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Differential Uptake Mechanisms of Fluorescent Substrates into Stem-Cell-Derived Serotonergic Neurons

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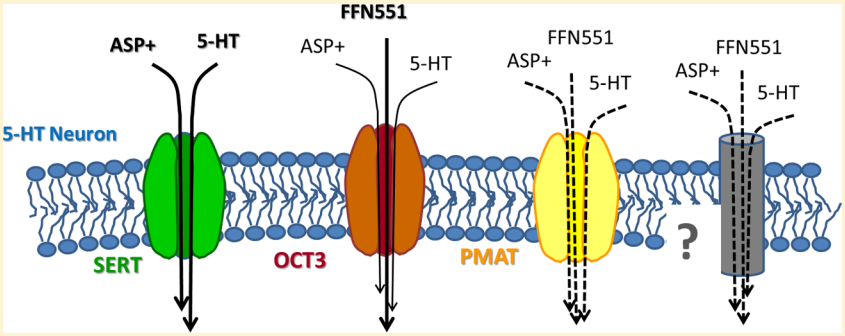

ABSTRACT: The actions of the neurotransmitters serotonin, dopamine, and norepinephrine are partly terminated by diffusion and in part by their uptake into neurons via the selective, high-affinity transporters for serotonin (SERT), dopamine (DAT), and norepinephrine (NET), respectively. There is also growing evidence that all three monoamines are taken up into neurons by low-affinity, high-capacity organic cation transporters (OCT) and the plasma membrane monoamine transporter (PMAT). Pharmacological characterization of these low-affinity recombinant transporter proteins in heterologous expression systems has revealed that they are not antagonized by classical inhibitors of SERT, DAT, or NET but that decynium-22 (D22) antagonizes OCT3 and PMAT, whereas corticosterone and progesterone selectively inhibit OCT3. Here, we show that SERT, PMAT, and OCT3, but not OCT1 and OCT2, are coexpressed in murine stem cell-derived serotonergic neurons. Using selective antagonists, we provide evidence that uptake of the fluorescent substrates FFN511, ASP+, and 5-HT into stem cell-derived serotonergic neurons is mediated differentially by these transporters and also involves an as yet unknown transport mechanism.

KEYWORDS: *serotonergic neurons in vitro, live cell imaging, fluorescent substrate transport*

Serotonergic neurotransmission plays an important role in neural development and memory formation, and serotonergic dysfunction is thought to play a role in various psychiatric disorders, such as depression and schizophrenia.[1] The existence of extrasynaptic receptors and transporters for serotonin (5-hydroxytryptamine, 5-HT) and 5-HT release sites without corresponding postsynaptic structures suggests that serotonergic neurotransmission modulates neuronal plasticity primarily via volume transmission in an autocrine and paracrine fashion rather than at synaptic terminals.[2−5] Consequently, vesicles that can accumulate and release 5-HT have been shown to exist in both axons and somatodendritic areas.[6−8]

Uptake of 5-HT from the extracellular space into neurons and blood platelets is mediated by a specific high-affinity serotonin transporter protein (SERT).[9−13] In the CNS, SERT plays a crucial role in serotonergic neurotransmission via regulation of extracellular neurotransmitter concentrations,[14,15] and thus is the molecular target for clinically effective drugs such as selective serotonin reuptake inhibitors (SSRIs).[16−19] In recent years, it has been shown that the monoaminergic neurotransmitters 5-HT, dopamine, and norepinephrine are also transported by the plasma membrane monoamine transporter (PMAT) and the organic cation transporters 1, 2, and 3 (OCT1, OCT2, OCT3).[20−22] Upon heterologous expression of the respective recombinant proteins, these transporters have been characterized as low-affinity, high-capacity monoamine transporters that are also implicated in antidepressant therapies.[23,24] In fact, OCT3 expression is upregulated in the brains of SERT-knockout mice,[25] and OCT3 blockade enhances the antidepressant effects of SSRIs in mice.[26] Moreover, treatment with antisense oligonucleotides against OCT3 has been shown to induce antidepressant-like effects in mice.[27]

Due to their low cell number and vastly branching axons, serotonergic neurons are difficult to cultivate in primary culture. Hence, studies on the mechanisms of drug action in vitro have been performed primarily on serotonergic neurons derived from the neuronal progenitor cell line (1C11$^{5\text{-HT}}$ neurons)[28] or serotonergic neurons obtained from mouse embryonic stem (mES) cells.[29,30] In a recent study, we quantified the uptake and release of fluorescent substrates in 1C11$^{5\text{-HT}}$ neurons and serotonergic neurons differentiated from mES cells.[31] The

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substrates in this study included (i) 4-(4-(dimethylamino)-styryl)-*N*-methylpyridinium (ASP+),[32] a fluorescent analog of the neurotoxin MPP+; (ii) the fluorescent monoamine analogue "fluorescent false neurotransmitter" (FFN511), which has been used to visualize dopamine release from individual presynaptic terminals in the striatum;[33] and (iii) 5-HT itself, which when excited at wavelengths from 320 to 460 nm can be detected at wavelengths from 390 to 540 nm.[34] We found previously that ASP+, as well as 5-HT were taken up, although not completely, by SERT, whereas FFN511 uptake was not mediated by SERT and thus occurred through an unknown mechanism.[31]

In the present study, we studied the uptake mechanisms of these fluorescent substrates in greater detail. To this end, we investigated the presence of other transporters coexpressed in 1C11-derived serotonergic neurons and to what extent they contribute to substrate uptake in these cells.

RESULTS AND DISCUSSION

To study mechanisms of fluorescent substrate uptake, we focused on $1C11^{5\text{-HT}}$ neurons, as previously reported.[31] Upon induction by dibutyryl cyclic AMP (Db_2-cAMP) and cyclohexane carboxylic acid (CCA), 1C11 cells differentiate within 4 days into a serotonergic phenotype ($1C11^{5\text{-HT}}$ neurons) that exhibits synthesis of 5-HT by tryptophan hydroxylase 2, SERT-driven uptake of serotonin, and VMAT2-driven 5-HT accumulation into synaptic vesicles.[28] To examine the potential expression of the low-affinity, high-capacity monoamine transporters OCT1, OCT2, OCT3, and PMAT, in addition to SERT, in $1C11^{5\text{-HT}}$ neurons, we performed an expression analysis of the respective transcripts. As shown in Figure 1,

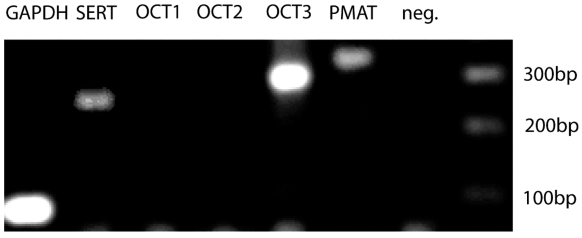

Figure 1. Representative PCR results from $1C11^{5\text{-HT}}$-derived cDNA. Lane 1 shows the internal control transcript for GAPDH at 94 bp. In lane 2, we detect a transcript for mSERT at 293 bp. The RT-PCR results for mOCT1 and mOCT2 in lanes 3 and 4 were negative. Transcripts for mOCT3 and mPMAT were detected at or above the 300 bp marker (lanes 5 and 6). The negative control (cDNA synthesis without reverse transcriptase) did not yield PCR products for all primer pairs used (not shown). A negative control for GAPDH transcript amplification is shown in lane 7.

transcripts for SERT, OCT3, and PMAT, but not OCT1 and OCT2 were detected in $1C11^{5\text{-HT}}$ neurons. These findings are consistent with in situ hybridization studies performed by Amphoux and colleagues[20] wherein OCT3 was the most readily detected OCT subtype in rat brain with substantial labeling observed in the dorsal raphe. In the Amphoux study, the pattern of OCT2 expression differed completely from OCT3 labeling and the mRNA encoding OCT1 was barely detectable.

Based on the time course and concentration dependence of uptake of the fluorescent substrates uncovered in our recent study,[31] here we allowed uptake of ASP+ at 10 μM to occur for 30 s and 5-HT uptake at 500 μM for 10 min, as these conditions had proven to be optimal for quantification by confocal microscopy; FFN511 uptake was allowed to occur at 10 μM for 5 min, to ensure optimal imaging conditions in case of the strong fluorescence-diminishing effects of putative FFN511 uptake inhibitors (Figure 2).

To discriminate between the involvement of SERT, OCT3, and PMAT, in the uptake of the three substrates, we performed transporter inhibition experiments in the presence or absence of selective uptake inhibitors. Since SERT, OCT3, and PMAT are coexpressed in 1C11-derived serotonergic neurons (Figure 1), we choose inhibitor concentrations that maximally block substrate translocation mediated by each respective target transporter and quantified residual uptake via the remaining transporters. Escitalopram has a K_i value of ~1 nM[35] and has been shown to induce approximately 50% SERT internalization from the cell surface when used at 500 nM for 4 h.[36] For SERT blockade, we used the SSRI escitalopram at 1 μM for 4 h. Under these conditions, escitalopram completely inhibits SERT-mediated but not OCT3-mediated transport as its K_i value for OCT3 is ~160 μM.[22] To selectively block OCT3-mediated transport, we preincubated $1C11^{5\text{-HT}}$ neurons for 10 min with 10 μM corticosterone or 10 μM progesterone, before visualizing accumulation of the fluorescent substrates. Corticosterone inhibits the recombinant OCT3 from different species with different inhibitory potencies. For instance, corticosterone inhibits human OCT3 with a K_i value of ~0.3 μM,[37] rat OCT3 with a K_i value of ~5 μM,[22] and mouse OCT3 with a K_i value of ~4 μM.[38] The human PMAT is blocked by corticosterone with a K_i value of 450 μM.[39] Moreover, we investigated the use of decynium-22 (D22), which selectively inhibits [^{3}H]MPP+ uptake by OCT3 and PMAT.[39,40] However, in accordance with reports from Inyushin and colleagues[41] and Beikmann et al.,[9] we also found that D22 at 1 μM exhibited a strong fluorescence. Therefore, D22 could not be used to block uptake of fluorescent substrates through OCT3 and PMAT in our confocal microscopy-assisted live cell imaging studies. To the best of our knowledge, there are no nonfluorescent compounds that selectively block PMAT-mediated transport; therefore for further analyses we restricted our experiments to the inhibitors described above.

As shown in Figure 3, FFN511 uptake into serotonergic neurons was not affected by escitalopram, either upon pretreatment with 1 μM escitalopram for 4 h or upon 1 h treatment with 100 nM escitalopram (under the latter conditions escitalopram blocks ~95% of SERT-mediated uptake but does not induce SERT internalization as shown in a heterologous expression system).[42] In contrast, FFN511 uptake was significantly reduced to approximately 50% of control values by both corticosterone and progesterone. These data suggest that at 10 μM, approximately 50% of FFN511 molecules enter $1C11^{5\text{-HT}}$ neurons by OCT3- but not by SERT-driven transport. Due to the lack of a suitable nonfluorescent inhibitor of PMAT, contributions to uptake of FFN511 by this protein cannot be ruled out. However, the finding that FFN551 derivatives, Mini102 and Mini202, are accumulated into vesicles in HEK293 cells which are transfected with VMAT2, but do not express SERT, OCT3, or PMAT, further suggests that FFN511 and its derivatives can enter cells through a yet unknown mechanism, in addition to uptake via OCT3.[43]

In contrast to FFN511, ASP+ uptake into serotonergic neurons was predominantly mediated by SERT, as revealed by ~70% uptake inhibition by escitalopram. Progesterone did not affect ASP+ uptake and corticosterone led to a significant

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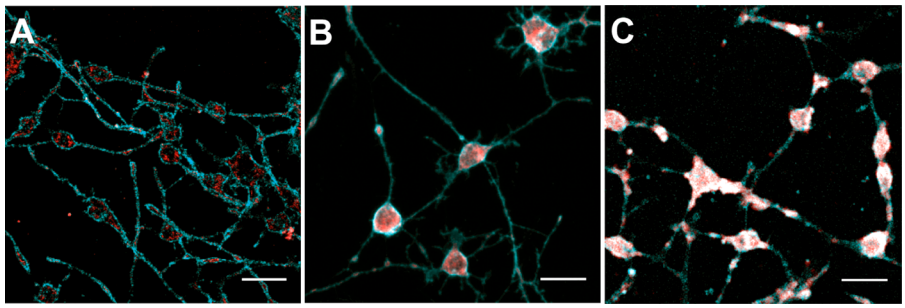

Figure 2. Representative fluorescence images of 1C11$^{5\text{-HT}}$ neurons with (A) 5 min incubation of 10 μM FFN511, (B) 30 s incubation of 10 μM ASP+, and (C) 10 min incubation of 500 μM 5-HT. Scale bars are 25 μm. In all images, cells were costained with choleratoxin subunit b, which labels the cell membrane. Choleratoxin is shown in cyan and FFN511, ASP+, and 5-HT are in red. Fluorescence, as described in Methods (image acquisition and data analysis), is shown here in the absence of inhibitors.

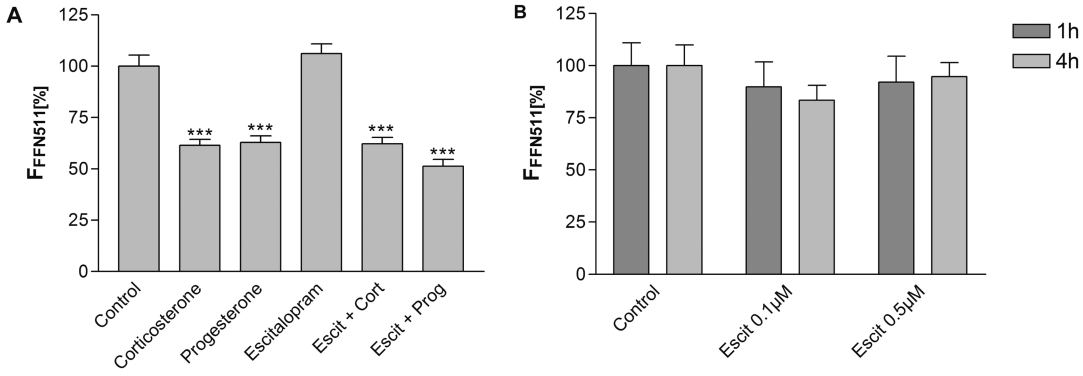

Figure 3. (A) Inhibition of FFN511 uptake into serotonergic neurons. The 1C11$^{5\text{-HT}}$ cells were treated with either 10 μM corticosterone, 10 μM progesterone, 1 μM escitalopram, or a combination of these compounds for the indicated incubation times. Subsequently, the cells were loaded with 10 μM FFN511 for 5 min in the presence of the respective inhibitors. Corticosterone and progesterone reduced FFN511 uptake to 62 ± 3% and 63 ± 3%, respectively, of control values; escitalopram did not signicicantly inhibit FFN511 uptake (110 ± 5%). The combinations of escitalopram with corticosterone or progesterone produced reductions similar to corticosterone or progesterone alone to 62 ± 3% and 51 ± 3%, respectively ($F = 35$, $p < 0.0001$, df 583). In (B), we tested whether lower concentrations and shorter incubation times of escitalopram could lead to a decreased uptake of FFN511. In all conditions, no significant change in FFN511 uptake was observed ($F = 1.1$, $p < 0.34$, df 145). Bars represent means ± SEMs of $N \geq 90$ cells taken from $N \geq 2$ independent experiments. ***$p \leq 0.001$.

elevation of ASP+ uptake as compared to the control and progesterone treatments (see Figure 4A). The ASP+ uptake was also increased by corticosterone in the presence of the protein synthesis inhibitor cycloheximide, and therefore increased ASP+ uptake is independent of de novo synthesis of SERT or other transporters. Corticosterone is a glucocorticoid receptor (GR) agonist, and it was previously shown that ligand-bound GR induces SERT trafficking, thus increasing localization of SERT to the cell surface by nongenomic means.[29] To address the question of whether the increase of ASP+ uptake is due to SERT externalization mediated by nongenomic corticosterone activation of GR, we quantified ASP+ uptake in the presence of corticosterone in combination with the GR inhibitor mifepristone (mif). As shown in Figure 4B, coapplication of mif abolished the SERT externalizing effect of corticosterone treatment.

Using heterologous expression systems, ASP+ has been shown to be a substrate for SERT (human) with a K_m value of ~160 nM,[44] and for OCT3 (mouse) with a K_m value of ~1.8 μM.[38] Together with our findings, these K_m values indicate that, in serotonergic neurons, which coexpress SERT and OCT3, ASP+ is predominantly taken up by the high-affinity SERT as compared to the low-affinity OCT3. Our data are also indicative of an enhanced ASP+ uptake via enhanced cell surface localization of SERT in the presence of corticosterone. The possible involvement of PMAT with respect to ASP+ transport awaits the development of nonfluorescent selective PMAT inhibitors and thus, remains to be elucidated.

Serotonin is taken up by SERT (rat) with a $K_m \sim 650$ nM,[45] by OCT3 (human) with a $K_m \sim 1$ mM,[21] and by PMAT (human) with a $K_m \sim 280$ μM.[21] Consequently, under our conditions at 500 μM, 5-HT uptake is largely driven by SERT, as indicated by significant transport inhibition by escitalopram (Figure 5). It should be noted here that with the 500 μM 5-HT concentration used, PMAT-driven 5-HT uptake is also possible. The unequivocal investigation of OCT3 in serotonin uptake in 1C11$^{5\text{-HT}}$ neurons will require additional studies at higher 5-HT concentrations that are closer to the Km value for OCT3 yet in the presence of both SERT and PMAT inhibitors.

In most studies to date, uptake characteristics and inhibitory profiles of the high-affinity SERT and the low-affinity, high-capacity monoamine transporters OCT3 and PMAT have been determined by quantification of [^{3}H]5-HT or [^{3}H]MPP+ influx in cellular systems expressing the respective recombinant transporter proteins separately. In contrast to these studies, we measured uptake of fluorescent substrates into serotonergic neurons that simultaneously coexpress native SERT, OCT3, and PMAT in the same cells. To this end, we differentiated serotonergic neurons from a precursor cell line 1C11 according to the report by Kellermann and colleagues.[28] Using this

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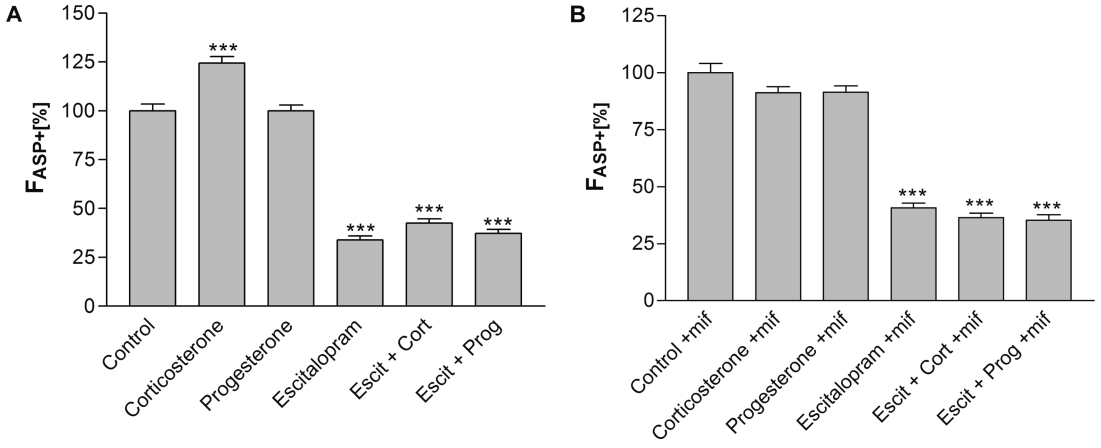

Figure 4. (A) Inhibition of ASP+ uptake into 1C11$^{5\text{-HT}}$ serotonergic neurons. (B) Inhibition of ASP+ uptake into 1C11$^{5\text{-HT}}$ serotonergic neurons in the presence of the GR antagonist mifepristone (mif; 10 μM). The 1C11$^{5\text{-HT}}$ cells were treated with either 10 μM corticosterone, 10 μM progesterone, 1 μM escitalopram, or a combination of compounds. Subsequently, the cells were loaded with 10 μM ASP+ for 30 s in the presence of the respective inhibitors. In (A), corticosterone and progesterone failed to inhibit ASP+ uptake, but escitalopram reduced uptake to 34 ± 2% compared to control. In contrast, corticosterone significantly increased the uptake of ASP+ into 1C11$^{5\text{-HT}}$ cells to 120 ± 4% compared to control, whereas progesterone had no effect to increase ASP+ uptake (100 ± 3%). The combinations of escitalopram with corticosterone or progesterone revealed decreases in uptake of 43 ± 2% and 37 ± 2%, respectively (F = 170, p < 0.0001, df 677), which were similar decreases to those observed for escitalopram alone. In (B), the increase of ASP+ uptake by corticosterone was prevented by the application of mifepristone (corticosterone + mif 91 ± 3%). Again, progesterone did not alter ASP+ uptake significantly (96 ± 3%). Escitalopram reduced ASP+ uptake to 41 ± 2%, and the combinations of escitalopram with corticosterone or progesterone revealed 36 ± 2% and 35 ± 2% inhibition of ASP+ uptake, respectively, compared to controls (F = 120, p < 0.0001, df 523). Bars represent means ± SEMs of $N \geq 90$ cells taken from $N \geq 2$ independent experiments. ***$p \leq 0.001$.

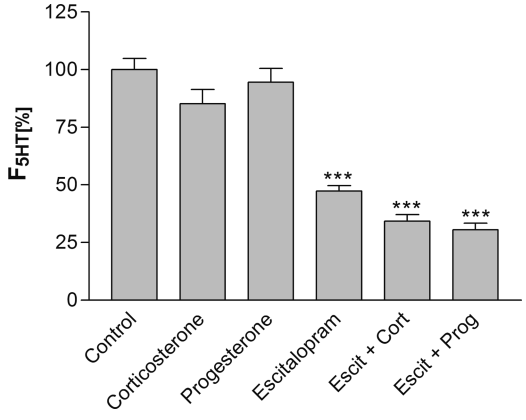

Figure 5. Inhibition of 5-HT (500 μM) uptake into 1C11$^{5\text{-HT}}$ neurons. The 1C11$^{5\text{-HT}}$ cells were treated with either 10 μM corticosterone, 10 μM progesterone, 1 μM escitalopram, or a combination of compounds. Subsequently, the cells were loaded with 500 μM 5-HT for 10 min in the presence of the respective inhibitors. Corticosterone and progesterone did not induce significant decreases in 5-HT uptake (84 ± 6% and 95 ± 6% of control, respectively). Escitalopram significantly reduced 5-HT uptake to 47 ± 2% of control. The combinations of escitalopram and corticosterone or progesterone reduced 5-HT uptake to a similar extent as escitalopram alone (i.e., 34 ± 3% or 31 ± 3%, respectively (F = 52, p < 0.0001, df 579)). Bars represent means ± SEMs of $N \geq 90$ cells taken from $N \geq 2$ independent experiments. ***$p \leq 0.001$.

protocol, 1C11 cells differentiate within 4 days into a serotonergic phenotype as revealed by the expression of the 5-HT synthesizing enzyme tryptophan hydroxylase 2, 5-HT(1B/D), 5-HT(2B), and 5-HT(2A) receptors, SERT-driven uptake of serotonin, as well as VMAT2-mediated substrate accumulation into synaptic vesicles.[46] Together with the finding that 1C11-derived neurons express OCT3 and PMAT together with SERT at native levels, this approach better reflects the in vivo situation than heterologous expression systems. One problem with transfected cells is that they overexpress the transfected protein, thus, protein levels are often much higher than the native levels. This drawback is even greater when cotransfecting two or three transporters into a non-neuronal cell type such as HEK293 cells. Moreover, regulation of the overexpressed proteins can be altered, (e.g., membrane localization) and in nonnative cell types, the regulatory machinery is likely to be different. In contrast to serotonergic neurons differentiated from embryonic stem cells[30] and in vivo, however, 1C11-derived serotonergic neurons do not exhibit a distinct neuronal polarity (T. Lau, unpublished observation) and, therefore, are not well suited for studies comparing somatodendritic versus axonal substrate uptake or release.

Interestingly, uptake of the substrates tested here was not blocked completely by application of escitalopram or corticosterone alone, nor by coapplication of these two antagonists. This suggests either participation of PMAT in substrate translocation, or an as yet unidentified transport mechanism by which substrates can enter 1C11-derived neurons. There are several findings that point to the latter possibility. For example, the pH-sensitive FFN551 derivatives, Mini102 and Mini202, have been shown to enter HEK293 cells, which are transfected with VMAT2 but do not express monoamine transporters on their cell surface. Inside HEK cells, Minis then are accumulated into vesicles by a proton-driven antiport mechanism mediated by the recombinant VMAT2.[43] Moreover, it has been shown that the fluorescent substrate IDT307 (another MPP+ derivative also referred to APP+ in the literature) partially accumulates into lymphocytes in the presence of SSRIs, as well as high concentrations of 5-HT, thus suggesting secondary, as yet unknown uptake mechanisms in addition to SERT, OCT3, or PMAT into cells.[9]

In summary, the results obtained from the 1C11-derived neuronal in vitro model show that serotonergic neurons coexpress SERT, PMAT, and OCT3, but not OCT1 and OCT2. Comparable to findings obtained from heterologous

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expression of these recombinant transporters separately, in 1C11$^{5\text{-HT}}$ neurons ASP+ and 5-HT are taken up preferentially by SERT. On the other hand, the false neurotransmitter FFN511, developed to label synaptic vesicles in dopaminergic neurons,[33] is taken up into serotonergic neurons by OCT3 but not by SERT. The development of nonfluorescent PMAT-selective antagonists will allow the involvement of this transporter protein in substrate accumulation in serotonergic neurons to be investigated in the future.

METHODS

Materials. Corticosterone (#C2505), progesterone (#P8783), 5-HT (serotonin creatinine sulfate monohydrate #H7752), and mifepristone (#M8046) were purchased from Sigma-Aldrich (Hamburg, Germany). The ASP+ was obtained from Life Technologies (#D288, Darmstadt, Germany), and FFN511 was acquired from Abcam Chemicals (#ab120331, Cambridge, United Kingdom). Escitalopram oxalate was a generous gift from Lundbeck (Valby, Denmark).

Cultures of 1C11 Cells. Murine 1C11 cells were obtained from Dr. Odile Kellermann (INSERM, Paris, France) and were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 mM MEM nonessential amino acids (NEAA, #11140-035), 2 mM L-glutamine (L-Gln, #25030-081), and 100 U/mL penicillin/streptomycin (#15240-062) (all from Life Technologies, Darmstadt, Germany). For the differentiation of 1C11 cells into 1C11$^{5\text{-HT}}$ serotonergic neurons, 1C11 cells were trypsinized and transferred to 1C11$^{5\text{-HT}}$ differentiation medium. This medium was supplemented with N6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (Db_2-cAMP, #D0627, 500 μg/mL) and cyclohexane carboxylic acid (CCA, #W353108, 500 μg/mL, both Sigma-Aldrich, Hamburg, Germany). For each differentiation, 5000 cells/well were seeded in 8-well μ-slides for high-end microscopy applications (#80826, Ibidi, Planegg, Germany). Serotonergic differentiation was complete after 4 days and resulted in 100% 1C11$^{5\text{-HT}}$-neurons, which were positive for serotonergic markers such as SERT, tryptophan hydroxylase 2 (TPH2), and VMAT. The 1C11$^{5\text{-HT}}$ cells do not express other monoamine transporters such as NET and DAT.[28]

Reverse Transcriptase PCR. Total RNA was extracted with Trizol (#10296028, Life Technologies, Darmstadt, Germany) from 1C11$^{5\text{-HT}}$ cells on day four of differentiation followed by isopropanol precipitation. The RNA was transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kits (#04896866001, Roche, Grenzach-Wyhlen, Germany). Subsequently, PCR reactions (35 cycles 30 s 95 °C, 30 s 62 °C, and 1 min at 72 °C, followed by a final elongation 10 min at 72 °C) were carried out with the following primers: hGAPDH fwd 5′-GCACCGTCAAGGCTGAGAAC-3′ and rvs 5′-CGCCCCACTTGATTTTGG-3′ (94bp); mSERT fwd 5′- AGCATCCACATTCTTTGCC-3′ and rvs 5′-AGAACCAAGACACGACGAC-3′ (293bp); mOCT1 fwd 5′-CCCAATAGCGGCATCAAATC-3′ and rvs 5′-CCAAAACCCCAAACAAAATGAG-3′ (296bp); mOCT2 fwd 5′-TGCCTTCATCATCATTCTCACC-3′ and rvs 5′-AGCAATAGCACAAGTCCCCC-3′ (372bp); mOCT3 fwd 5′-TGGGACTTATCGGAGGCAAC-3′ and rvs 5′-CCAGCCGAAAGAGCAGAAAC-3′ (360bp); mPMAT fwd 5′-ACTATCTTCACCACAAGTACCC-3′ and rvs 5′-CTCTCTCCAGTCATCACACC-3′ (341bp). Primers were from Eurofins Genomics (Ebersberg, Germany).

Blocking Experiments with Escitalopram, Progesterone, and Corticosterone. The 1C11$^{5\text{-HT}}$ serotonergic neurons on day four of differentiation were incubated for 4 h with escitalopram 1 μM to inhibit SERT activity at 37 °C. In order to inhibit de novo protein synthesis, cycloheximide (#7698, 30 μg/mL, Sigma-Aldrich, Hamburg, Germany) was added to the medium. Subsequently, the cells were additionally treated for 10 min with 10 μM corticosterone or 10 μM progesterone. Finally, they were loaded with either ASP+ (10 μM, 30 s), FFN511 (10 μM, 5 min), or 5-HT (500 μM, 10 min). The cells were washed twice with differentiation medium devoid of dyes or inhibitors to avoid background staining. The ASP+ was applied at 10 μM to avoid fluorescence saturation that can interfere with quantitative microscopy. Compared to our previous study (Lau et al.[31]), FFN511 uptake was extended to ensure optimal imaging conditions in case of strong fluorescence-diminishing effects of putative FFN511 uptake inhibitors.

Mifepristone was applied at 10 μM for 30 min prior and in the presence of corticosterone treatment of 1C11$^{5\text{-HT}}$ cells. At this concentration, mifepristone was shown to inhibit GR-dependent nongenomic redistribution of SERT in serotonergic neurons in vitro.[29] All drugs and fluorescent dyes used were applied in pH- and temperature-adjusted bath solutions to avoid temperature or pH shifts that might interfere with protein function or transport. To label the plasma membrane, cells were coincubated with 1 μg Alexa488- or Alexa555-conjugated choleratoxin subunit b for 5 min (#C34775 and #C34776, Life Technologies, Darmstadt).

Image Acquisition and Data Analysis. The 1C11$^{5\text{-HT}}$ cells were kept at 37 °C during image acquisition using a microscope microheating system (Ibidi, Planegg, Germany). For 5-HT uptake, epifluorescence image acquisition was carried out using a Neurolucida system (MBF Bioscience, Williston, VT, United States) with a 63× oil-immersion objective mounted Zeiss Axio Imager 2 (Zeiss, Jena, Germany). Excitation range was set to 358 nm by a DAPI filter.

All images for ASP+ and FFN511 uptake were taken on a Leica TCS SP confocal imaging system attached to a DM IRE2 microscope using a HCX PL APO 63× oil planchromat lens with a NA 1.40 (Leica, Mannheim, Germany). Excitation lasers were either an argon-ion laser for FFN511 (458−514 nm) or a DPSS laser for ASP+ (561 nm). The Z-stacks were acquired with sections taken every 0.5 μm and all images were exported as tiff-files.

Data analysis was performed according to Lau et al.[31] The tiff-files were imported to NIH ImageJ (version 1.45s; National Institutes of Health, Bethesda, MD), and Z-projections of each image sequence were calculated. With the plugin MultiMeasure in NIH ImageJ, cells were selected with the freehand selection tool and imported into the region of interest (ROI) manager. For all ROIs, the integrated densities of the fluorescence were determined. At least 90 cells per treatment were included in the analysis in $N \geq 2$ independent experiments. Statistical analysis was performed with GraphPad Prism 3.0 (GraphPad Software Incorporated, San Diego, CA) using one-way ANOVA and post hoc Tukey tests. The results are displayed in bar graphs as means ± SEMs. In every set of experiments, control and inhibition assays were performed in parallel and the fluorescence was determined in at least 90 and not more than 140 individual cells. Each set of experiments was carried out at least two times with similar results.

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Notes

The authors declare no competing financial interest.

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