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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 EC_{50} 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.

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 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

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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 FLAG-tagged 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 min 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 5́-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-myc-hSERT 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*g*, and pellets dissociated by pipetting in dissection medium with soybean trypsin inhibitor (Sigma). Following centrifugation at 100*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 at 16–20 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 h prior to experiments. Cells were equilibrated in pre-heated 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*g*, 15 min at 4 °C), the biotinylated proteins were separated from non-biotinylated 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 sulfate 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

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