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Electrical coupling between the human Serotonin Transporter and Voltage-Gated Ca²⁺ Channels

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Abstract

Monoamine transporters have been implicated in dopamine or serotonin release in response to abused drugs such as methamphetamine or ecstasy (MDMA). In addition, monoamine transporters show substrate-induced inward currents that may modulate excitability and Ca²⁺ mobilization, which could also contribute to neurotransmitter release. How monoamine transporters modulate Ca²⁺ permeability is currently unknown. We investigate the functional interaction between the human serotonin transporter (hSERT) and voltage-gated Ca²⁺ channels (Ca_V). We introduce an excitable expression system consisting of cultured muscle cells genetically engineered to express hSERT. Both 5HT and S(+)-MDMA depolarize these cells and activate the excitation-contraction (EC)-coupling mechanism. However, hSERT substrates fail to activate EC-coupling in Ca_V1.1-null muscle cells, thus implicating Ca²⁺ channels. Ca_V1.3 and Ca_V2.2 channels are natively expressed in neurons. When these channels are co-expressed with hSERT in HEK293T cells, only cells expressing the lower-threshold L-type Ca_V1.3 channel show Ca²⁺ transients evoked by 5HT or S(+)-MDMA. In addition, the electrical coupling between hSERT and Ca_V1.3 takes place at physiological 5HT concentrations. The electrical coupling between monoamine neurotransmitter transporters and Ca²⁺ channels such as Ca_V1.3 is a novel mechanism by which endogenous substrates (neurotransmitters) or exogenous substrates (like ecstasy) could modulate Ca²⁺-driven signals in excitable cells.

Keywords

calcium channels; calcium imaging; L-type calcium channel; N-type calcium channel; monoamine transporters; neurotransmitter transport; skeletal muscle; excitability

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1. Introduction

Monoamine neurotransmitter transporters belong to the solute carrier 6 (SLC6) gene family formed by structurally related proteins that use the Na^+ driving force to concentrate substrates against their chemical gradient [1]. Their known physiological functions are to limit the neurotransmitter signaling by decreasing its extracellular concentration and to cycle the transmitter molecules back to the presynaptic cells [2]. Small molecules that target monoamine transporters are therapeutically significant; amphetamine and methylphenidate which act on the dopamine transporter (DAT) are used as treatment for attention deficit hyperactivity disorder, whereas serotonin transporter (SERT) inhibitors like fluoxetine or citalopram are effective drugs to treat mood disorders [3, 4]. Drugs that interfere with DAT activity, e.g. cocaine, amphetamine, and certain amphetamine derivatives are stimulants that have high addictive liability [5]. In particular 3,4-methylenedioxy-*N*-methylamphetamine (MDMA or ecstasy), which is highly abused, share characteristics of both stimulant and empathogen and targets both DAT and SERT [6-8].

Most psychoactive amphetamines that are substrates of monoamine transporters compete with neurotransmitter uptake and are believed to disrupt vesicular neurotransmitter storage and induce efflux of neurotransmitters through the transporter; therefore increasing the extracellular concentration of the neurotransmitter in the brain [9, 10]. Monoamine transporters show significant inward currents when challenged with substrates. These substrate-induced currents are viewed as uncoupled from the actual transport and although they have not been directly implicated in Ca^{2+} channel activation, they can increase excitability, which presumably further contributes to neurotransmitter release [11].

Voltage-gated Ca^{2+} channels are well known modulators of cell excitability and the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in excitable cells [12]. The lower-threshold L-type Ca^{2+} channel $\text{Ca}_v1.3$ is involved in dendritic serotonin release from serotonergic neurons [13] and pace-making in dopaminergic neurons [14]. Ca^{2+} channels in neurons open briefly leading to local events that are not efficiently amplified by intracellular Ca^{2+} channels from internal stores compared to muscle cells; in addition, neurons express a variety of Ca^{2+} channels that make the connection between drug-induced currents and Ca^{2+} channel activation difficult to interpret. To overcome these problems, we have devised a new model in which we express the human SERT (hSERT) in an excitable cell that contains a reliable Ca^{2+} amplification system that allows us to explore the effect of serotonin (5HT) and S(+)-MDMA (the most potent enantiomer of ecstasy) on cytosolic Ca^{2+} signals. We showed in engineered cultured muscle cells (myotubes) expressing hSERT that both S(+)-MDMA and 5HT mobilize Ca^{2+} evidenced by Ca^{2+} transients. In addition, hSERT substrates failed to induce Ca^{2+} signals in $\text{Ca}_v1.1$ -null myotubes, suggesting that hSERT substrate-induced currents depolarize the plasma membrane sufficiently, to the level of voltage-gated Ca^{2+} channel activation. To test whether the hSERT-mediated depolarization activates voltage-gated Ca^{2+} channels that are natively expressed in neurons, we co-expressed hSERT either with $\text{Ca}_v1.3$ or $\text{Ca}_v2.2$ in HEK293T cells. Both 5HT and S(+)-MDMA induce Ca^{2+} signals in $\text{Ca}_v1.3$ - but not in $\text{Ca}_v2.2$ -expressing cells, suggesting that hSERT-mediated depolarization is within the range of activation of Ca^{2+} channels that are more sensitive to membrane depolarization. In addition, 5HT showed comparable potency for both, activating

the $\text{Ca}_v1.3$ -mediated Ca^{2+} signal, and for inducing its transport; suggesting that 5HT transport and hSERT-coupling to voltage-gated Ca^{2+} channels may coexist in the physiological range of 5HT concentration.

In summary we demonstrate that the hSERT-mediated depolarization activates voltage-gated Ca^{2+} channels, in particular those more sensitive to membrane depolarization. These findings support a mechanism by which hSERT activation may modulate Ca^{2+} permeability in excitable cells.

2. Results

To study whether the membrane depolarization driven by substrate-induced currents of hSERT can couple to the activation of voltage-gated Ca^{2+} channels, we expressed hSERT in cultured muscle cells (myotubes). Skeletal muscle cells were selected for these experiments because they have the excitation-contraction (EC)-coupling mechanism which is characterized by a tight control of the $[\text{Ca}^{2+}]_i$ as function of the membrane potential. In addition since EC-coupling is driven by $\text{Ca}_v1.1$ activation, it is a suitable model to assess voltage-gated Ca^{2+} channel activation by substrate-induced hSERT currents. The response of EC-coupling in skeletal muscle cells is in the millisecond time scale, thus any depolarization of the membrane potential is almost instantaneously followed by increase in $[\text{Ca}^{2+}]_i$ [15]. In this study we expressed hSERT in wild type (Wt) and dysgenic (MDG) myotubes to study the depolarization evoked by hSERT substrates. MDG myotubes were used as controls because they do not express $\text{Ca}_v1.1$ which is the voltage sensor of EC-coupling, therefore they are insensitive to voltage changes [16]. Myotubes do not express hSERT natively, but after transduction with hSERT-IRES-Puromycin retrovirus they expressed this protein as shown in Fig. 1. The hSERT immuno-staining visualized by confocal microscopy showed a plasma membrane localization of the protein in transduced cells (Fig. 1A and 1C). Western blot analysis showed a single band only in transduced cells (Fig. 1E). Moreover, fluoxetine-sensitive APP^+ uptake was detected only in myotubes transduced with the hSERT-encoding retrovirus (Fig. 1F and 1G).

Interestingly, Wt myotubes expressing hSERT showed a fast and reversible increase of $[\text{Ca}^{2+}]_i$ during exposure to S(+)-MDMA, 5HT, or high external K^+ (Fig. 2). Conversely, MDG myotubes expressing hSERT did not respond to S(+)-MDMA, 5HT or high external K^+ , but did respond to caffeine, which opens the intracellular Ca^{2+} channel directly, releasing Ca^{2+} from internal stores (Fig. 2). The refractoriness of MDG+hSERT myotubes to 5HT or S(+)-MDMA strongly suggests that the depolarization, and not any other secondary effect, causes the increase in $[\text{Ca}^{2+}]_i$ in Wt+hSERT myotubes. Accordingly, $[\text{Ca}^{2+}]_i$ was unchanged when non-transduced Wt or MDG cells were challenged with 5HT or S(+)-MDMA (Fig. 2). Furthermore, the Ca^{2+} transients induced either by 5HT or by S(+)-MDMA were blocked completely by fluoxetine in WT+hSERT myotubes, demonstrating that hSERT-mediated depolarization exceeds the threshold for EC-coupling activation (Fig. 3). In contrast, Ca^{2+} signals induced by both hSERT substrates are insensitive to tetrodotoxin (TTX, $3\mu\text{M}$), whereas the TTX batch used in this study completely blocked action potential-induced Ca^{2+} transients in myotubes (Fig. 4). Together these data suggest that voltage-gated Na^+ channel activity does not contribute in the overall

membrane depolarization induced by hSERT currents in myotubes expressing the transporter.

To test the hypothesis that either 5HT- or S(+)-MDMA-induced depolarization can activate the lower threshold L-type Ca^{2+} channel $\text{Ca}_V1.3$ - or as a control, the high-voltage-activated $\text{Ca}_V2.2$ Ca^{2+} channel - were co-expressed with hSERT in HEK293T cells. As shown by others [17] and confirmed here the Ca^{2+} current (I_{Ca}) of $\text{Ca}_V1.3$ has a ~ 30 mV left shift in its voltage dependence curve compared to the I_{Ca} of $\text{Ca}_V2.2$ (Fig. 5). Therefore, the activation threshold of I_{Ca} is ~ -50 mV and ~ -15 mV for $\text{Ca}_V1.3$ and $\text{Ca}_V2.2$, respectively (Fig. 5). To study the electrical coupling between hSERT and these Ca^{2+} channels, the co-transfected cells were subjected to intracellular $[\text{Ca}^{2+}]$ determination by fluorescence microscopy as described in Materials and methods. When hSERT was expressed without either Ca_V channel, the cells did not respond to hSERT substrates or to high K^+ -depolarization (Fig. 6A). In contrast, 5HT and S(+)-MDMA evoked fast Ca^{2+} transients when $\text{Ca}_V1.3$ (but not $\text{Ca}_V2.2$) was co-expressed with hSERT: nevertheless, high K^+ depolarization evoked Ca^{2+} transients when either Ca^{2+} channel was expressed (Fig. 6B and 6C), demonstrating that hSERT-mediated depolarization activates $\text{Ca}_V1.3$ but not $\text{Ca}_V2.2$. Finally, cells transfected with $\text{Ca}_V1.3$ in the absence of hSERT only have a positive response to high K^+ depolarization, whereas S(+)-MDMA or 5HT are without effect (Fig. 6D).

The over-expression of hSERT in transient transfection experiments would lead to non-physiological levels of depolarization upon challenging the cells to hSERT substrates. To address this concern, a stable cell line expressing the hSERT-IRES-DsRED construct was generated using the Flp-InTM T-RexTM expression system as described in Materials and methods. Since these cells are restricted to a single copy of the gene of interest, the expression level of the inserted gene is lower than in transient transfection experiments. Using the APP⁺ assay, the Flp-In cells showed 60% less hSERT maximal activity than HEK cells subjected to transient transfection with the hSERT-IRES-DsRED plasmid, both the transient transfection of HEK cells and the induction of Flp-In cells were carried out 3 days prior to the experiment (Fig. 7A). Similarly the DsRed signal was reduced 93% in Flp-In cells (Fig. 7A). Although hSERT activity is reduced in Flp-In cells, when they were transfected with $\text{Ca}_V1.3$, 5HT induced Ca^{2+} signals in a dose-dependent manner (Fig. 7B) whereas untransfected Flp-In cells (no Ca^{2+} channel but expressing hSERT) are refractory to 5HT treatment (not shown). Interestingly, dose-response experiments for [³H]-5HT uptake done in Flp-In cells showed an EC_{50} of 1.68 ± 0.38 μM ($n = 5$, Fig. 7C), whereas the EC_{50} for the Ca^{2+} signal induced by 5HT in these cells was 0.90 ± 0.08 μM ($n > 57$, Fig. 7C) ($p < 0.01$, t -test). The comparable potency of both actions of 5HT, as a substrate of hSERT, and driving hSERT-mediated depolarization toward $\text{Ca}_V1.3$ activation, suggests that the coupling between hSERT-mediated depolarization and Ca_V channel opening is significant in the physiological range of 5HT concentration.

3. Discussion

Most if not all transporters forming the SLC6 gene family show substrate-induced inward ionic currents when challenged near rest. In particular for monoamine neurotransmitter

transporters, such as the electroneutral serotonin transporter or the electrogenic dopamine and norepinephrine transporters, the charge measured during substrate flux exceeds by several fold what is predicted by their transport rate and stoichiometry [18-24]. Single channel events have been described in monoamine neurotransmitter transporters, mimicking bona fide ion channels. To see whether the depolarizing currents evoked by hSERT substrates are physiologically relevant in unclamped cells, we expressed hSERT in cultured muscle cells (myotubes). This heterologous expression system has a sensitive built-in machinery to measure membrane depolarization, which is the EC-coupling mechanism. EC-coupling of skeletal muscle cells is based in the physical coupling between the voltage-gated Ca^{2+} channel ($\text{Ca}_v1.1$) in the plasma membrane and the ryanodine receptor 1 (RyR1), which is the Ca^{2+} channel of the sarcoplasmic reticulum [25, 26]. The membrane depolarization induces a conformational change of $\text{Ca}_v1.1$ that physically opens the RyR1, releasing Ca^{2+} from internal stores [27]. Thus, MDG myotubes that lack $\text{Ca}_v1.1$ expression are insensitive to depolarization (Fig. 2). Accordingly, Wt myotubes expressing hSERT activate the EC-coupling machinery upon exposure to 5HT, S(+)-MDMA or high K^+ external solution (40 mM), whereas these agents have no effect on MDG expressing hSERT myotubes (Fig. 2), suggesting that 5HT and S(+)-MDMA depolarize hSERT-expressing myotubes. Although a low density of T-type Ca^{2+} channels ($\text{Ca}_v3.2$) can be expressed in differentiated myotubes [28], no increase of cytosolic Ca^{2+} was detected in MDG myotubes when exposed to K^+ -depolarization (Fig. 2), suggesting that the T-type channel expression is negligible in our experimental conditions. However, voltage-gated Na^+ channel conductance could work as an intermediary between hSERT-mediated depolarization and EC-coupling. This seems not to be the case because TTX did not affect Ca^{2+} signals mediated by hSERT-mediated depolarization (Fig. 4). Conceivably, the depolarization evoked by hSERT activity is not fast enough to induce coherent activation of voltage-gated Na^+ channels and/or the depolarization is too long, and thus Na^+ channels become inactivated. Further experiments are needed to address the modulation of voltage-gated Na^+ channels by hSERT currents. $\text{Ca}_v1.1$ is an atypical voltage-gated Ca^{2+} channel; its Ca^{2+} current (L-type) is activated at high voltages (threshold ~ 0 mV) [29], whereas the $\text{Ca}_v1.1/\text{RyR1}$ complex is activated at low voltages (threshold ~ -30 mV) [29]. Thus the voltage dependence of the EC-coupling activation approximates more that of a low-voltage-activated Ca^{2+} channel than that of a high-voltage-activated Ca^{2+} channel in myotubes. The dose-response curve of the Ca^{2+} release induced by K^+ -depolarization in myotubes has been described previously [29, 30]. Using the Goldman-Hodgkin-Katz equation (Eq. 3 in Materials and methods) we estimate that at rest (4 mM $[\text{K}^+]_e$), threshold (~ 15 mM $[\text{K}^+]_e$), half response (~ 20 mM $[\text{K}^+]_e$), or maximal Ca^{2+} release response (~ 40 mM $[\text{K}^+]_e$) in myotubes, the membrane potentials are -60 (known resting membrane potential of myotubes [31]), -43 , -38 , and -24 mV, respectively. This approximation of the voltage dependency of the Ca^{2+} release is left shifted ~ 10 mV compared to patch-clamp recordings reported in myotubes likely due to uncorrected liquid-junction potential [29]. Since 5HT and S(+)-MDMA showed Ca^{2+} release in hSERT-expressing myotubes that is between threshold and half of the maximal K^+ response (Fig. 2), the substrate-induced current of hSERT should depolarize the cells to -43 or -38 mV, then taking into account that the resting membrane potential of myotubes is -60 mV, the magnitude of the depolarization induced by these hSERT substrates is about 17 to 22 mV.

Monoamine neurotransmitter transporters coexist with Ca^{2+} channels, a variety of Cl^- and K^+ channels, receptors, and other transporters in excitable cells. Since the relative proportions of monoamine transporters to other current inducing membrane proteins are likely to be highly variable among cell types, it is impossible to predict if the substrate-induced current will be sufficient to significantly depolarize native cells. For instance K^+ conductance activated by autoreceptors (GIRK channels) [32, 33], TASK channels [34] and Ca^{2+} -activated K^+ conductance [35] would oppose and neutralize the depolarization evoked by monoamine transporters current. Case specific studies are required to establish resting membrane potential changes upon monoamine transporter activation. Nevertheless, dopamine-, amphetamine- and methamphetamine-induced DAT current increases action potential firing frequency in dopaminergic neurons from the midbrain [11, 36], demonstrating that the depolarization evoked by DAT activation modulates excitability in native cells. In addition, it has been shown that dopamine induces cytosolic Ca^{2+} signals in cultured neurons from midbrain, cortex and hippocampus and using pharmacological tools the authors suggest that monoamine transporter-mediated depolarization could activate voltage-gated Ca^{2+} channels [37].

In the present work, we hypothesize that the depolarization evoked by hSERT activation is sufficient to activate voltage-gated Ca^{2+} channels. As a proof of concept, we tested this hypothesis in a controlled environment such as the one provided by the heterologous expression systems described in this study. In particular we focused our study in the lower-threshold L-type Ca^{2+} channel, $\text{Ca}_v1.3$, because it is expressed in serotonergic and dopaminergic neurons and has been shown to modulate some aspects of their function. SERT is expressed broadly through the cell bodies and along neurite extensions in cultured serotonergic neurons. In addition, SERT is expressed in both serotonergic fibers and cells bodies of the raphe nucleus in the brain [38, 39]. $\text{Ca}_v1.3$ is also present in the dorsal raphe nucleus and is involved in the AMPA-mediated dendritic serotonin release in serotonergic neurons [13], which is implicated in depression [40, 41]; thus altogether these data suggest that SERT and $\text{Ca}_v1.3$ may co-localize in the soma and dendrites of serotonergic neurons. In dopaminergic neurons, by means of its voltage dependence characteristics, $\text{Ca}_v1.3$ drives pacemaking and bursts of electrical activity [14]. In addition, $\text{Ca}_v1.3$ activity is implicated in selective death of dopaminergic neurons in the substantia nigra in Parkinson's disease [42]. $\text{Ca}_v1.3$ has ~ 30 mV left shift in the peak current – voltage curve compared to the high-voltage-activated Ca^{2+} channel, $\text{Ca}_v2.2$ (Fig. 5). The threshold of activation that we measured for $\text{Ca}_v1.3$ is about –50 mV when 5 mM external Ca^{2+} is used as the charge carrier (Fig. 5), similar to that found by others [17, 43]. Taking into account the sensitivity of $\text{Ca}_v1.3$ to voltage changes, the estimated 20 mV depolarization achieved by hSERT activation should activate $\text{Ca}_v1.3$ but not $\text{Ca}_v2.2$ in physiological conditions. Indeed, when we co-expressed either $\text{Ca}_v1.3$ or $\text{Ca}_v2.2$ with hSERT in HEK239T cells, both 5HT and S(+)-MDMA evoked clear activation of $\text{Ca}_v1.3$ (Fig. 6C) but not of $\text{Ca}_v2.2$ (Fig. 6B) despite that, both types of cells showed convincing Ca transients induced by strong K^+ -depolarization (Fig. 6B and 6C).

Since over-expression of hSERT due to transient transfection could undermine the physiological relevance of our findings, the expression of hSERT was reduced using the Flp-In expression system which inserts a single copy of hSERT cDNA into the cell's

genome by targeted recombination. This procedure decreased hSERT activity by 60% when compared to transiently transfected HEK cells (Fig. 7A). Despite the significant reduction in hSERT expression, the Flp-In cells showed robust coupling between hSERT activity and $\text{Ca}_V1.3$ opening. Moreover, concentrations as low as 100 nM are sufficient to induce Ca^{2+} signals implying that a relatively low number of transporters would depolarize the membrane sufficiently to activate $\text{Ca}_V1.3$ channels (Fig. 7B and 7C). Accordingly, 5HT showed comparable potency for inducing its own transport and for activating $\text{Ca}_V1.3$ -mediated Ca^{2+} signals, implying that the electrical coupling between hSERT and Ca_V channels can coexist with 5HT transport in a physiological range of 5HT concentrations.

As mentioned above, one consequence of $\text{Ca}_V1.3$ activation is the increase of action potential firing in excitable cells such as dopaminergic neurons [14]. Although the participation of $\text{Ca}_V1.3$ has not been previously implicated, it was suggested that substrate-induced currents mediated by DAT produce bursts of electrical activity in midbrain dopaminergic neurons [11] and Ca^{2+} currents may be implicated in DA release mechanisms. Therefore, the electrical coupling between a monoamine transporter and $\text{Ca}_V1.3$ described in the present study supports the hypothesis that monoamine transporter-mediated modulation of cell excitability requires $\text{Ca}_V1.3$ activation. Further research effort is needed to assess this new hypothesis which is beyond the scope of the present study.

In summary, we demonstrate that hSERT-mediated depolarization activates voltage-gated Ca^{2+} channels in three independent heterologous expression systems: 1) myotubes engineered to express hSERT undergo ~20 mV depolarization, as estimated by partial activation of the $\text{Ca}_V1.1/\text{RyR1}$ complex when exposed to 5HT or MDMA, 2) in HEK293T cells co-expressing hSERT with voltage-gated Ca^{2+} channels, the hSERT-mediated depolarization is within the range of activation of $\text{Ca}_V1.3$ but not $\text{Ca}_V2.2$, and 3) in Flp-In cells co-expressing hSERT and $\text{Ca}_V1.3$, despite the lower expression of hSERT, $\text{Ca}_V1.3$ activation is coupled to hSERT-mediated depolarization. In addition, the 5HT transport and $\text{Ca}_V1.3$ activation, both mediated by hSERT, occur with similar 5HT potency, suggesting that hSERT/ $\text{Ca}_V1.3$ coupling ensues at physiological concentrations of 5HT.

The trans-activation of Ca^{2+} channels by the monoamine transporters demonstrated in our models illustrates a new mechanism by which endogenous substrates (neurotransmitters) or artificial substrates (amphetamine-related agents) could modulate excitability and signaling mechanisms directed by the rise of intracellular Ca^{2+} in excitable cells.

4. Materials and methods

Expression of hSERT and voltage-gated Ca^{2+} channels in HEK293T cells

The hSERT cDNA (accession number L05568) was subcloned into the bicistronic pIRES2-DsRed expression vector (Clontech) to generate the hSERT-IRES-DsRed plasmid. The $\text{Ca}_V2.2$ (α_{1B}), $\text{Ca}_V1.3$ (α_{1D}), β_3 and $\alpha_2\gamma_1$ expression plasmids were obtained from Addgene (cat# 26570, 26571, 26574 and 26575, respectively) were kindly provided by Dr. Diane Lipscombe (Department of Neuroscience, Brown University, Providence, Rhode Island, USA). HEK293T cells were cultured on 96 well imaging plates or 12 well plates with 18 mm round glass coverslips inside each well. The culturing media used was Dulbecco's

modified Eagle medium supplemented with 10% fetal bovine serum. The cells were transfected with the hSERT-pIRES-DsRed plasmid using lipofectamine 2000 (Life Technologies) following the instructions of the manufacturer. When the hSERT-pIRES-DsRed plasmid was co-transfected with Ca^{2+} channels, Fugene 6 (Promega) was used as transfection reagent. The ratio of DNA used was $\alpha_1:\beta_3:\alpha_2\gamma_1:\text{hSERT-DsRed} = 3:3:3:0.4$. The transfected cells were identified by the DsRed fluorescent signal.

Generation of hSERT in the Flp-In™ T-Rex™ expression system

To produce stable inducible cell lines using the *Flp-In™ T-Rex™ expression system* (Invitrogen), the hSERT-IRES-DsRed DNA fragment was subcloned into the pcDNA5/FRT/TO vector to generate the pcDNA5/hSERT-IRES-DsRed/FRT/TO plasmid. The inducible expressing cells were made following the manufacturer's protocol. Briefly, the *Flp-In™ T-rex™* host cells lines are HEK cells with a single FRT recombination site and a Tet repressor gene. These cells were co-transfected with the pcDNA5/hSERT-IRES-DsRed/FRT/TO and the pOG44 plasmids. The latter encodes the Flp recombinase. Clones that have inserted a single copy of the gene of interest into the recombination site acquire a hygromycin resistance. These cells were transiently transfected with the $\text{Ca}_v1.3\text{-Ca}^{2+}$ channel using Fugene 6 (Promega). The ratio of DNA used was $\alpha_1:\beta_3:\alpha_2\gamma_1:\text{EGFP} = 3:3:3:0.4$. The transfected cells were identified by the EGFP fluorescent signal. hSERT and DsRed were induced adding doxycycline 1 $\mu\text{g/mL}$ to the culture media for 3 days.

Permanent expression of hSERT in mouse myoblasts

The hSERT cDNA was inserted in the retroviral expression vector pCMMP-MCS-IRES-Puro (Addgene 36952) that in addition to the inserted cDNA it encodes a puromycin resistance. This plasmid was kindly provided by Dr. Bill Sugden (McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin, USA). The retroviral particles were packaged in HEK293T helper cells as previously described [44]. Wild type (Wt) and dysgenic (MDG) mouse myoblasts were kindly provided by Drs. Paul D. Allen and José R. López (Department of Molecular Biosciences, School of Veterinary Medicine, University of California–Davis, Davis, California, USA). These cells were transduced with the retroviral particles and selected as previously described [44]. Myoblast culture and differentiation to myotubes was done as reported previously [44].

Immunofluorescence

Immunofluorescence was done as previously described [45] using as primary antibody a monoclonal anti-hSERT antibody (mAb Technologies, Stone Mountain, GA) and secondary antibody anti-mouse Alexa Fluor 488 antibody (Invitrogen). The low magnification images were acquired in an epifluorescence microscope (Olympus IX70) equipped with a digital camera (Andor Technology, Belfast, UK) and the proper filter set to detect alexa 488 and DAPI staining. High magnification images were acquired in a confocal microscope (Zeiss LSM 710) using the 63X 1.4NA objective. The confocal images have an optical section of 0.8 μm for both channels.

Western Blot

Total protein extracts were run in SDS-PAGE in duplicates; one gel was subjected to Coomassie blue staining (not shown) and the other was electrically transferred to a PVDF membrane. To detect hSERT expression, the membrane was incubated with anti-hSERT monoclonal mouse antibody and then exposed to a secondary antibody conjugated with horseradish peroxidase. Then the hSERT band was visualized by chemiluminescence using RapidStep ECL Reagent (Calbiochem) and the images were acquired in a digital imaging system (FluorChem E, Protein Simple, Santa Clara, CA).

Measurement of Ca^{2+} currents

Macroscopic Ca^{2+} currents (I_{Ca}) were measured as previously described [29] with the following modifications: the external solution used was 155 mM tetraethylammonium (TEA)-Cl, 5 mM CaCl_2 and 10 mM Hepes, pH 7.4, with TEA-OH. The patch pipette internal solution consisted of 135 mM CsCl, 10 mM $\text{Cs}_2\text{-EGTA}$, 1 mM CaCl_2 , 4 mM MgCl_2 , and 10 mM Hepes, pH 7.4 with CsOH. The whole-cell patch-clamp parameters of the recordings were: cell capacitance = 27 ± 3 pF, access resistance = 5.5 ± 0.5 M Ω , τ = 153 ± 26 μ s (n=14). The effective series resistance was compensated and corrected by 80 % using the Axopatch circuit. In these conditions the remaining voltage error is < 1.5 mV. The leak current was subtracted using a -P/6 protocol before each sweep. The pipettes were fire polished, Sylgard coated and had a resistance of ~ 2.5 M Ω when filled with the internal solution. The liquid-junction potential was not corrected. The recorded signals were acquired at 10 kHz and filtered at 5 kHz.

The voltage dependence of the I_{Ca} was fit to the following equation:

$$I_{\text{Ca}} = G_{\text{max}} (V - V_r) / \left[1 + \exp \left(\left(V_{1/2} - V \right) / k \right) \right] \quad (\text{Eq.1})$$

Where G_{max} is the maximal conductance, V is the test potential, $V_{1/2}$ is the potential at which $G=1/2$ G_{max} , k represents a slope parameter and V_r is the reversal potential.

Measurements of intracellular Ca^{2+}

The Ca^{2+} sensitive dyes Fluo-4AM and Fura-2AM (Life Technologies) were dissolved in DMSO pluronic F-127 20% and then were diluted in imaging solution (IS: 130 NaCl, 4 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 Hepes, 10 glucose, pH 7.4, in mM) to get a final concentration of 5.5 μ M. The cells were loaded for 12 min with Fluo-4AM or 25 min with Fura-2AM all at 37°C. Then the cells were washed twice with IS and placed on the stage of an epifluorescence microscope. All the compounds used were dissolved in IS and when high K^+ solution was used equimolar amount of NaCl was substituted by KCl. 5HT was purchased from Sigma and S(+)-MDMA hydrochloride was a gift from the National Institute on Drug Abuse NIDA (NIH). The setup consists of a Olympus IX70 microscope equipped with a Polycrome V (Till Photonics, Gräfelfing, Germany) as a light source, a Luca S digital camera (Andor Technology, Belfast, UK), and an automatic perfusion system (AutoMate Scientific, Berkeley, CA). The imaging system was controlled by the Live Acquisition Software from Till Photonics. The measurements were done under constant perfusion at RT (23°C) or at 35°C using a ThermoClamp-1 heater (AutoMate Scientific, Berkeley, CA). The objective

used was an Olympus 20X 0.8NA Oil. Measurements were done using an excitation wavelength of 490/10 nm, a dichroic mirror 505LP and an emission wavelength of 535/50 nm for Fluo-4. The Fura-2 signal was acquired switching the excitation wavelength between 340/10 nm and 380/10 nm at 6Hz, the dichroic mirror used was LP490 and the emission wavelength was 510/40 nm. The transfected cells were identified by visualization of DsRed (excitation 550/15 nm, dichroic mirror 570LP and emission 620/60 nm) or EGFP fluorescence (excitation 490/10 nm, dichroic mirror 505LP, emission 535/50 nm). The reported values are the fluorescence values divided by the basal level of each cell and reported as $\gamma F/F_0$ for Fluo-4 and $\gamma F_{(340/380)}/F_0$ for Fura-2. All signals were background subtracted. The Ca^{2+} signal was acquired at 50 or 100 Hz as indicated in the figure legends for Fluo-4 or 3Hz for Fura-2. Electrical stimulation was delivered by 2 platinum wires positioned close to the cells using a micromanipulator; square 0.5 ms pulses were applied at 1 Hz using a Grass S48 stimulator.

The dose-response relationships for Ca^{2+} and [3H]-5HT uptake experiments were fit by the equation:

$$Y(X) = Y_{max} / [1 + 10 \exp(\{ \log EC_{50} - \log X \} * n)] \quad (\text{Eq.2})$$

Where X is the concentration of 5HT or S(+)-MDMA applied, $Y(X)$ is the response measured, Y_{max} is the maximal response, EC_{50} is the concentration that produces half-maximal response, and n is the Hill slope parameter.

To estimate the depolarization evoked by external $[K^+]$ the Goldman-Hodgkin-Katz equation was used:

$$E_m = RT/F \ln \{ (P_{Na} [Na]_e + P_K [K]_e + P_{Cl} [Cl]_i) / (P_{Na} [Na]_i + P_K [K]_i + P_{Cl} [Cl]_e) \} \quad (\text{Eq. 3})$$

Where E_m is the membrane potential; P_{ion} permeability of the ion; $[ion]_e$ and $[ion]_i$ are the extracellular and intracellular concentration of the ion, respectively; R is the ideal gas constant, T is the temperature, F is the Faraday's constant. The parameters used in the equation are as follows: relative permeability ($K^+ = 100$; $Na^+ = 5$ and $Cl^- = 10$). In contrast to adult fibers, cultured myotubes have low Cl^- permeability at rest because of their underdeveloped T-tubule system [46, 47], external and internal concentration in mM (K^+ variable, 105; Na^+ 130, 7 and Cl^- 130, 20, respectively) at 35°C.

APP⁺ uptake assay

4-(4-(dimethylamino)phenyl)-1-methylpyridinium (APP⁺), a fluorescent substrate of hSERT, was used to monitor hSERT activity by fluorescence microscopy as previously described [48]. Cells grown on imaging plates were placed on the stage of the fluorescence microscope describe above. The wavelengths used to detect the APP⁺ signal were 460/10 nm for excitation and 535/50 nm for emission.

[3H]-5HT uptake

Flp-In cells expressing hSERT were counted and 2×10^6 cells were exposed to different concentrations of 5HT where 3% of a given concentration consisted of [3H]-5HT. The 5HT

uptake was carried out for 10 min at 37°C in IS (see composition above). Non-specific uptake was measured adding 5 μ M fluoxetine to the radioactive cocktail. Then the cells were centrifuged, washed once with cold 1X PBS, centrifuged again, cell pellets were resuspended in Ecoscint H (National Diagnostics, Atlanta, GA, USA), and the radioactivity counted using a liquid scintillation counter. The mean $\pm$ s.e.m. of 5 independent experiments done in duplicates are reported.

Statistics

All data are presented as mean $\pm$ s.e.m. unless indicated in the figure legend. Comparisons were made by unpaired, two-tailed *t*-test, with $p < 0.05$ considered significant.

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Highlights

S(+)-MDMA (ecstasy) and 5HT (serotonin) induce Ca^{2+} mobilization in cultured muscle cells expressing hSERT.

hSERT-mediated depolarization activates $\text{Ca}_v1.3$ but not $\text{Ca}_v2.2$ in HEK293 cells.

The electrical coupling between hSERT and $\text{Ca}_v1.3$ takes place at physiological concentrations of 5HT.

hSERT-mediated depolarization activates voltage-gated calcium channels.

Figure 1. Expression of hSERT in cultured skeletal muscle cells

Myoblasts isolated from wild type (Wt) or dysgenic (MDG) mice were transduced with the hSERT encoding retrovirus. The transduced cells were selected as described in Materials and methods. The cells were differentiated into myotubes and the hSERT expression was evaluated by immunofluorescence visualized by confocal microscopy (scale bar = 20 μ m). The hSERT signal is shown in green and the DAPI nuclear staining is shown in blue for Wt-transduced (A), Wt-control (B), MDG-transduced (C), and MDG-control myotubes (D). E, Western blot that shows the expression of hSERT in samples from myotubes transduced with the hSERT-retrovirus (+) or uninfected cells (-). (F and G) The hSERT activity was evaluated measuring the uptake of APP⁺ by fluorescence microscopy. F, Wt transduced myotubes (black trace) and the effect of fluoxetine (gray trace, FLX). Non-transduced Wt myotubes (red trace) and the effect of fluoxetine (FLX, blue trace) are plotted. G is the same as F but performed in dysgenic (MDG) myotubes. The traces shown are mean $\pm$ s.e.m. of n 25 cells for each condition.

Figure 2. S(+)-MDMA and 5HT activate EC-coupling in skeletal muscle cells expressing hSERT
Differentiated muscle cells in culture (myotubes) were loaded with the permeable Ca^{2+} sensitive dye Fluo-4AM. The intracellular Ca^{2+} concentration was monitored at 35°C under constant perfusion by fluorescence microscopy. The acquisition frequency used was 100 Hz. The wild type (Wt) and the Wt myotubes transduced with hSERT retrovirus (Wt+hSERT) were exposed to 10 μ M S(+)-MDMA, 10 μ M 5HT, or high K^+ solution (40 mM) as indicated in the figure. The dysgenic (MDG) and the MDG myotubes transduced with hSERT retrovirus (MDG+hSERT) were exposed to the same protocol plus an additional pulse of caffeine 20 mM (Caff). Average traces $\pm$ s.e.m. are shown (n = 36).

Figure 3. Fluoxetine inhibits the EC-coupling activation induced by hSERT substrates

Differentiated muscle cells in culture (myotubes) were loaded with the permeable Ca^{2+} sensitive dye Fluo-4AM. The intracellular Ca^{2+} concentration was monitored at 35°C under constant perfusion by fluorescence microscopy. The acquisition frequency used was 100 Hz. Ca^{2+} signal of Wt myotubes expressing hSERT exposed to $10\text{ }\mu\text{M}$ S(+)-MDMA, $10\text{ }\mu\text{M}$ 5HT and $1\text{ }\mu\text{M}$ fluoxetine as indicated in the figure. The traces shown are the mean $\pm$ s.e.m. of $n = 30$ cells. The peak of the Ca^{2+} increase induced by S(+)-MDMA ($0.95 \pm 0.102\text{ }\gamma\text{F/F}_0$, $n = 37$) is no different from the 5HT-induced peak ($1.0 \pm 0.093\text{ }\gamma\text{F/F}_0$, $n = 36$, $p > 0.05$ *t*-test).

Figure 4. EC-coupling activation mediated by hSERT substrates does not involve voltage-gated Na^+ channels

(A) Cultured myotubes were loaded with Fluo4-AM and the intracellular Ca^{2+} concentration was monitored by fluorescence microscopy at room temperature under constant perfusion. The acquisition frequency was 50 Hz. The representative trace shows a myotube electrically paced at 1 Hz; the Ca^{2+} transient was completely blocked by 3 μM TTX. (B). Intracellular Ca^{2+} concentration was measured by fluorescence microscopy at 35°C under constant perfusion. The acquisition frequency was 100 Hz. After dye loading, hSERT-expressing myotubes were pre-incubated with TTX (3 μM) for 20 min, and TTX was kept in the

perfusion solutions during the whole experiment. Control hSERT-myotubes were subjected to the same protocol but in absence of TTX. The traces shown are the mean $\pm$ s.e.m. of n = 39 cells. TTX did not affect the amplitude of the Ca^{2+} signal induced by MDMA (10 μM) or 5HT (10 μM) in myotubes expressing hSERT.

Figure 5. Voltage-dependence of Ca_V1.3- and Ca_V2.2- mediated Ca²⁺ currents

HEK293T cells were cotransfected with hSERT-IRES-DsRed, β_3 , $\alpha_2\gamma_1$ and alternatively Ca_V1.3 (closed black circles) or Ca_V2.2 (open gray circles) plasmids. The experiments were done under constant perfusion at 23°C. The currents were evoked by a trend of pulses starting at a holding potential of -70 mV in steps of 10 mV for Ca_V1.3 or steps of 5 mV for Ca_V2.2. Representative family of responses for Ca_V1.3 (closed black circle) or Ca_V2.2 (open gray circle), and the value of the test potential are shown for each sweep. The current-voltage dependence of the peak current densities were fit according to Eq.1 (Materials and methods) for Ca_V1.3 (black trace) and Ca_V2.2 (gray trace). The data are expressed as mean $\pm$ s.e.m. The fitting parameters are the following: G_{max} , 458 ± 84 and 737 ± 178 (pS/pF); $V_{1/2}$, -20 ± 1.9 and $7^{***} \pm 0.7$ (mV); k , 7.9 ± 0.37 , and $5.1^{***} \pm 0.1$ (mV) for Ca_V1.3 ($n = 6$) and Ca_V2.2 ($n = 8$), respectively. *** = $p < 0.0001$, t -test.

Figure 6. The depolarization induced by hSERT activation is electrically coupled to $\text{Ca}_V1.3$ opening

HEK293T cells were loaded with the permeable Ca^{2+} sensitive dye Fluo-4AM and the intracellular Ca^{2+} concentration was measured by fluorescence microscopy at an acquisition rate of 50 Hz. All measurements were done at 35°C under constant perfusion. The tested cells were transfected with the following plasmids: (A) hSERT-IRES-DsRed (without Ca_V channel); (B) α_{1B} ($\text{Ca}_V2.2$) and hSERT-IRES-DsRed; (C) α_{1D} ($\text{Ca}_V1.3$) and hSERT-IRES-DsRed; (D) α_{1D} ($\text{Ca}_V1.3$) and DsRed (without transporter). In all cases the β_3 and $\alpha_2\gamma_1$ plasmids were included in the transfection mix. The transfected cells were identified by their

DsRed fluorescence. (A-D) As indicated in each trace, the cells were exposed to 10 μ M S(+)-MDMA (light gray box) or 10 μ M 5HT (dark gray box) for 5 s. After wash the cells were exposed to 200 nM valinomycin (V, white box) for 2 s to increase the K^+ permeability and were immediately exposed to 130 mM K^+ solution for 5 s (K^+ , black box) to depolarize the cells toward positive potentials, then after wash the cells were exposed to 3 μ M 4Br-A23187, a Ca^{2+} ionophore. The traces shown are mean $\pm$ s.e.m. of n = 20 cells.

Figure 7. The electrical coupling between hSERT and Ca_v1.3 take place in the physiological concentration range of 5HT

(A) Flp-InTM T-RexTM cells (Flp-In, open circles) expressing the hSERT-IRES-DsRed construct were generated as described in Materials and methods, and HEK293 cells (closed circles) were subjected to transient transfection using the hSERT-IRES-DsRed plasmid. To compare the activity of hSERT among cell types, the maximal rate of APP⁺ uptake was measured computing the maximum of the first derivative of APP⁺ signal as function of time. In addition, the DsRed signal was measured for each cell. The maximal rate of APP⁺ uptake (y axis) and DsRED fluorescence (x axis) is plotted for each individual cell (note that the

axis are in logarithmic scale) and the values shown are the mean $\pm$ standard deviation. The hSERT activity was $44.8^{***} \pm 15.4$ (a.u./s), and 110.4 ± 55.0 (a.u./s) for Flp-In cells ($n = 202$) and HEK293 cells ($n = 107$), respectively ($^{***} = p < 0.0001$, t -test). The DsRed signal was $119^{***} \pm 60$ (a.u.) and $1,821 \pm 1,310$, for Flp-In cells ($n = 202$) and HEK293 cells ($n = 107$), respectively ($^{***} = p < 0.0001$, t -test). (B) Flp-In cells were transiently transfected with $\text{Ca}_v1.3$ as described in Materials and methods, the transfected cells were identified by the EGFP fluorescence and the Ca^{2+} signal was determined using Fura-2AM. Measurements were done under constant perfusion at 35°C and the acquisition frequency was 3Hz. The cells were exposed to a variable concentration of 5HT followed by $30\mu\text{M}$ 5HT to record the maximal response. The traces shown are mean $\pm$ s.e.m. of $n = 57$ cells for each condition. (C) The dose-response experiments shown in B were fit to Eq. 2 (Materials and methods) and yield the following fitting parameters for the 5HT- dependent Ca^{2+} signal: Maximal Response = 0.519 ± 0.013 ($[\text{F}_{340/380}/\text{F}_0]$), $\text{EC}_{50} = 0.90 \pm 0.08$ (μM) and Hill Slope = 1.15 ± 0.12 ($n > 57$). The dose response of $[\text{}^3\text{H}]\text{-5HT}$ uptake in Flp-In cells was fit to eq.2 (Materials and methods) and yield the following parameters. Maximal Response = $55,155 \pm 5,512$ (cpm), $\text{EC}_{50} = 1.68 \pm 0.38^{**}$ (μM) and Hill Slope = 1.55 ± 0.43 ($n = 5$, $^{**} = p < 0.01$ vs EC_{50} of Ca^{2+} experiments, t -test). The fitted curves of the Ca^{2+} signal and $[\text{}^3\text{H}]\text{-5HT}$ uptake experiments were normalized and superimposed for better comparison.