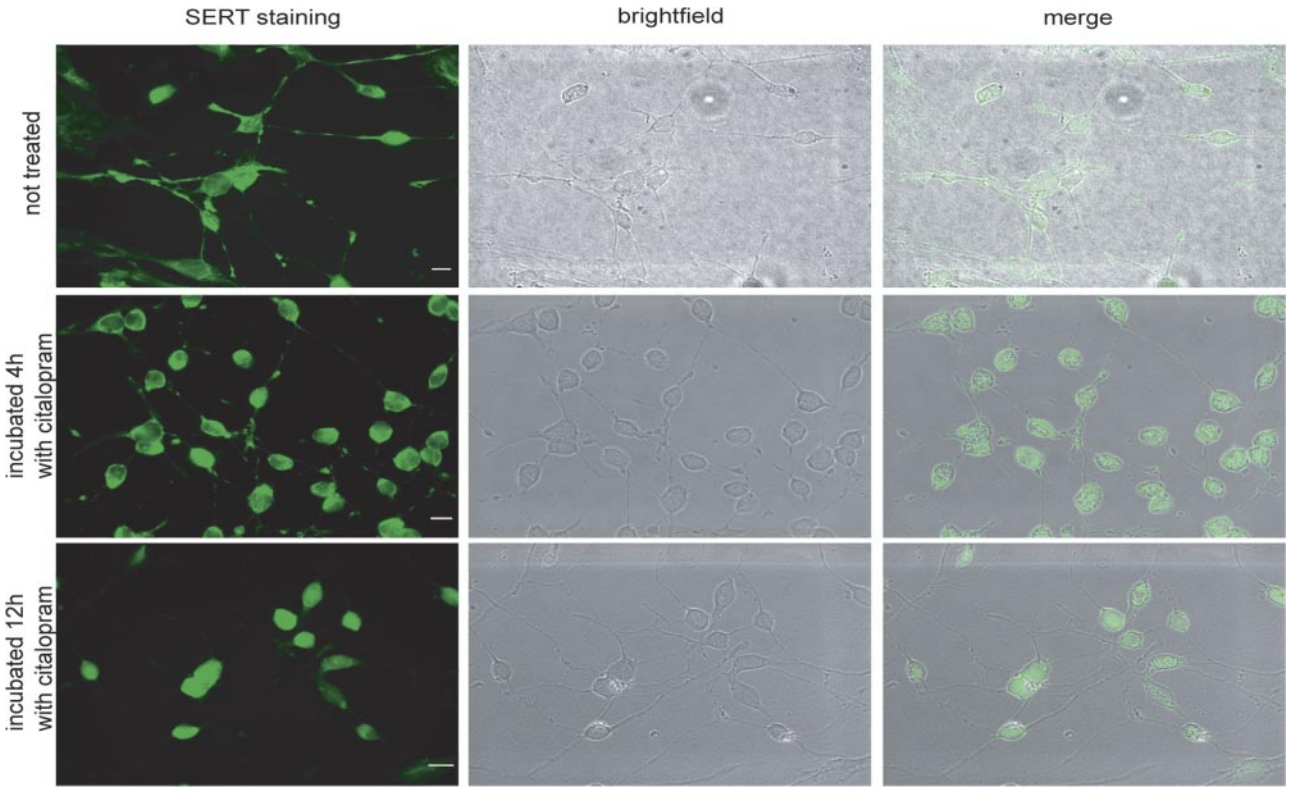

Figure 6. Effect of citalopram on the morphology and neurite outgrowth of 1C11^{5HT} cells. Cells were incubated with 500 nM citalopram as indicated and stained with the anti-SERT antibody in the presence of saponin. Column 1: immunostaining images; column 2: brightfield images; column 3: merged images. Scale bars = 20 μm.

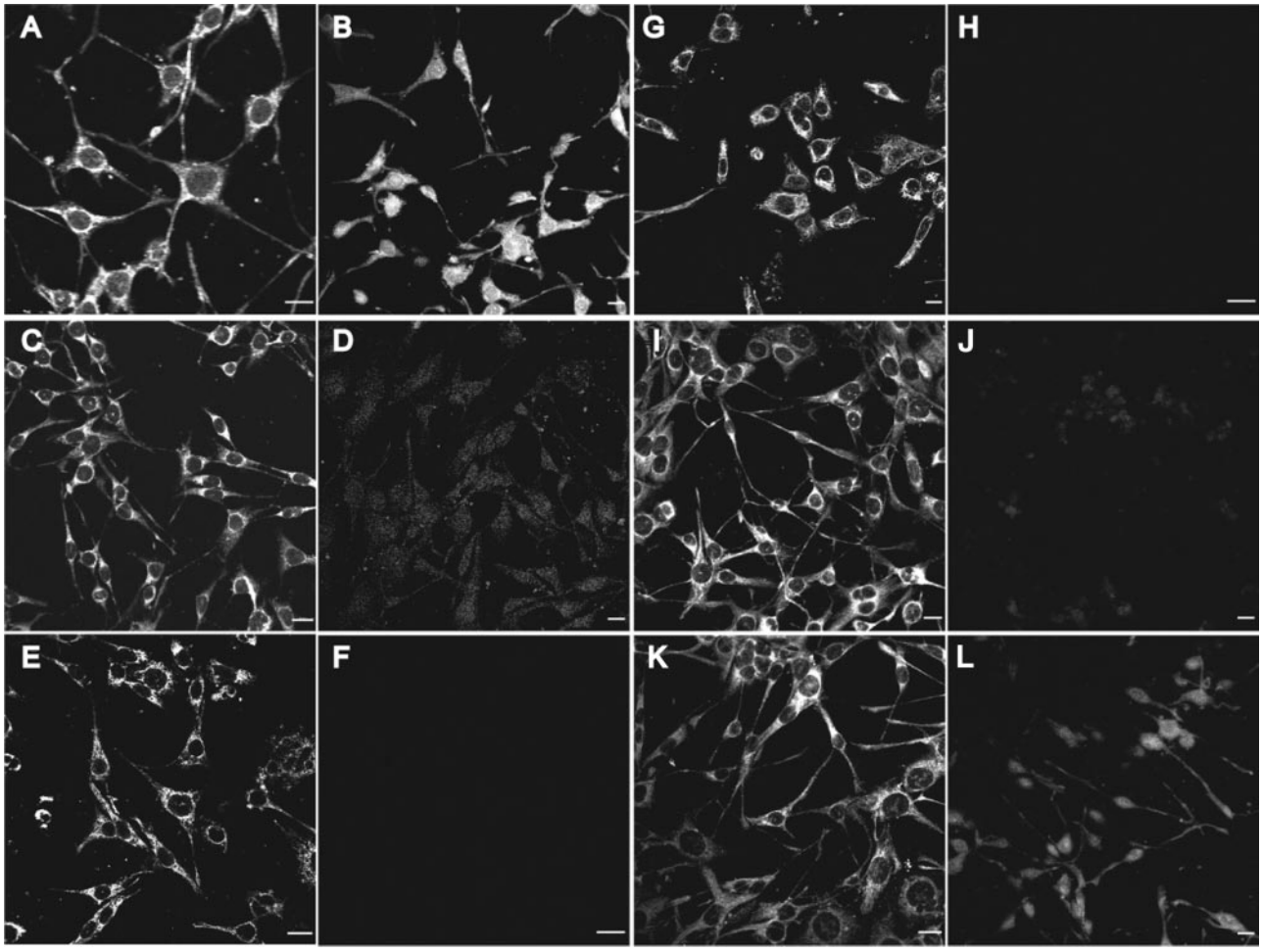

Figure 7. Time kinetics of citalopram treatment in 1C11^{5HT} cells. 1C11^{5HT} cells were incubated for different time periods with 500 nM citalopram or vehicle: *A*, *B*) control (no citalopram); *C*, *D*) 3 h; *E*, *F*) 6 h; *G*, *H*)12 h. Thereafter, the medium was removed; the cells were gently washed twice with medium at 37°C, fixed, and immunostained as described in Materials and Methods. *I–L*) Cells were treated with citalopram for 24 h, the SSRI containing medium was removed, and the cells were kept in fresh medium in the absence of citalopram but in the presence of the protein synthesis inhibitor cycloheximide for 3 h (*I*, *J*) and 6 h (*K*, *L*) before staining for SERT. Staining with the anti-SERT antibody was performed in the presence (*A*, *C*, *E*, *G*, *I*, *K*) and absence (*B*, *D*, *F*, *H*, *J*, *L*) of saponin. Scale bars = 20 μm.

demonstrated by quantitative autoradiography of brain sections using [^{125}I]RTI-55. In contrast, on infusion of citalopram for 2 wk a decrease in [^{125}I]RTI-55 ligand overlays was observed, with no change in SERT mRNA expression (32). To ensure that citalopram treatment shown here in cell culture only affects SERT trafficking but does not interfere with SERT gene expression, we analyzed mRNA expression in citalopram-treated neurons.

Treating pharmacologically defined neurons with highly selective drugs creates homogeneous mRNA samples for array-based analysis of drug-induced regulation of gene expression. Therefore, we performed microarray analysis on mRNA expression in citalopram-treated cells as compared to control cells. After differentiation into serotonergic neurons 1C11^{5HT} cells were treated either with citalopram for 24 h or cultivated in medium without citalopram. mRNA was isolated and analyzed using the Affymetrix expression platform. The analysis of the expression profiles revealed that the treatment with citalopram induced little change in global gene expression. By applying a threshold for fold change of 1.6-fold, only 31 misregulated genes could be identified (**Table 1**). The concentration of SERT-RNA is changed minimally (1.2-fold), indicating that the observed internalization of SERT is regulated on the posttranscriptional level.

DISCUSSION

The major finding of this study is that exposure to the SSRI citalopram leads to an internalization of the serotonin transporter from the cell membrane to intracellular compartments, both in a heterologous expression system and in serotonergic neurons. In all cell systems analyzed, that is, HEKhSERT cells, HEK-hSERT-GFP cells, and serotonergic neurons, treatment

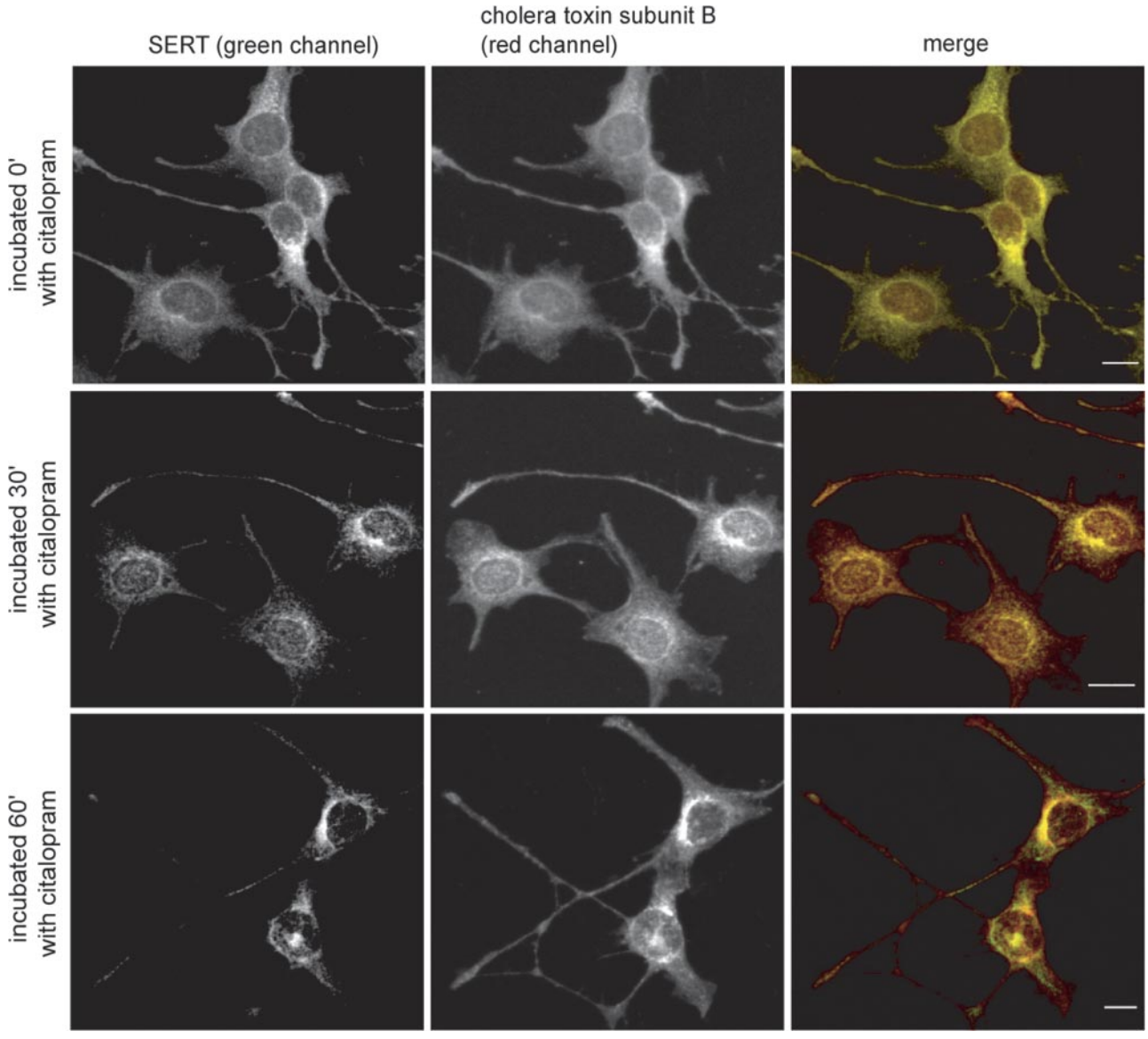

Figure 8. Early citalopram-induced retrograde trafficking of SERT. Serotonergic neurons were incubated with 500 nM citalopram for the indicated time points in the presence of cholera toxin subunit B (CTSB). Note that after 1 h of exposure, CTSB (present in the medium throughout the experiment) is continuously incorporated in the neurites, thus labeling the whole cell, whereas SERT immunoreactivity has already started to accumulate at the cell soma. For optimal presentation of fluorochrome signals, confocal images are shown in grayscale. For merged channels, green and red colors for the respective channels are applied. Scale bars = 20 μm.

with 500 nM citalopram led to a time-dependent internalization of SERT molecules to intracellular compartments. The concentration of 500 nM was chosen because at this concentration, maximal effects had been observed when analyzing the regulatory effect of citalopram treatment on recombinant SERT transport parameters in HEK293 cells with [^{3}H]5HT transport assays and [^{3}H]citalopram-binding studies (18). This concentration is well within the range of steady-state plasma levels (95–720 nM) in patients treated with citalopram at doses of 30–60 mg/day (33). In serotonergic neurons, we observed that citalopram not only causes an internalization of SERT molecules from the cell surface but also induces trafficking of SERT from the neurite extensions into the somata of the neurons. This process was reversible on removal of the drug. As seen in the presence of the protein synthesis inhibitor cycloheximide, reappearance of the transporters on the cell surface is not due to newly synthesized SERT protein but rather results from trafficking from an intracellular pool to the cell surface. This explanation is supported by the fact that microarray analyses performed on RNAs prepared from citalopram-treated cells and control cells showed no change in SERT mRNA levels. Interestingly, it recently has been shown that in humans, exposure to the SSRI fluoxetine alters the expression of SERT in blood platelets known to be devoid of a nucleus and mRNA transcription (34). Here, we want

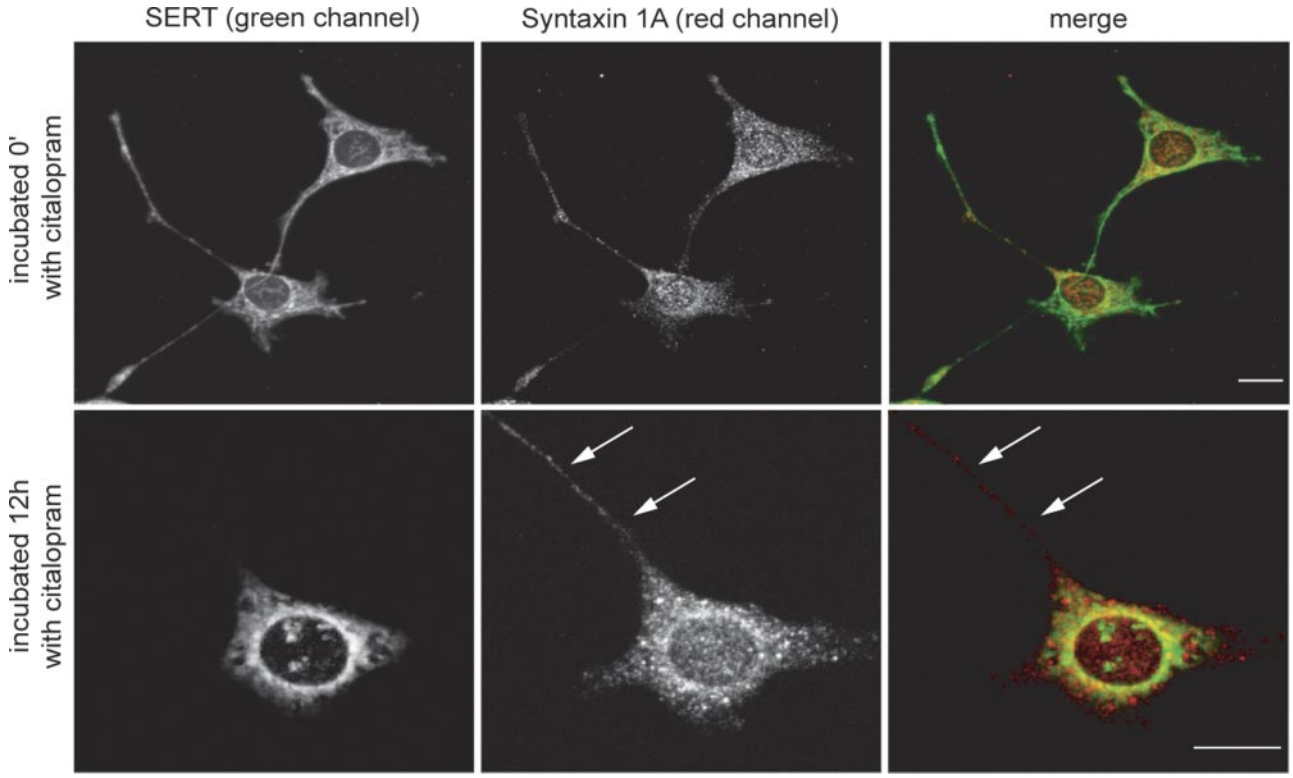

Figure 9. Internalized SERT is not distributed to recycling vesicles. After 12 h, SERT has accumulated in perinuclear structures without colocalization with an antibody against syntaxin 1A. Note the much more punctuate staining of syntaxin 1A, which is still visible in the long neurite after 12 h drug exposure (arrows). For optimal presentation of fluorochrome signals, confocal images are shown in grayscale. For merged channels, green and red colors for the respective channels are applied. Scale bars = 20 μm.

to point out possible differences in the molecular mechanisms by which SERT molecules are internalized in non-neuronal cells as compared to neurons, where SERT trafficking may also be regulated by interactions with neuron-specific proteins. Monitoring SERT trafficking in serotonergic neurons with cell compartment-specific markers revealed that, during the internalization process, SERT is not distributed to recycling vesicles but partly to the trans-Golgi network. Colocalization of SERT during internalization with CTSB suggests that lipid rafts may play an important role in the regulation of SERT trafficking from the cell surface to intracellular compartments. This idea is supported by the findings that SERT-containing complexes reside in detergent-insoluble membrane fractions (35) and that the activity of SERT is regulated by membrane cholesterol content (36).

Immunostaining on serotonergic neurons had revealed that SERT localization is not restricted to synaptic nerve endings. In the central nervous system, serotonergic neurons produce their effects "hard-wired" at synaptic boutons, as well as in a paracrine fashion *via* extrasynaptic axonal and somatodendritic release (37, 38). Because 5HT is cleared in these regions exclusively by SERT, it is not surprising that in the rat brain and spinal cord, dense SERT expression has been found not only at synaptic boutons but also on serotonergic cell bodies in the raphé nuclei, as well as concentrated in varicosities along axonal fibers projecting into different areas of the brain and into the spinal cord (27, 39). Consistently, we also observed SERT immunoreactivity all over the cell body and neurite extensions of the differentiated serotonergic neurons used in the present study.

Our *in vitro* data are consistent with the *in vivo* findings that serotonin clearance in the CA3 region of the hippocampus is reduced on SSRI treatment to a greater extent by antidepressant-induced down-regulation of SERT than by acute blockade of the transporter (14, 20). Comparably to the studies of Benmansour and colleagues (14, 20), which did not report reduced SERT gene expression as revealed by *in situ* hybridization measurements in the rat brain, we did not observe changes in SERT gene expression using microarray analyses of neuronal cell cultures. This suggests that *in vivo* and *in vitro* SERT trafficking is regulated by exposure to an SSRI on the posttranscriptional level. Assuming that this process takes longer *in vivo* (several days, ref. 20) than in the cell culture (several hours, present study), this mechanism may be comparable to the internalization of 5HT2A receptor on clozapine treatment, which has been shown to occur within a short time *in vitro*, but takes several days *in vivo* (40).

Regulation of neurotransmitter transporter cell

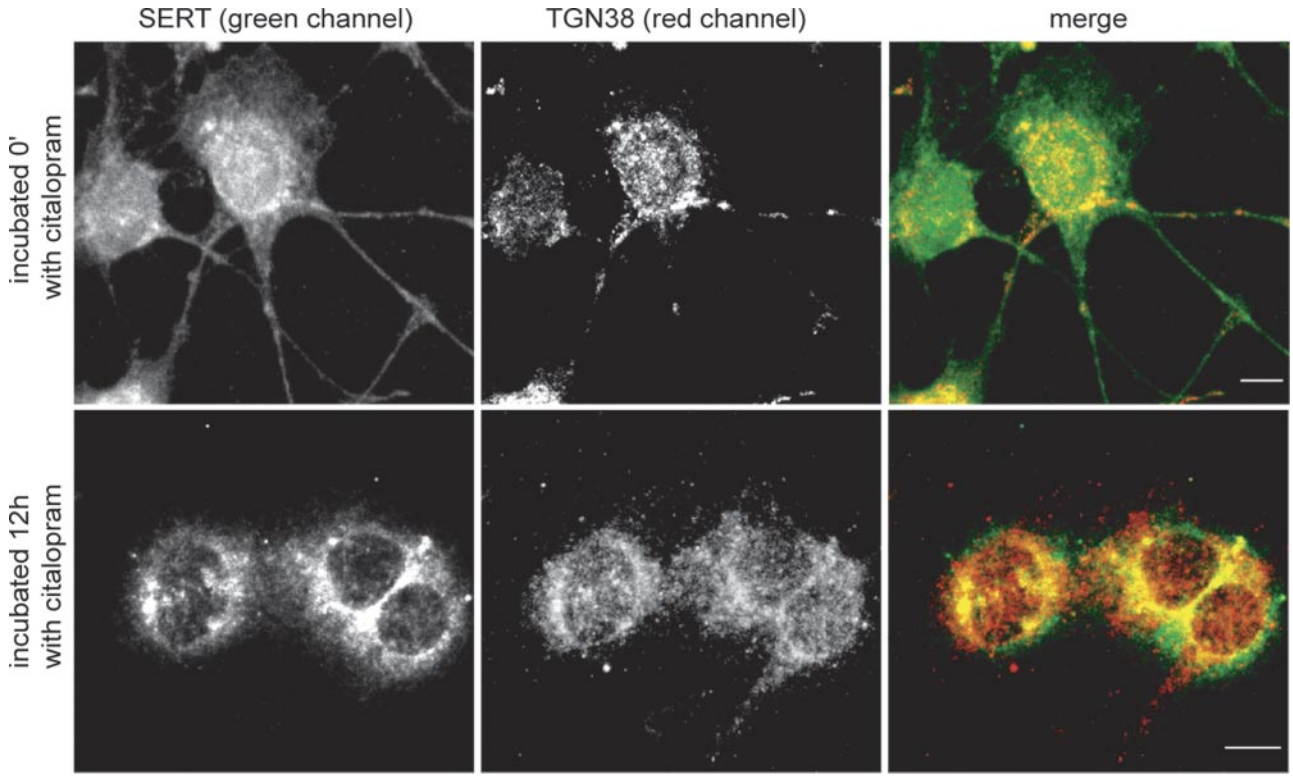

Figure 10. Internalized SERT is partly distributed to the trans-Golgi-network. After 12 h, SERT had accumulated in perinuclear structures, where it partly colocalized with an antibody against TGN38. For optimal presentation of fluorochrome signals, confocal images are shown in grayscale. For merged channels, green and red colors for the respective channels are applied. Scale bars = 20 μm.

surface expression is known to be regulated by various therapeutic drugs and drugs of abuse, as well as by multiple protein kinase interactions (41, 42). In this respect, it has been shown that activation of the mitogen-activated protein kinase (MAPK) p38 MAPK leads to enhanced SERT surface expression, whereas activation of protein kinase C down-regulates SERT surface expression (22, 43). Substrates of SERT, such as 5HT and amphetamines, protect SERT from PKC-dependent internalization, whereas SERT antagonists, such as antidepressants and cocaine, block this substrate-mediated protection (44). Interestingly, cocaine alone up-regulates the cell surface expression of SERT (45). Comparably, it has been shown that the dopamine transporter (DAT) also is down-regulated by PKC activation and up-regulated by exposure to cocaine (46–48). In case of the norepinephrine transporter (NET), longer exposure to the selective norepinephrine (NE) reuptake inhibitors (SNRIs) has been shown to down-regulate NET expression on the cell surface, thereby decreasing [^{3}H]NE reuptake capacity in a heterologous expression system, as well as in neuronal cell lines endogeneously expressing the transporter (17, 19, 49). In these studies, the antidepressant-induced down-regulation of NET could not be achieved by SSRI treatment and *vice versa*;SNRI treatment did not affect SERT expression *in vivo* (14, 20) or *in vitro* (18, this study). Thus, one may assume that in contrast to acute treatment, prolonged administration of an SSRI or SNRI leads to an overall reduction of SERT or NET molecules on monoaminergic neurons, resulting in an overall reduction of monoamine reuptake and longer-lasting elevation of extracellular 5HT or NE. This implies that drug-induced transporter internalization may be one important regulatory step in the highly complex interplay of neuronal correlates during the treatment of depression (9).

Adversely, one might speculate that during the pathogenesis of depression, the density of the target molecules for antidepressants—the serotonin and/or norepinephrine transporter—may increase on the cell surface, resulting in enhanced clearance of the monoamines out of the extracellular space. Indeed, it recently has been shown by measuring the binding potential of SERT using [^{11}C]DASB and positron emission tomography that SERT levels were increased in distinct brain areas of patients with bipolar disorder as compared to healthy subjects (50). In line with this idea, it also has been reported that *in vivo*, chronic stress increases the plasmalemmal distribution of the norepinephrine transporter in noradrenergic axons in the prefrontal cortex of rats (51). In addition, *in vitro* administration of the stress hormone dexamethasone, mimicking stress, increases SERT expression in immortalized human B-lymphoblastoid cells (52). Thus, one

TABLE 1. *Misregulated genes after treatment with citalopram*

Affymetrix ID	Gene description	Fold change
1427760_s_at	proliferin	2.286
1443586_at	FIP1 like 1 (*Saccharomyces cerevisiae*)	1.914
1453688_at	CWF19-like 2, cell cycle control (*Schizosaccharomyces pombe*)	1.89
1428839_at	WD repeat domain 53	1.871
1434067_at	uw45e08. x1 Soares_mammary gland_NMLMG	1.869
1457534_at	Ubiquitin associated protein 2-like	1.863
1422967_a_at	Transferrin receptor	1.851
1438271_at	Mortality factor 4 like	1.821
1456091_at	SEC22 vesicle trafficking protein-like 3 (*S. cerevisiae*)	1.804
1453282_at	Coxsackievirus and adenovirus receptor	1.799
1443526_at	PHD finger protein 21A	1.796
1435083_at	Cortexin	1.795
1443587_at	cDNA sequence AK129128	1.789
1437566_at	Guanine nucleotide binding protein-like 2	1.782
1458056_at	Splicing factor, arginine/serine-rich 12	1.737
1447845_s_at	Vanin 1	1.737
1428771_at	RIKEN cDNA 2410127E18 gene	1.731
1449161_at	Endothelin 2	1.681
1456303_at	Transcribed sequence	1.669
1441880_x_at	Hypothetical protein MGC30332	1.663
1448552_s_at	RIKEN cDNA 2310028N02 gene	1.66
1436590_at	Protein phosphatase 1, regulatory (inhibitor) subunit 3B	1.647
1440641_at	Vacuolar protein sorting 29 (*S. pombe*)	1.62
1438972_x_at	RIKEN cDNA 2810410L24 gene	1.619
1453457_at	Sorcin	1.618
1438222_at	Formin binding protein 3	1.612
1436317_at	RIKEN cDNA D230012E17 gene	1.608
1451913_a_at	Hypoxia up-regulated 1	1.601
1447802_x_at	AV099323 C57Bl/6 J ES cell	−1.647
1455204_at	Phosphatidylinositol transfer protein, cytoplasmic 1	−1.724
1457823_at	BB533736 RIKEN full length enriched	−1.869

long-term action of SSRIs might be to counteract enhanced SERT surface expression observed in depression. More information about the molecular mechanisms involved in SSRI-induced transporter internalization *in vitro* and *in vivo* may allow us to develop new strategies to shorten this process in the pharmacotherapy of depression leading to a faster clinical response and remission of the patients. FJ

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