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Serotonin-induced down-regulation of cell surface serotonin transporter

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Abstract

The serotonin transporter (SERT) terminates serotonergic signaling and enables refilling of synaptic vesicles by mediating reuptake of serotonin (5-HT) released into the synaptic cleft. The molecular and cellular mechanisms controlling SERT activity and surface expression are not fully understood. Here we demonstrate that the substrate 5-HT itself causes acute down-regulation of SERT cell surface expression. To assess surface SERT expression by ELISA, we used a SERT variant (TacSERT) where the N-terminus of SERT was fused to the intracellular tail of the extracellularly FLAG-tagged single-membrane spanning protein Tac. In stably transfected HEK293 cells, 5-HT caused a dose-dependent reduction in TacSERT surface signal with an EC50 value equivalent to the K_m value observed for 5-HT uptake. The 5-HT-induced reduction in surface signal reached maximum within 40-60 min and was blocked by the selective SERT inhibitor S-citalopram. 5-HT-induced reduction in SERT expression was further supported by surface biotinylation experiments showing 5-HT-induced reduction in wild type SERT plasma membrane levels. Moreover, preincubation with 5-HT lowered the V_{max} for 5-HT uptake in cultured raphe serotonergic neurons, indicting that endogenous cell-surface resident SERT likewise is down-regulated in the presence of substrate.

Keywords

serotonin transporter; monoamine transporters; neurotransmitter:sodium symporters; serotonin; trafficking; internalization; ELISA; raphe neurons

1. Introduction

In the brain, the serotonin transporter (SERT) is localized to serotonergic neurons where it mediates reuptake of the neurotransmitter serotonin (5-HT) following its release from the

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presynaptic nerve terminal (Broer and Gether, 2012; Kristensen et al., 2011). SERT plays a major role in regulating serotonergic signaling and alterations in its function have been linked to several psychiatric disorders such as depression, anxiety, OCD (obsessive compulsive disorder), autism and alcohol abuse. Furthermore, SERT has been subject to intensive research efforts as the target for antidepressant drugs such as citalopram (Cipralex/Lexapro) and fluoxetine (Prozac), as well as for psychostimulant drugs such as cocaine and 3,4-methylenedioxymethamphetamine ('Ecstasy') (Broer and Gether, 2012; Kristensen et al., 2011). SERT is a member of the gene family of solute carrier 6 (SLC6) transporters (also referred to as neurotransmitter/sodium symporters) that also includes plasma membrane transporters for other neurotransmitter such as dopamine, norepinephrine, λ-aminobutyric acid and glycine (Broer and Gether, 2012; Kristensen et al., 2011). These transporters share a predicted topology of 12 transmembrane domains, a large extracellular loop containing multiple glycosylation sites and N-and C-termini at the cytoplasmic side (Broer and Gether, 2012; Kristensen et al., 2011). The predicted topology is supported by the crystal structure of a homologous bacterial leucine transporter (Yamashita et al., 2005) and recently the drosophila DAT (Penmatsa et al., 2013). The transport of 5-HT in SERT proceeds by an alternating access mechanism where binding of the substrate a long with co-transported ions, Na^+ and Cl^-, induce a conformational change in SERT from an outward facing conformation to an inward facing conformation (Broer and Gether, 2012; Kristensen et al., 2011).

Because SERT is an important determinant in shaping the magnitude and duration of serotonergic signaling, it is important to understand the molecular and cellular mechanisms that control the availability of SERT in the plasma membrane. The level of surface SERT can be acutely regulated in various ways. Activation of protein kinase C (PKC) by phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), leads to a redistribution of the transporter away from the plasma membrane. This has been demonstrated in heterologous cell lines (Qian et al., 1997) and in rat brain synaptosomes (Samuvel et al., 2005). A similar effect of PKC activation has been observed for other SCL6 neurotransmitter transporters (for review see (Kristensen et al., 2011)). In addition, the activity of kinases like cGMP-dependent protein kinase (PKG) and p38 mitogen-activated protein kinase (MAPK) have been shown to have an impact on the amount of SERT molecules available at the cell surface (Samuvel et al., 2005; Zhu et al., 2004). Furthermore, several interacting partners have been identified; many of which are believed to alter SERT surface expression (Bauman et al., 2000; Carneiro and Blakely, 2006; Chanrion et al., 2007; Haase et al., 2001; Muller et al., 2006). Interestingly, substrates have been shown to mediate internalization of the closely related dopamine transporter (Chi and Reith, 2003; Saunders et al., 2000). However, co-incubation of SERT-expressing HEK293 cells with PMA and 5-HT abolished the PMA-induced redistribution of SERT away from the cell surface, suggesting that 5-HT has a stabilizing effect on SERT surface expression (Ramamoorthy and Blakely, 1999).

In the present study we address the effect of 5-HT alone at SERT surface expression. Using a SERT variant in which the N-terminus of human SERT was fused to the intracellular tail of the FLAG-tagged single-membrane spanning protein Tac (TacSERT), we measured changes in surface SERT by cellular ELISA in HEK293 cells. We observed a significant decrease in surface expressed TacSERT following 30-60 minutes application of the substrate

5-HT. The decrease in SERT surface expression following acute 5-HT application was confirmed by surface biotinylation in HEK293 cells expressing wild type SERT. Finally, preincubation of primary serotonergic cultures with 5-HT prior to uptake experiments results in a marked reduction of V_{max} indicating that endogenous cell surface resident SERT is likewise down-regulated in response to substrate.

2. Materials and Methods

2.1. cDNA constructs

The construct TacSERT was described in (Sucic et al., 2010). For the construct c-myc SERT, the c-myc tag (EQKLISEEDL) was added the N-terminus of hSERT using PCR with a single 5′-primer containing the c-myc tag. The resulting c-myc-SERT was subcloned into the bicistronic expression vector pCIHygro containing the hygromycin resistence gene (Saunders et al., 2000). Fusion of the c-myc epitope at the N-terminus position has previously been demonstrated not to alter binding or uptake properties of the transporter (Tate and Blakely, 1994).

2.2. HEK293 cell culturing and transfection

HEK293 cells (ATCC, number CRL-1573) were grown in Dulbecco's modified Eagles medium (DMEM) with Glutamax-I supplemented with 10 % fetal bovine serum (FBS), 5 mM sodium pyruvate and penicillin/streptomycin (100 μg/ml) at 37 °C in a humidified incubator with 5% CO_2. All products for cell culturing were purchased from Invitrogen. For stable transfection of TacSERT was carried out using Lipofectamine 2000 (Invitrogen). Following selection with G418 (500 μg/ml), 6 clones were isolated and the expression of the serotonin transporter was confirmed by [^{3}H]5-HT uptake. Two clones, the one with the highest and the one with the lowest expression levels, were chosen for further experiments.

For biotinylation experiments, HEK293 cells were stably transfected with the c-mychSERT and stably transfected clones were isolated following selection with hygromycin (250 μg/mL). All stably transfected cells were grown in medium with dialyzed FBS to minimize the amount of 5-HT in the media.

2.3. Primary cultures of serotonergic raphe neurons

The rhombencephalon (midbrain and brainstem) were dissected from fetal Sprague-Dawley rats (Charles River, Germany) at E14 as described in (Lautenschlager et al., 2000). Meninges were carefully removed and the neural tube was opened from the dorsal side. Fine dissection was performed along the midline of the mesencephalon giving tissue strips of about 1-2 mm in width. Tissue was immediately placed in sterile ice cold Krebs Buffer with 2% (v/v) HEPES, 3 g/L bovine serum albumin and 1.2 nM magnesium sulfate (dissection medium). Tissue was incubated in trypsin (Sigma) for 6 min at 37°C, centrifugated at 100 x *g*, and pellets dissociated by pipetting in dissection medium with soybean trypsin inhibitor (Sigma). Following centrifugation at 100 x *g* for 10 min, cells were resuspended in 37°C Neurobasal medium (Invitrogen) supplemented with 0.2 % penicillin/streptomycin (Invitrogen) and 2 % B27 (Invitrogen). Cells were plated on poly-ornithine coated 96 well plates (Corning, NY) and after 5-6 days in vitro (DIV) medium was exchanged with medium

supplemented with 5-fluorodeoxyuridine. Half of the medium was afterwards exchanged every 4-5th day. Experiments were carried out at16-20 DIV.

2.4. Surface ELISA

ELISA was performed on whole cells plated in black-walled clear-bottom 96- well plates (Wallac, PerkinElmer). HEK293 cells were washed once and equilibrated 30 min in DMEM (without serum) and subsequently treated with or without 5-HT at the indicated concentrations. Following 60 min incubation with anti-FLAG M1 primary antibody (Sigma) in DMEM on ice, cells were fixed in 4 % paraformaldehyde. Nonspecific binding was blocked in 1% BSA/PBS and a secondary HRP-conjugated antibody was applied for 30 min at RT. HRP activity was measured using the fluorescent substrate Amplex Red (Molecular Probes). The level of nonspecific signal was measured on non-transfected cells.

2.5. Cell surface biotinylation

HEK293 cells stably expressing c-myc tagged hSERT was seeded in poly-D-lysine coated 6-well plates at a density of 450.000 cells/well 48 hours prior to experiments. Cells were equilibrated in preheated DMEM without serum and subsequently incubated with 5-HT at the indicated concentrations for 30 min at 37°C. On ice, cells were treated with membrane-impermeant sulfo NHS-SS-Biotin (Pierce) (1 mg/mL freshly prepared in PBS) for 40 min. To quench unreacted biotin, cells were subsequently washed in 100 mM glycine/PBS. Proteins were solubilized in solubilization buffer (25 mM Tris, pH7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM *N*-ethylmaleimide and protease inhibitors) and incubated for 20 min with end-over-end rotation at 4 °C. After centrifugation (16.000 x *g*, 15 min at 4 °C), the biotinylated proteins were separated from nonbiotinylated proteins using avidin beads (Pierce) (200 μg protein/175 μL beads). Biotinylated protein was eluted from beads by incubation in loading buffer containing 100 mM DTT for 30 min at 37°C with shaking and subsequently subjected to SDS-PAGE and western blotting. SERT proteins were visualized using anti-c-myc antibody clone 9E10 (1:1000, Sigma). The ECL+ chemiluminiscent substrate (GE healthcare) was used for detection. Immunoreactive band intensities were quantified using Adobe Photoshop 6.0 (Adobe Systems). The biotinylated fractions were normalized to the total cell solubilizates.

2.6. [^{3}H]5-HT uptake experiments

Cells were washed once in 37°C uptake buffer (25 mM HEPES, 120 mM sodium chloride, 5 mM potassium chloride, 1.2 mM calcium chloride and 1.2 mM magnesium sulphate supplemented with 10 mM D-glucose, 1 mM ascorbic acid and 0.1 mM pargyline, pH 7.4), and equilibrated in uptake buffer for 30 min at 37°C before the addition of 10 μM 5-HT (or buffer for control) for 30 min at 37°C. After 5-HT pretreatment, cells were washed three times in buffer at room temperature. Uptake was initiated by adding serial dilutions (final conc. 6.4 μM to 0.05 μM) of [^{3}H]5-HT/5-HT (for determination of saturation kinetics) or a fixed concentration (17-35 nM) of [^{3}H]5-HT. [^{3}H]5-HT (Hydroxytryptamine creatine sulphate, 5-[1,2-^{3}H[N]]) was purchased from PerkinElmer. Uptake was allowed at room temperature for 5 or 10 min in HEK293 cells or neurons, respectively, and terminated by washing twice in ice cold uptake buffer. For HEK293 cells (in 24 well plates), cells were

lysed in 1% sodium dodecyl sulphate (SDS) and subsequently transferred to cell counting plates (PerkinElmer) where Optiphase HiSafe scintillation fluid (PerkinElmer) was added. For neurons (in 96 well plates) scintillation fluid was added directly to the wells. Radioactivity was measured in a Wallac β scintillation counter (PerkinElmer). Nonspecific uptake (defined as background) was determined in the presence of 1 μM paroxetine added during washing steps and [^{3}H]5-HT/5-HT uptake. Background corrected data were analyzed in GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). All determinations were performed in triplicates or more.

3. Results

3.1. Cell surface expression of TacSERT is reduced in response to 5-HT

To examine changes in surface expression of SERT, clones of HEK293 cells stably expressing TacSERT were generated and a cellular surface ELISA based on the extracellular FLAG-epitope was performed. The experiments were performed on two distinct cell clones with different expression levels to ensure that the effects observed were not clone specific. Following pretreatment of HEK293 TacSERT cells with varying concentrations of 5-HT at 37°C for various time points, a significant reduction in cell surface TacSERT was observed at 10 μM and 100 μM 5-HT already after 10 minutes (Fig. 1A). The decrease in surface TacSERT was increased over time. No significant decrease in surface TacSERT was observed after pretreatment with 1 μM 5-HT at any time points. A concentration-response curve of substrate-induced downregulation revealed a half-maximal response to 5-HT at 3.1 ± 1.1 μM 5-HT (Fig. 1B). This corresponds to the K_m-value of 5-HT uptake previously reported for TacSERT expressed in HEK293 cells (3.2 ± 1.2 μM (Sucic et al., 2010)).

3.2. 5-HT-induced alterations in TacSERT cell surface expression is mediated via a direct interaction of 5-HT with SERT

To test if the reduction in surface SERT following pretreatment with 5-HT was mediated via a direct interaction of 5-HT with the SERT, the selective serotonin reuptake inhibitor (SSRI) citalopram was employed. As shown in figure 2A, the effect of 5-HT was abolished at all concentrations in the presence of 10 μM of citalopram. Furthermore, S-citalopram inhibited the effect of 10 μM 5-HT in a concentration-dependent manner (Fig. 2B), supporting that the change in surface SERT is mediated directly by 5-HT interacting with SERT. Citalopram alone (10 μM) did not alter TacSERT surface expression (Fig. 2A) and neither did S-citalopram at any concentration ranging from 1 nM to 100 μM (Fig. 2B).

3.3. Surface biotinylation confirm reduction of SERTcell surface expression in response to 5-HT

To validate, that the 5-HT-induced reduction of surface TacSERT observed in the ELISA is not an artifact of the tethering of the SERT N-terminus by the extra transmembrane domain introduced in TacSERT, cell surface biotinylation was performed in HEK293 cells stably expressing hSERT N-terminally tagged with c-myc. In accordance with data obtained in the ELISA, biotinylation experiments revealed a significant reduction in surface SERT following pretreatment with 5-HT for 30 min at concentrations of 10 μM and above (Fig. 3). No change was observed for total SERT protein levels at any 5-HT concentration.

3.4. V_{max} is reduced in primary cultures of raphe neurons pretreated with 5-HT

We next wished to examine if endogenous SERT in cultured serotonergic raphe neurons undergo a similar mechanism. After preincubation with 10 μM 5-HT for 30 min at 37°C, neurons were rapidly washed three times to remove excessive 5-HT, and the [^{3}H]5-HT uptake was measured. Washing steps and uptake were performed at room temperature (20°C) at which limited trafficking takes place. We found that pre-incubation with the substrate induced a significant decrease (~34 %) in [^{3}H]5-HT uptake (Fig. 4A). Saturation kinetics revealed a corresponding reduction in V_{max} whereas no significant change was observed for the K_m value (Fig. 4B). The effect of 5-HT preincubation on [^{3}H]5-HT uptake was confirmed in the HEK293 cells stably expressing TacSERT. Consistent with the above results, 5-HT induced a significant decrease in [^{3}H]5-HT uptake (Fig. 4C) corresponding with a decrease in V_{max} determined by saturation kinetics (Fig. 4D).

4. Discussion

In the present study, the effect of 5-HT on SERT surface expression in HEK293 cells was characterized at 5-HT concentrations ranging from 0.1 μM to 100 μM. A concentration dependent reduction in surface SERT was observed with a half-maximal response to 5-HT at 3.1 ± 1.1 μM. The reduction was significant already after pretreatment for 10 min and became more profound over time. The effect of 5-HT on TacSERT surface expression is mediated via a direct interaction of 5-HT with SERT, since it was blocked by the SERT specific inhibitor citalopram. Data obtained with surface ELISA on TacSERT was supported by surface biotinylation experiments on c-myc tagged SERT. Furthermore, 5-HT uptake experiments performed in primary cultures of raphe serotonergic neurons reveal a decrease in V_{max} following 5-HT preincubation, indicating that 5-HT also induce down-regulation of endogenously expressed surface SERT.

In HEK293 cells as well as in rat brain synaptosomes, the dopamine transporter (DAT) has been reported to undergo endocytosis in response to dopamine with a concentration- and time dependency corresponding to what we observed for SERT (Chi and Reith, 2003; Saunders et al., 2000). Likewise, substrate-induced trafficking was observed for the GABA transporter GAT1, however, unlike SERT and DAT, GAT1 was found to be upregulated at the surface in response to GABA (Bernstein and Quick, 1999).

A logistic fit to the concentration-response data estimates an EC50 for SERT down-regulation by 5-HT to be approximately 3 μM, which is comparable to the Km value for 5HT uptake and indicates that the process is associated with substrate translocation. This is further supported by the blocking effect of SERT uptake inhibitor citalopram on substrate induced down-regulation. A similar correlation between GABA induced GAT1 up-regulation and GABA uptake was observed (Bernstein and Quick, 1999). Furthermore, whereas delivery of the substrate amphetamine directly into the cell was sufficient to mediate DAT trafficking of a transport-deficient variant of DAT (Kahlig et al., 2006), the same did not apply for dopamine-induced redistribution of DAT (Kahlig et al., 2006), supporting that transport of dopamine is necessary to mediate dopamine induced DAT down-regulation.

In HEK293 cells, SERT has been shown to internalize in response to activation of PKC by PMA. A surface biotinylation study subsequently showed that PMA induced trafficking was abolished by the substrate 5-HT when co-applied at a concentration of 1 μM (Ramamoorthy and Blakely, 1999). This indicates that 5-HT promotes retention of SERT at the membrane, which is in contradiction to our data pointing towards a decreased stability at the membrane induced by the substrate. However, Ramamoorthy *et al*. (1999) examined the effect of 5-HT when co-incubated with PMA, whereas our finding is based on the effect of 5-HT alone. Interestingly, they found an EC_{50}-value for the effects of 5-HT on PMA-induced phosphorylation of SERT that was more than 10-fold lower than K_m for 5-HT uptake, indicating that the effect potentially could be independent of substrate translocation (Ramamoorthy and Blakely, 1999). Of further interest, chronic treatment with 5-HT of cultured thalamocortical neurons that express SERT during development was found to upregulate SERT levels in the plasma membrane. The upregulation was blocked by SERT inhibitors indicating that the effect was SERT-mediated (Whitworth et al., 2002). Possibly, 5-HT might have a different effect when exposed to SERT over an extended time period and/or SERT might be subject to differential regulation when expressed during development in thalamocortical neurons.

In blood platelets endogenously expressing SERT, 10 μM 5-HT was also found to induce an increase in surface expressed SERT (Carneiro and Blakely, 2006). However, this effect could not be blocked by citalopram and hence represents an event suggested to be induced by activation of endogenous 5-HT2A receptors (Carneiro and Blakely, 2006). Another study performed with blood platelets report of changes in surface SERT following nM concentrations of 5-HT (Brenner et al., 2007). The changes were shown to be biphasic with an upregulation of plasma membrane SERT after pretreatment of isolated platelets with 0.75 nM 5HT and a decrease in membrane SERT following pretreatment with 1.5 nM 5-HT.

Studies on acute regulation of SERT have been performed mostly in immortalized cell lines transfected to heterologously express the transporter. In addition, studies have been performed on endogenous SERT in blood platelets or in synaptosomal preparations. Attempts to examine acute regulation of endogenous SERT in serotonergic neurons remain essentially unexploited. Surface ELISA could not be applied due to the lack of an extracellular antibody epitope tag in the endogenous transporter. Furthermore, attempts to detect changes in SERT in the neuronal plasma membrane by surface biotinylation were unsuccessful due to the low levels of SERT protein in the neuronal cell culture (data not shown). Accordingly, we measured changes in SERT uptake capacity in raphe serotonergic neurons following preincubation with 5-HT. A major concern, using this approach, is that the 5-HT applied during the preincubation step would interfere with the 5-HT uptake assay. However, the cells were washed extensively before initiation of uptake. Moreover, several previous studies have used the same approach to address substrate-induced regulation of neurotransmitter transporter surface levels in cell lines as well as in synaptosomes. Whereas dopamine and choline decrease uptake capacity of DAT and the choline transporter, respectively (Chi and Reith, 2003; Okuda et al., 2011), GABA induced up-regulation of GAT1 mediated GABA transport (Bernstein and Quick, 1999) indicating that a decrease in uptake capacity is not merely the result of competition with excessive substrate from the

preincubation step. In summary, we conclude that although not a direct measurement of surface expression, the reduction in V_{max} observed following pre-incubation with 10 μM 5-HT, indicate that substrate induced down-regulation of SERT is not a phenomenon restricted to SERT expressed in heterologous cell lines but is likewise a phenomena observed for endogenous SERT in serotonergic neurons.

Acknowledgments

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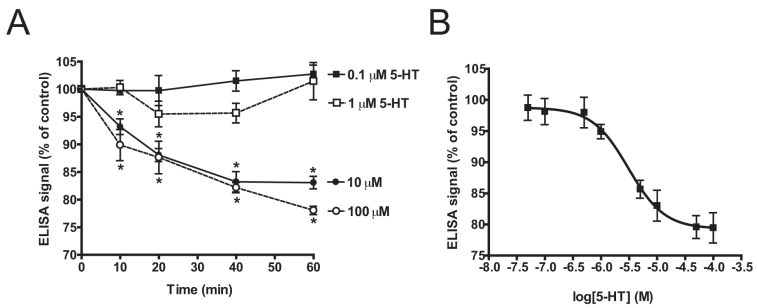

Figure 1.

Surface ELISA on HEK293 cells stably expressing TacSERT. (A) Prior to the assay cells were incubated with the indicated concentrations of 5-HT for various time points. 5-HT-induced trafficking of TacSERT was expressed relative to unstimulated cells at time 0 min. Solid squares represent 0.1 μM, open squares 1 μM, solid circles 10 μM and open circles 100 μM. Data represent the mean (± SEM) of 4 experiments performed in triplicates. (B) Concentration-response curve of 5-HT-induced change in surface expression of TacSERT. The half-maximal response to 5-HT is 3.1 ± 1.1 μM. Data represents the mean (± SEM) of 4 experiments performed in triplicates.

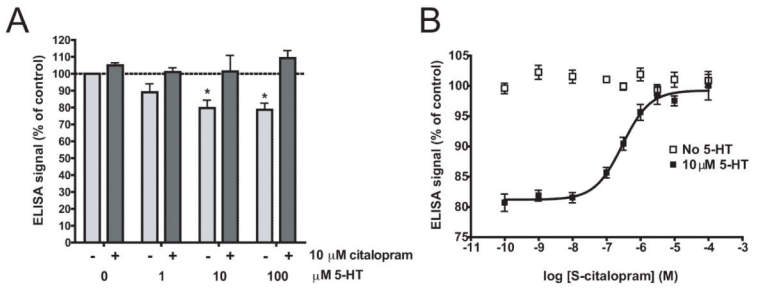

Figure 2.

Surface ELISA on HEK293 cells stably expressing TacSERT. (A) Cells were pretreated with the indicated concentrations of 5-HT prior to the assay. Co-application of 10 μM citalopram was conducted in parallel. Data represents the mean (± SEM) of 3 experiments performed in triplicates. * denotes significant different from control using one-sample t-test ($P < 0.05$). (B) Cells were co-incubated with 10 μM 5-HT and increasing concentrations of S-citalopram prior to the assay (solid squares). Incubation with S-citalopram alone was conducted in parallel (open squares). Data represents the mean (± SEM) of triplicates. The experiment was repeated once with equivalent results.

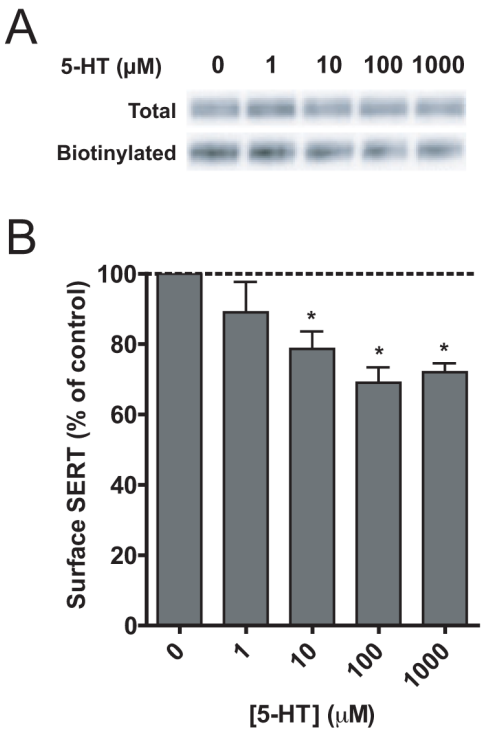

Figure 3.

Biotinylation of surface proteins in HEK293 cells stably expressing c-myc hSERT. Cells are preincubated with or without 5-HT at the indicated concentrations for 30 min at 37 °C. (A) Representative SERT immunoblot of three separate experiments. (B) Quantitive analysis of SERT band densities using Adobe Photoshop. Biotinylated fractions (surface protein) are normalized to total protein and subsequently expressed as percentage of control (preincubation without 5-HT). Data represents the mean (± SEM) of 3 independent experiments. * denotes significant different from control using one-sample t-test ($P < 0.05$).

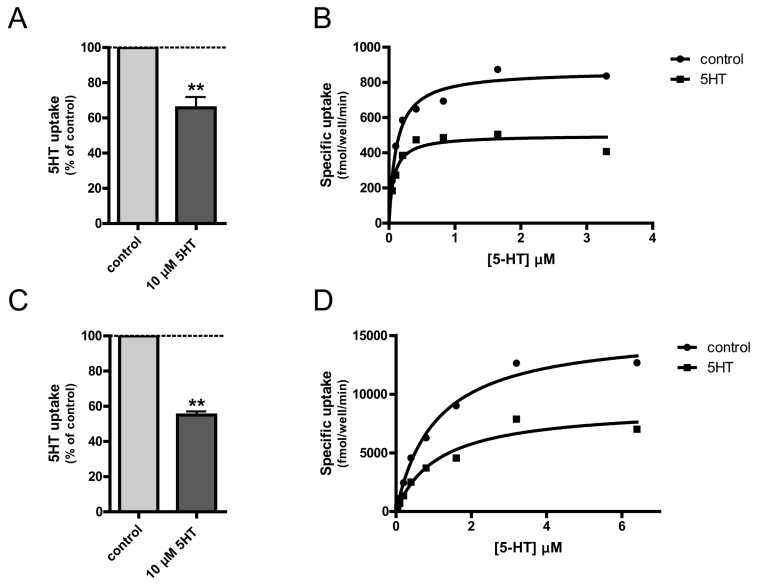

Figure 4.

5-HT uptake on primary serotonergic raphe neurons and on HEK293 cells stably expressing TacSERT. Cells are preincubated with or without 5-HT for 30 min at 37 °C and subsequently washed three times in buffer at RT before the initiation of 5-HT uptake. (A) [^{3}H]5-HT uptake in primary serotonergic raphe neurons. Data represents the mean (± SEM) of 6 independent experiments. ** denotes significant different from control using paired t-test ($P < 0.01$). (B) Kinetic measurements of [^{3}H]5-HT/5-HT uptake in serotonergic raphe neurons preincubated with (K_m: 0.07μM, V_{max}: 500 fmol/well/min) or without 5-HT (K_m: 0.11 μM, V_{max}: 870 fmol/well/min). The data are representative of two independent experiments. (C) [^{3}H]5-HT uptake in HEK293 cells. Data represents the mean (± SEM) of 3 independent experiments. ** denotes significant different from control using paired t-test ($P < 0.01$). (D) Kinetic measurements of [^{3}H]5-HT/5-HT uptake in HEK293 cells pre-incubated with (K_m: 1.11 μM, V_{max}: 9000 fmol/well/min) or without 5-HT (K_m: 1.06 μM, V_{max}: 15500 fmol/well/min). The data are representative of three independent experiments.