Studies of monoamine transporters

Phosphorylation of the serotonin transporter and interactions of antidepressants with the serotonin and norepinephrine transporters

**PhD thesis**
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Preface

The present thesis is submitted as a part of the requirements for obtaining the PhD degree at University of Copenhagen. The experimental work was performed during the period of May 2008 to February 2011 at Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen.

The presented studies concern molecular aspects of monoamine transporter regulation and interactions with antidepressants. Specifically, Chapter 2 describes identification of phosphorylation sites in the serotonin transporter (SERT) using and in vitro phosphorylation approach and Chapter 3 deals with possible functional consequences of phosphorylation at the identified sites. Purification and phosphoproteomic analysis of SERT is described in Chapter 4, whereas Chapter 5 covers studies of molecular determinants for interactions of antidepressants with the serotonin and norepinephrine transporters. A general introduction to monoamine transporter structure, function and regulation is given in Chapter 1, serving as a background for the experimental work presented in the following chapters.

The majority of data presented in Chapter 2-3 and 5 are included in paper I and II, respectively. Paper I has been submitted to Molecular Pharmacology and paper II has been prepared for submission to Journal of Biological Chemistry.


Additional work performed within the PhD period not included in the thesis:


Lena Sørensen
Copenhagen, May 2011
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As a part of the PhD programme, I have co-supervised MSc students Mette Thomsen, Stinna Hansen and Troels Eskildsen, and I would like to thank them for their contributions to the project. I also want to thank post doc Alicia Lundby, Centre for Protein Research, University of Copenhagen, for conduction of phosphoproteomic analyses. Furthermore, I thank the Biostructural Research group at the Department of Medicinal Chemistry for providing me access to their protein purification equipment. Special thanks are dedicated to my friend and former colleague Simone Vink for proofreading the thesis.

Finally, a heart-felt thanks to my family and friends, especially to my boyfriend Stephen for coming along to Denmark, which enabled our life together and for his continuous love and support.
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine, serotonin</td>
</tr>
<tr>
<td>ADHD</td>
<td>attention deficit hyperactivity disorder</td>
</tr>
<tr>
<td>AdiC</td>
<td>arginine-agmatine antiporter</td>
</tr>
<tr>
<td>ApcT</td>
<td>H⁺-coupled amino acid symporter</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>β-PMA</td>
<td>4-β-phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>BetP</td>
<td>sodium-betaine symporter</td>
</tr>
<tr>
<td>B&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximal binding capacity</td>
</tr>
<tr>
<td>BRET</td>
<td>bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>CaiT</td>
<td>carnitine-butyrobetaine antiporter</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>cdk5</td>
<td>cyclin-dependent kinase 5</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>dopamine transporter</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagles medium</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DRI</td>
<td>selective dopamine reuptake inhibitor</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-fluorenylmethoxycarbonyl</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GAT</td>
<td>GABA transporter</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GluA1</td>
<td>ionotropic glutamate receptor A1</td>
</tr>
<tr>
<td>GLYT</td>
<td>glycine transporter</td>
</tr>
<tr>
<td>GSK3</td>
<td>glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HBTU</td>
<td>O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>hDAT</td>
<td>human dopamine transporter</td>
</tr>
<tr>
<td>hNET</td>
<td>human norepinephrine transporter</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>hSERT</td>
<td>human serotonin transporter</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>concentration of inhibitor producing 50% inhibition</td>
</tr>
<tr>
<td>IL</td>
<td>intracellular loop</td>
</tr>
<tr>
<td>INSR</td>
<td>insulin receptor</td>
</tr>
<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
</tr>
</tbody>
</table>
LC-MS/MS  liquid chromatography-tandem mass spectrometry
LeuT  leucine transporter
MADAM  2-(2-dimethylaminomethyl-phenylsulphanyl)-5-methyl-phenylamine
MAPK  mitogen-activated protein kinase
MDD  major depressive disorder
MDMA  3,4-methylenedioxymethamphetamine, “ecstasy”
Mhp1  sodium-benzylhydantoin transporter
MS  mass spectrometry
MS/MS  tandem mass spectrometry
NE  norepinephrine
NET  norepinephrine transporter
NHS  N-hydroxysuccinimide
nNOS  nitric oxide synthase
NO  nitric oxide
NRI  selective norepinephrine reuptake inhibitor
NSS  neurotransmitter/sodium symporter
NTT  neurotransmitter transporter
OCD  obsessive-compulsive disorder
ON  over night
p38MAPK  p38 mitogen-activated protein kinase
PBS  phosphate buffered saline
PBSCM  phosphate buffered saline supplemented with CaCl₂ and MgCl₂
PDB  protein data bank
PKA  protein kinase A
PKC  protein kinase C
PKG  protein kinase G
PP1  protein phosphatase 1
PP2A  protein phosphatase 2A
pSer/pS  phosphoserine
pThr/pT  phosphothreonine
pTyr/pY  phosphotyrosine
Rluc  Renilla luciferase
rSERT  rat serotonin transporter
RT  room temperature
S1  central substrate binding site
S2  second substrate binding site
SCAM  substituted cysteine accessibility method
SDS-PAGE  sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.E.M.  standard error of mean
SERT  serotonin transporter
SLC  solute carrier
SNRI  serotonin and norepinephrine reuptake inhibitor
Src  sarcoma tyrosine kinase
SSRI  selective serotonin reuptake inhibitor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syn1A</td>
<td>syntaxin1A</td>
</tr>
<tr>
<td>TCA</td>
<td>tricyclic antidepressant</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane helix</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>maximal transport capacity</td>
</tr>
<tr>
<td>vSGLT</td>
<td>sodium-galactose symporter</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
</tbody>
</table>

Standard one and three letter abbreviations for proteinogenic amino acids are used throughout the report.
Abstract

The transmembrane transporters for serotonin, norepinephrine and dopamine (SERT, NET and DAT) play key roles in the chemical neurotransmission in the brain by mediating reuptake of released transmitters into the presynaptic neurons. The transporters are highly exploited drug targets for treatment of psychiatric diseases, including depression, anxiety disorders and attention deficit hyperactivity disorder (ADHD).

The transporters are phosphoproteins, and it is well-described that kinase-dependent signalling pathways can lead to acute up- or down-regulation of transporter function. However, the molecular mechanisms behind these processes are poorly understood, including the influence and location of specific transporter phosphorylation sites. In the present study, we have performed \textit{in vitro} phosphorylation assays with peptides corresponding to the entire intracellular regions of human SERT (hSERT) using a panel of nine selected protein kinases. Hereby, five residues located in the N- and C-termini and in the intracellular loops 1 and 2 were identified as sites for phosphorylation; Ser149, Ser277 and Thr603 for PKC, Ser13 for CaMKII and Thr616 for p38MAPK.

Possible regulatory roles for SERT transport activity and surface expression of these potential phosphoacceptors were investigated using phospho-mimicking mutations and co-transfection or pharmacological activation of kinases in a heterologous expression system. Our results suggest that Ser277 is involved in an initial phase of PKC-mediated down-regulation of SERT. Furthermore, a bioluminescence resonance energy transfer (BRET) assay was used to study the interaction between hSERT and syntaxin1A. Our set of data does not support a suggested role on the interaction of CaMKII-mediated phosphorylation of Ser13 in hSERT.

A combination of affinity purification and liquid chromatography-tandem mass spectrometry (LC-MS/MS) was employed to identify possible phosphorylation sites in full-length SERT. Two purification procedures: nickel- and (S)-citalopram-based affinity chromatography, were successfully established for purification of SERT from heterologous expression systems and native tissue, respectively. No phosphorylation sites were identified in the LC-MS/MS analysis of heterologously expressed hSERT, which covered 21 out of 38 intracellular Ser/Thr/Tyr residues.

A separate study focused on the interactions of antidepressants with SERT and NET. We determined the effect of mutating six key residues in the S1 substrate binding pocket of hSERT and hNET on the potency of 15 prototypical SERT/NET inhibitors from different antidepressant drug classes. Analysis of the resulting drug sensitivity profiles provides novel information about drug binding modes and identifies specific S1 residues as important molecular determinants for inhibitory potency and SERT NET selectivity.
Resumé (Danish)

De transmembrane transportere for serotonin, noradrenalin og dopamin (SERT, NET og DAT) spiller centrale roller i den kemiske neurotransmission i hjernen ved at transportere frigivne neurotransmitterstoffer tilbage ind i de presynaptiske neuroner. Transporterne er hyppigt udnyttede mål for lægemidler brugt i behandling af psykiske lidelser såsom depression, angstlidelser og ADHD (attention deficit hyperactivity disorder).

Transporterne er fosforproteiner, og det er velkendt at cellulære signaleringsveje, som er afhængige af kinaseaktivitet, kan føre til akut op- eller nedregulering af transportaktiviteten. De underliggende molekylære mekanismer, inklusiv indflydelse og placering af direkte fosforyleringssites i transporterne, er dog stort set ukendte. I dette studie har vi udført in vitro fosforylerings-assay med peptider svarende til alle intracellulære regioner af human SERT (hSERT) ved brug af ni udvalgte protein kinaser. Herved blev fem residier, som er placeret i N- og C-terminalerne og i de intracellulære loops 1 og 2, identificeret som fosforyleringssites; Ser149, Ser277 og Thr603 for PKC, Ser13 for CaMKII og Thr616 for p38MAPK.

Disse potentielle fosforacceptorers mulige indflydelse på reguleringen af transportaktivitet og overfaldeudtryk af SERT blev undersøgt ved introduktion af fosfor-efterlignende mutationer og co-transfektion eller farmakologisk aktivering af kinaser i et heterologt ekspressionssystem. Vores resultater tyder på, at Ser277 er involveret i en initiel fase af PKC-medieret nedregulering af SERT. Derudover blev interaktionen mellem SERT og syntaxin1A studeret ved brug af BRET (bioluminescence resonance energy transfer) assay. Vores data understøtter ikke en tidligere foreslået indflydelse på interaktionen af CaMKII-medieret fosforylering af Ser13 i hSERT.

En kimation af affinitetsoprensning og væskekromatografi-tandem massespektrometri (LC-MS/MS) blev anvendt til identificering af mulige fosforyleringssites i fuldlængde SERT. To oprensningsprocedurer; nikkel- og (S)-citalopram-baseret affinitetskromatografi, blev etableret til oprensning af SERT fra henholdsvis et heterologt ekspressionssystem og nativt væv. For heterologt udtrykt SERT blev ingen fosforyleringssites identificeret i LC-MS/MS-analyserne, som dækkede 21 ud af 38 intracellulære Ser/Thr/Tyr residier.

Et separat studie fokuserede på interaktionerne mellem antidepressiva og SERT og NET. Vi undersøgte effekten af at mutere seks nøgle-residier i S1 substratebindingslommen i hSERT and hNET på potensen af 15 prototypiske SERT/NET inhibiterer tilhørende forskellige klasser af antidepressiva. Analyse af de resulterende sensitivitetsprofiler for de undersøgte stoffer tilvejebringer ny information om måden hvorpå stofferne binder og identificerer specifikke residier i S1, som er vigtige molekylære determinanter for inhibitorisk potens og SERT/NET-selektivitet.
1 Introduction

The cell membrane is the physical boundary that separates the interior of a cell from its surroundings. While permeable to certain substances, the cell membrane is impermeable to others and the selective permeability is the key to maintenance of a proper internal environment and is essential for all life. Transport of impermeable solutes, such as ions, nutrients and signalling molecules across the membrane is carried out by integral membrane proteins functioning as either channels or transporters. While channels allow substances to pass the membrane by passive diffusion only, transporters enable translocation of substances against their chemical gradient via energetic coupling of the process.

In the central nervous system (CNS), transporters play a major role in the chemical neurotransmission. Proper synaptic signalling requires the precise control of the duration and concentration of available neurotransmitters in the synaptic cleft. An important control mechanism is reuptake of transmitters into the presynaptic neuron or surrounding glia cells by a group of transporter proteins belonging to the solute carrier 6 (SLC6) family. The SLC6 transporters belong to a larger family of membrane-bound transporters called the solute carrier superfamily, and the SLC6 family contains 20 genes encoding a group of highly similar transporter proteins, of which nine are neurotransmitter transporters (NTTs)\(^1\). These include four transporters for \(\gamma\)-aminobutyric acid (GABA): GAT1, GAT2, GAT3 and BGT1, two transporters for glycine: GLYT1 and GLYT2, and three transporters for the biogenic monoamines serotonin (5-hydroxytryptamine; 5-HT), norepinephrine (NE) and dopamine (DA): SERT, NET and DAT, respectively\(^3\). The SLC6 proteins perform transport of substrates into cells against their electrochemical gradient using co-transport of extracellular Na\(^+\) as a driving force for substrate translocation, and the SCL6 NTTs are often called “neurotransmitter/sodium symporters” (NSS)\(^3\). The use of the electrochemical potential difference of Na\(^+\) across the cell membrane as energy source for transport defines the SLC6 transporters as secondary active transporters. The monoamine transporters, primarily SERT and to a smaller extent NET, are the focus of the present thesis.

1.1 Monoamine transporters

1.1.1 Physiology

The biogenic monoamines, 5-HT, NE and DA, are common neurotransmitters in the mammalian CNS and they play essential roles in a range of behavioral, physiological and cognitive functions, including memory, mood, sleep, appetite, attention, arousal, anxiety and reward\(^5\). SERT, NET and DAT appear to be exclusively expressed in their corresponding neurons and the distribution of the monoamine transporters in the brain therefore correlates with the distribution of the respective neurotransmitter systems. In the individual neurons, monoamine transporters are widely distributed both in dendrites and axons with a predominant extrasynaptic localization\(^4\). The central role of monoamine NTTs is to
mediate rapid uptake of synaptically released neurotransmitters from the extracellular space into the presynaptic neurons, and the uptake can be maintained against very large concentration gradients\textsuperscript{4,8} (Figure 1.1.A). NTT-mediated uptake kinetics follows the Michaelis-Menten model with substrate $K_m$ values in the lower micromolar range and maximal turnover rates of around 1 substrate molecule per second\textsuperscript{12-18}. Apart from the mandatory requirement for extracellular Na\textsuperscript{+}, transport is also dependent on extracellular Cl\textsuperscript{-}, which is believed to be co-transported with substrate and Na\textsuperscript{+}, and as the only monoamine transporter, SERT performs counter-transport of K\textsuperscript{+}\textsuperscript{4,19,20} (Figure 1.1.B).

**Figure 1.1. Monoaminergic synapse and endogenous substrates for SERT, NET and DAT**

A. Schematic representation of a monoaminergic synapse. Following vesicular release, neurotransmitters exert their effects on pre- and postsynaptic receptors (for clarity, only postsynaptic receptors are depicted in the figure). SERT, NET and DAT are crucial for the termination of neurotransmission by performing reuptake of the neurotransmitters from the synaptic cleft into presynaptic terminals, as well as for maintaining low tonic neurotransmitter concentrations outside synapses. Once inside the presynaptic terminal, vesicular monoamine transporters (VMATs) sequester 5-HT, NE and DA into synaptic vesicles. B. Chemical structures of the endogenous substrates (5-HT, NE and DA) for SERT, NET and DAT and ion coupling stoichiometry for neurotransmitter reuptake.

By mediating the rapid removal of neurotransmitters from the synapses, monoamine NTTs are major determinants in the regulation of synaptic signaling. Due to their involvement in multiple aspects of brain function, they are widely used targets for studies of neurotransmission at the molecular and cellular level and are established as important drug targets for treatment of a range of brain diseases, including depression, anxiety, obsessive compulsive disorder (OCD), attention deficit hyperactivity disorder (ADHD) and Parkinson’s disease, and they are also targets for some of the most widely used drugs of abuse, such as cocaine, 3,4-methylenedioxymethamphetamine (MDMA or “ecstasy”) and amphetamine\textsuperscript{19,21-25}.

### 1.1.2 Structure

Knowledge of the membrane topology of the monoamine transporters has existed since the 1990’s where the transporters were cloned\textsuperscript{26-30}. Sequence analysis combined with biochemical studies revealed a
topology with 12 membrane-spanning helices combined with intracellular and extracellular loops and with long intracellular N- and C-terminal tails\textsuperscript{19} (Figure 1.2).

\begin{center}
\includegraphics[width=0.5\textwidth]{membrane_topology.png}
\end{center}

**Figure 1.2. Membrane topology of monoamine transporters**

Monoamine transporters contain 12 membrane-spanning regions connected by intra- and extracellular loops with the N- and C-termini located intracellularly. The extracellular loop between TM3 and TM4 contains sites for N-linked glycosylation.

Human SERT, NET and DAT are comprised of 630, 617 and 620 amino acids, respectively, and they all contain a large extracellular loop between the third and fourth transmembrane helix (TM) with N-linked glycosylation sites, which are important for expression of the transporters at the cell surface\textsuperscript{31-34}. Furthermore, the transporters are thought to exist as homo-oligomeric complexes\textsuperscript{35-42}.

1.1.2.a **Crystal structure of LeuT**

The tertiary structure of SLC6 transporters has to date not been determined. However, a major breakthrough in the understanding of the architecture and transport mechanism of these proteins came in 2005 with the crystallization of the prokaryotic homologue, LeuT, an amino acid transporter from the thermophilic bacteria *Aquifex aeolicus*\textsuperscript{43}. The first crystal structure was in complex with the substrate Leu and two Na\textsuperscript{+} ions, and besides confirming the 12 TM membrane topology, it provided several novel insights into to the structure of this class of proteins.

The 12 TMs are entirely $\alpha$-helical except from short unwound segments in TM1 and TM6, and they pack as an intertwined helical bundle into a cylindrical shape that can be divided into an interior and an outer part. The inner ring is formed almost exclusively by TM1, TM3, TM6 and TM8 and holds the central substrate binding site (S1) where the substrate and the two Na\textsuperscript{+} ions are accommodated (Figure 1.3).
Figure 1.3. Topology and X-ray structure of LeuT

A. Topology of LeuT with the inner ring (TM1, TM3, TM6 and TM8) that forms the substrate binding site shown in blue. The red triangles highlight the inverted repeats, formed by TM1 to TM5 and TM6 to TM10, that are related by an apparent two-fold symmetry. Also shown are the substrate (yellow sphere) and the two bound Na⁺ ions (magenta spheres). B. Structure of LeuT in the substrate bound outward-facing occluded conformation (PDB 2A65) seen from side view. The substrate binding site is located at the core of the transporter and the substrate and two Na⁺ ions are shown as van der Waals spheres in yellow and magenta, respectively. TMs that form the inner ring are highlighted in blue.

The overall structure of LeuT is asymmetric, but a pseudo-two-fold axis of symmetry is found in the protein within TM1-10. This arises from the two V-shaped 5-helix bundles containing TM1-TM5 and TM6-TM10, respectively, which are related by rotational symmetry around an axis through the center of the membrane plane. The fold of this structural motif has subsequently been identified in structures of other secondary active transporters with no sequence relationship to LeuT or SLC6 transporters, which suggests that a larger family of transporters operate via a conserved structural mechanism.

Since 2005, several structures of LeuT have been solved in complex with different substrates and various competitive and non-competitive inhibitors. In all but one of the solved structures, the protein exists in a conformation, where the substrate binding pocket is occluded from both the external and internal medium (Figure 1.4). Access to the S1 binding pocket from the extracellular medium is blocked by an interaction network formed by side chains from TM1, TM3, TM6 and TM10, and the aromatic side chains of Tyr108 on TM3 and Phe253 on TM6 form a hydrophobic lid across the top of the pocket (Figure 1.4). Access from the substrate binding pocket towards the intracellular medium is blocked by a much larger protein structure containing a tight network of interactions formed mainly by tight packing of the intracellular halves of TM1, TM6 and TM8 (Figure 1.4). These regions, which prevent access to the substrate from either side of the membrane, have been proposed to act as external and internal gates that undergo a series of conformational changes during the transport cycle. One structure of LeuT in an open-to-out conformation, which is stabilized by binding of the competitive inhibitor Trp, has been solved. In general, structures of the transporter in different conformations are valuable tools in the elucidation of structural changes in the protein that underlie transport mechanism (section 1.1.3).
The S1 pocket is made up of amino acid side chains from all four TMs surrounding the pocket (TM1, TM3, TM6 and TM8) and from backbone amide groups from the unwound regions of TM1 and TM6\(^43\). The pocket can be divided into two regions: a polar region formed exclusively by the unwound regions of TM1 and TM6 that accommodates the \(\alpha\)-amino and \(\alpha\)-carboxylate groups of the amino acid substrates, and a hydrophobic pocket formed by aliphatic side chains from TM1, TM3 and TM6 that accommodates the hydrophobic substrate side chain\(^43\) (Figure 1.4). The \(\alpha\)-carboxylate group of the substrate interacts directly with one of the Na\(^+\) ions, thus providing a structural basis for the tight coupling between transport of substrate and ions. It has been suggested that the extracellular pathway (often referred to as the extracellular vestibule) that forms the solvent-accessible path from the extracellular medium towards the S1 site harbours a second substrate binding site in LeuT, designated the S2 site, and it has been proposed that occupation of the S2 site is required to trigger conformational changes that release substrate from the S1 to the intracellular side\(^55\). While substrate has not been observed in the S2 site in any crystal structure of LeuT, co-crystals with certain LeuT inhibitors or detergents have found these binding in the S2 site and hereby preventing the conformational changes that are necessary for substrate translocation\(^49,51-53\). This might be relevant for the mechanism of action of SLC6 NTT inhibitors.

![Figure 1.4](image)

**Figure 1.4. Cross-sectional illustration of LeuT in the outward-facing occluded conformation**

A. View of the entire protein (PDB 2A65). Leu is located in the substrate binding site (S1) in the core of the transporter and shown as van der Waals spheres in yellow. The extracellular and intracellular gating regions are highlighted with dashed lines, and the water-accessible pathway from the extracellular space towards S1 is denoted with an orange arrow. B. Close-up view of the S1 and S2 regions. The TMs forming the inner ring (TM1, TM3, TM6 and TM8) in addition to TM10, which lines the extracellular vestibule, are shown. The extracellular gate, which is formed by a water-mediated salt bridge between R30 and D404 in addition to the two aromatic residues Y108 and F253, separate the substrate binding site from the extracellular vestibule. C. Close-up view of the substrate binding pocket. TMs forming the substrate binding site (TM1, TM3, TM6 and TM8) are shown as blue helices, the substrates in yellow ball-and-stick representation and Na\(^+\) ions as magenta spheres. Side chains of residues interacting directly with the substrate are shown as grey stick representations and labelled in green.
1.1.2.b Structural aspects of monoamine transporters

Although the overall sequence identity between LeuT and the monoamine transporters is only 20-25%, the core region encompassing the inner ring with the substrate and Na\(^+\) binding site is remarkably more conserved, making the crystal structures of LeuT solid templates for construction of three dimensional homology models of the mammalian SLC6 transporters\(^{43,56}\). Accordingly, LeuT based models of SERT, NET and DAT have been generated and used in computational as well as functional studies of the transporters\(^{12,56-63}\). The high degree of similarity between the core region of LeuT and the monoamine transporters generates models with very similar structure regarding arrangement of the TMs. In contrast, the intracellular and extracellular loops are considerably less conserved and therefore confined with less accuracy in the models\(^{56}\). Additionally, the N- and C-termini are much shorter in LeuT than in the SLC6 transporters, and these cannot be modelled at all.

The S1 site is most likely structurally conserved between LeuT and the mammalian SLC6 transporters. Not only are 7 of the 11 residues in LeuT that interact directly with the substrate (Figure 1.4) conserved across all nine mammalian SLC6 NTTs, experimental evidence has also identified residues in S1 to control substrate affinity in the mammalian transporters\(^{56,64,65}\). Models of 5-HT and DA in the S1 site in SERT and DAT, respectively, have obtained quite similar poses of the substrates\(^{59,60,66}\). The aromatic moieties of the substrates are accommodated in a hydrophobic region of the pocket formed by hydrophobic and aliphatic residues in TM1, TM3 and TM6, similarly to the hydrophobic side chain of Leu in LeuT. The alkylamine side chains of 5-HT and DA both occupy a region equivalent to the polar region in LeuT that accommodates the substrate \(\alpha\)-carboxylate group. An important difference in S1 is the presence of an Asp (Asp98 in SERT) instead of the corresponding Gly24 in LeuT. An acidic side chain in this position is required for function of the monoamine transporters, and both biochemical studies and docking models of substrates into SERT and DAT suggest that the acidic side chain interacts directly with the amino group of the substrate\(^{12,59,66-68}\). Additionally, as the monoamine substrates lack a negatively charged carboxylate group, the unique presence of an Asp residue in this position is believed to compensate for the inability of the monoamine substrates to coordinate with the Na\(^+\) ion that coordinates directly to the \(\alpha\)-carboxylate group of Leu in the LeuT structures\(^{12,59,61,66,69,70}\).

1.1.3 Transport mechanism

Translocation of substrate and ions through SCL6 transporters is believed to occur via the “alternating access” mechanism. The model was put forward by Jarretzky in 1966, and implies that the transporter adopts at least three conformational states, which seal of access for the substrate to either the intracellular or extracellular environment through a conformation where access is blocked from both sides\(^{71}\). Binding of substrate and ions to an outward-facing conformation triggers structural rearrangement in the protein ultimately leading to a conformation where substrate and ions have access to the interior and are released. Subsequently, the transporter switches back to an outward-facing conformation and a new transport cycle
can begin (Figure 1.5). As mentioned earlier, transport of substrate is strictly coupled to transport of Na⁺, which provides the energy required for the thermodynamically unfavorable substrate transport. Additional ions are required for the function of the monoamine transporters, but the nature and stoichiometry of these are not shared by SERT, NET and DAT. NET and DAT are believed to translocate two Na⁺ and one Cl⁻ per substrate molecule, whereas SERT translocates one Na⁺ and one Cl⁻ while performing antiport of one K⁺ (Figure 1.5). In addition, SERT has been proposed to perform antiport of H⁺. Although SERT translocates only one Na⁺, it is feasible that a second Na⁺ binding site exists, although the role of occupation of this site for substrate binding and transport remains to be elucidated. The role of Cl⁻ for the function of the monoamine transporters has recently been questioned. The consensus has been that Cl⁻ is translocated along with Na⁺ and the substrate, but a recent study on DAT found that both intracellular and extracellular Cl⁻ facilitates transport turnover, which suggests that Cl⁻ plays a role in the return of the transporter to an outward-facing conformation following substrate release.

**Figure 1.5. Alternating access mechanism of monoamine transporters**

Schematic representation of the conformational states that the transporters have to shuttle between in order to move substrate from the extracellular space to the cytoplasm. Binding of substrates and ions to the core of the protein in the outward-facing open conformation induces a series of conformational changes, which involve closure of access to the extracellular medium and subsequently opening of access to the cytoplasm. From the inward-facing open conformation, substrates and ions are released to the inside of the cell, and conformational changes return the transporter to an outward-facing conformation ready for a new transport cycle. Shown is the ion stoichiometry for SERT. NET and DAT co-transport two Na⁺ ions per substrate molecule and do not perform counter-transport of K⁺. Also indicated in the figure are members of the prokaryotic “inverted repeat superfamily” of transporters, for which crystal structures in the given conformational state currently exist (labeled on each conformation in blue ovals). Mhp1, the sodium-benzylhydantoin transporter; AdiC, the arginine-agmatine antiporter; BetP, the sodium-betaine symporter; vSGLT, the sodium-galactose symporter; ApcT, the H⁺-coupled amino acid symporter; CaiT, the carnitine-butyrobetaine antiporter.
Prior to crystallization of LeuT, structural rearrangements of the transporter protein during the translocation cycle was mainly studied by the substituted cysteine accessibility method (SCAM), and numerous residues in extra- and intracellular loops and TM regions were identified, which undergo rearrangement during transport\textsuperscript{19,80}. However, it was not until the emergence of the crystal structures that the mechanism of neurotransmitter transport was put into a structural perspective. The currently available LeuT structures represent rather similar outward-facing or outward-facing occluded conformations with no available inward-facing conformation. In the recent years, however, a number of prokaryotic transporters have been crystallized, which have the same key structural features of the transporter core with the inverted repeat architecture, although they are not sequentially homologous to the SLC6 transporters. They are believed to operate via a highly conserved mechanism, which allows the use of these as templates for models of the molecular events underlying transport in the mammalian NTTs\textsuperscript{81-83}. These structures provide “snapshots” of different transporter conformations captured along what appears to be a transport cycle consistent with an alternating access mechanism. Thus, structures of transporters from this “inverted repeat superfamily” now exist in outward-facing open, outward-facing occluded, fully occluded, inward-facing occluded and inward-facing open conformations\textsuperscript{43-49,84-86} (Figure 1.5). Several proposals have been made for the principal movements of individual elements in the transporter core during the outward-to-inward transition. In one model, the unwound segments in the middle of TM1 and TM6 act as flexible “hinges”, around which the extra- and intracellular halves of TM1 and TM6 can move independently in sequential transitions to open and close the extra- and intracellular pathways\textsuperscript{43,49,81}. Another model has proposed that the helix bundle formed by TM1 and TM6 together with TM2 and TM7 constitute a rigid body that by a single “rocking” motion can account for the transition between the outward- and inward-facing conformations\textsuperscript{61,87}.

Using molecular dynamics (MD) simulations, changes in the interaction network that form the extra- and intracellular gates in LeuT have been studied. A study followed the formation of the proposed extracellular gate in LeuT, which is suggested to be a key event during the transition from the outward-facing open to the outward-facing occluded conformation\textsuperscript{88}. Upon movement from the extracellular vestibule towards the S1, Leu slides through the space between Arg30 and Asp404, which concurrently form a water-mediated salt bridge, and upon movement of substrate deeper into the S1 binding pocket, the aromatic lid is formed by Tyr108 and Phe253\textsuperscript{88} (Figure 1.4 and Figure 1.6). The residues in LeuT that form the extracellular gate are conserved across all SLC6 NTTs and have also been shown to be critical for monoamine transporter function\textsuperscript{59,67,89}. Also, MD simulation on a model of DAT was used to study the effect of disrupting the interaction network that is thought to stabilize the SLC6 NTTs in an outward-facing state by tight packing of the intracellular halves of the inner ring helices TM1, TM6 and TM8\textsuperscript{90}. Disruption of this tight network of interactions is predicted to be a major determinant for the transition into the inward-facing state\textsuperscript{43,49}. In the structures of LeuT, this network includes interactions between Arg5, Ser267, Tyr268 and Asp369 (Figure 1.6), all of which are strictly conserved in the SLC6 NTTs.
Figure 1.6. Gating regions in LeuT

**Middle.** Cross-sectional illustration of LeuT (PDB 2A65) in the outward-facing occluded conformation with Leu (shown as yellow van der Waals spheres) located in the substrate binding site (S1). **Left.** Close-up view of the external gate in LeuT in the outward-facing occluded conformation. The external gate is formed by a water-mediated salt bridge between R30 and D404 and the aromatic lid formed by Y108 and F253. The substrate is shown as yellow van der Waals spheres. **Right.** Close-up view of the internal gate in LeuT formed by an ionic network between R5, S267, Y268 and D369.

Recently, a study achieving real-time monitoring of protein dynamics of the intracellular gating region of LeuT was reported\(^9\). Using single-molecule FRET\(^9\), it was found that movement of TM1a is associated with intracellular gating in LeuT, and that this movement is regulated by substrate and inhibitor binding\(^9\). The same group has recently proposed a model for the entire substrate translocation pathway of both LeuT and DAT\(^5,93\). In this model, binding of a second substrate molecule in S2 is required to trigger the transition from the outward-facing occluded state to the inward-facing state.

Although the above mentioned models provide insight into structural dynamics of the mammalian NTTs, much still needs to be elucidated regarding the transport mechanism for monoamine and other SLC6 transporters.

### 1.2 Pharmacology of monoamine transporters

The central role of the monoamine transporters in regulation of neurotransmission makes them attractive targets for pharmacological manipulation of neural activity, both in studies of basal neurophysiology as well as in the treatment of CNS diseases, and more than 30 drugs targeting the monoamine transporters are in current clinical use\(^21\). Additionally, some of the most widely used drugs of abuse, such as cocaine, amphetamine and ecstasy have SERT, NET and DAT as their primary targets\(^19,21,94\).

#### 1.2.1 Antidepressants: inhibitors of monoamine transporters

The discovery of monoamine transporters as important drug targets for treatment of mood disorders was made more than four decades ago, and extensive drug discovery efforts in the area have provided a plethora of compounds, which are now used to treat major depressive disorder (MDD). The first generation of monoamine transporter inhibitors were the tricyclic antidepressants (TCAs), which were developed in the 1950’s with imipramine as the first drug on the market\(^21,22\). However, in addition to
targeting the monoamine transporters, the TCAs display activity across a variety of different receptors\textsuperscript{95-97}, which associates these drugs with a number of severe side effects. Subsequently, new generations of monoamine transporter inhibitors with little or no affinity for other proteins have been developed. These inhibitors are classified according to their selectivity profile, comprising the selective serotonin reuptake inhibitors (SSRIs), which today are the most prescribed class of antidepressants\textsuperscript{98}, prominent examples being citalopram, fluoxetine and paroxetine; selective norepinephrine reuptake inhibitors (NRIs), such as reboxetine and atomoxetine; and the selective dopamine reuptake inhibitor (DRI), bupropion. Dual-acting inhibitors with affinity for two of the three monoamine transporters include the classes of serotonin and norepinephrine reuptake inhibitors (SNRIs), such as duloxetine and desvenlafaxine, and the NET/DAT inhibitor, nomifensine. Prototypes of drugs from different classes are displayed in Figure 1.7.

**Figure 1.7. Structures of antidepressant drugs**
Chemical structures of prototypical antidepressants belonging to different drug classes.

Although the immediate effect of the marketed monoamine transporter inhibitors is increased concentration of extracellular monoamines in the brain\textsuperscript{99}, the onset of the therapeutic effect occurs only after weeks of treatment, suggesting that they work through slower, adaptive changes in the neurotransmitter systems\textsuperscript{100}. Novel treatment strategies are currently being pursued in order to overcome the shortcomings of the current antidepressant treatment. These include development of triple action inhibitors that target all three monoamine transporters\textsuperscript{101} and a so-called SRI+ drug, which apart from inhibiting SERT also acts on 5-HT\textsubscript{1A} and 5-HT\textsubscript{3} receptors\textsuperscript{102}. However, these types of drugs have yet to reach the market.

Despite the fact that antidepressant drug therapy has existed and been developed for more than 50 years, the drawbacks, such as late onset of action, frequent side effects, the relatively high percentage of non-responders (~30\%) and the fact that many responders relapse, underlie that there is still room for major improvements\textsuperscript{22,100,104}, especially when considering that the lifetime prevalence of MDD is estimated to 15-20\%\textsuperscript{22,105,106}. A deeper understanding of the disease biology of depression, including the role and
regulation of monoamine transporters in sick and healthy conditions, as well as a better understanding of how the current antidepressant drugs act, would greatly aid in the development of new, improved therapeutic regimens for treatment of MDD.

1.2.2 Drug binding sites in monoamine transporters

Prior to the structure of LeuT, insight into interactions of antidepressant drugs with the monoamine transporters had been provided by investigation of pharmacological properties of the drugs and mutagenesis studies. However, the absence of structural data on the monoamine transporter proteins hampered the understanding of the structural and mechanistic details underlying the actions of the drugs. Major advances are made in the present years, where homology models of the monoamine transporter proteins derived from the LeuT crystal structures are allowing structural interpretation of functional data on drug modulation. As previously mentioned, two binding sites have so far been identified in the crystal structures of inhibitor-bound LeuT: the centrally located substrate binding pocket S1 and the extracellular vestibule, which contains the putative S2 binding site. The equivalent regions in the monoamine transporters are likely candidates for harbouring ligand binding sites. Indeed, re-evaluation of extensive mutational analysis shows that the majority of residues that are critical for recognition of inhibitors are located in the TMs and loop regions that contribute to the formation of the extracellular permeation pathway and the substrate binding pocket. There is still, however, a significant proportion of the residues that by mutagenesis studies have been suggested to interact directly with transporter ligands, which are not located within the putative binding pockets. This indicates that these residues are more likely to influence ligand binding in an indirect manner; an effect that is difficult to differentiate from a direct interaction when conducting mutagenesis studies. Pharmacological studies of numerous inhibitors belonging to the TCAs, SSRIs and NRIs have shown that these act competitively on SERT, being displaced by substrate in a concentration-dependent manner. The central substrate binding pocket is an obvious candidate site for binding of competitive inhibitors, as inhibitor binding sites overlapping with the S1 site offers a straightforward structural mechanism for competitive inhibition. In support of this, mutagenesis studies have shown that the sensitivity towards a wide range of competitive inhibitors, including fluoxetine, paroxetine, citalopram and imipramine can be decreased by several orders of magnitude by mutation of S1 residues. Additionally, LeuT structures have been used as templates for construction of SERT models in complex with imipramine, (S)-citalopram and fluoxetine as well as a DAT model in complex with cocaine. These models have revealed a common structural model for competitive inhibition: inhibitors compete with substrate for binding in the S1 site and thereby trap the transporter in an outward-facing occluded or outward-facing open conformational state. The inhibitors are accommodated within the inner ring helices TM1, TM3, TM6 and TM8, and the amino group found in virtually all SERT, NET and DAT inhibitors is coordinated by the Asp (Asp98 in SERT) located in the helical break of TM1, which, as previously mentioned, interacts directly with the substrate.
The structures of LeuT co-crystallized with TCAs and SSRIs\textsuperscript{50-52} revealed that these compounds can bind in the extracellular S2 vestibule of LeuT, suggesting that the equivalent vestibule in the monoamine transporters may also accommodate inhibitors\textsuperscript{51,52}. However, it should be noted that in contrast to their action on monoamine transporters, TCAs and SSRIs interact non-competitively with LeuT and require micromolar concentration for inhibition of transport. This suggests that these findings from the LeuT structures cannot readily be transmitted to the mammalian transporters. A role, however, for a second binding site in the vestibule of the monoamine transporters might exist. Several studies have found that TCAs and SSRIs, including imipramine, paroxetine, citalopram and sertraline, also bind to a low-affinity allosteric site in SERT, and that binding to the low-affinity site distorts the dissociation kinetics of the inhibitor bound to the high-affinity site\textsuperscript{120-127}. Although studied to a lesser degree, NET and DAT have also been suggested to harbour a low-affinity allosteric binding site\textsuperscript{128}. It is tempting to speculate that the allosteric site in the monoamine transporters could be situated in the S2 vestibule outside the primary S1 binding pocket.

While several studies have investigated the interactions of antidepressants with SERT, less information exist about how these drugs interact with NET. A couple of studies of interactions of the TCAs nortriptyline and desipramine with NET have identified residues scattered across several TMs (TM2, TM5, TM6, TM7, TM8 and TM10) as important for potency of the drugs, many of which are outside putative binding pockets\textsuperscript{14,62,129-131}. To date, no homology model of NET in complex with antidepressants has been constructed, which limits the structural understanding of the binding pocket(s) for antidepressant drugs in NET.

In summary, although evidence exist that the high-affinity binding sites for antidepressants in monoamine transporters overlap with the S1 substrate binding site, for the majority of the drug compounds, limited information about their binding mode is available. The knowledge about why some antidepressant drugs show selectivity to one transporter or the other is scarce, and the structural parameters in SERT and NET as well as in the compounds that determine selectivity are largely unknown. Such information would aid in the understanding of the action of current drugs as well as in the development of novel compounds targeting the monoamine transporters.

1.3 Regulation of monoamine transporters

The important role of SERT, NET and DAT for neurotransmitter availability in the CNS means that posttranslational processes that dynamically regulate transporter surface density, localization and intrinsic transport capacity are critical for spatiotemporal regulation of monoaminergic signalling. Numerous studies have shown that the activity of monoamine transporters is subject to dynamic modulation by a range of cellular processes; transporter phosphorylation and protein-protein interactions are discussed below.
1.3.1 Phosphorylation

Analysis of the amino acid sequences of the SERT, NET and DAT proteins reveals multiple consensus sites for protein kinases\textsuperscript{132-135}, and numerous studies have shown that the monoamine transporters are phosphoproteins, which undergo regulation by phosphorylation. This provides a mechanism for rapid modulation of transmitter clearance in response to changing physiological demands. Phosphorylation of the transporter protein might change the intrinsic transport activity (trafficking-independent regulation) or change plasma membrane insertion or sequestration via exocytotic/endocytotic mechanisms and/or interaction with other proteins (trafficking-dependent regulation). Studies of direct transporter phosphorylation, which have resulted in localization of phosphorylation, are summarized in Table 1.1, and a schematic overview of phosphorylation- and protein-protein interaction-dependent SERT regulation is illustrated in Figure 1.8.

Table 1.1. Phosphorylation of monoamine transporters

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Kinase</th>
<th>Phosphorylation site/region</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERT</td>
<td>PKA\textsuperscript{a}</td>
<td>N- and C-termini</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>PKC\textsuperscript{a}</td>
<td>N- and C-termini</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>PKG\textsuperscript{b}</td>
<td>Thr276 (IL2)</td>
<td>137</td>
</tr>
<tr>
<td>NET</td>
<td>PKC\textsuperscript{a}</td>
<td>Thr258-Ser259 (IL2)</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>CaMKII</td>
<td>C-terminus</td>
<td>139</td>
</tr>
<tr>
<td>DAT</td>
<td>PKC\textsuperscript{a}</td>
<td>N-terminal Ser cluster (Ser2, Ser4, Ser7, Ser12, Ser13)</td>
<td>140-144</td>
</tr>
<tr>
<td></td>
<td>PKC\textsuperscript{b}</td>
<td>Ser262 (IL2), Ser586, Thr 613 (C-terminus)</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>CaMKII</td>
<td>N-terminus</td>
<td>144,145</td>
</tr>
<tr>
<td></td>
<td>ERK1/2\textsuperscript{a}</td>
<td>Thr53 (N-terminus)\textsuperscript{d}</td>
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</tr>
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<td>JNK\textsuperscript{a}</td>
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<td>p38 MAPK\textsuperscript{a}</td>
<td>Thr53 (N-terminus)\textsuperscript{d}</td>
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<td></td>
<td>Cdk5\textsuperscript{c}</td>
<td>N-terminus</td>
<td>144</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Phosphorylation of functional transporter and \textit{in vitro} phosphorylation. \textsuperscript{b} Putative; direct transporter phosphorylation by the kinase not confirmed. \textsuperscript{c} \textit{In vitro} phosphorylation only. \textsuperscript{d} Experiments performed with rDAT, the corresponding residue in hDAT is a Ser.

1.3.1.a PKC-mediated phosphorylation

Protein kinase C (PKC) is the most extensively studied kinase with a profound role in regulating the phosphorylation state and activity level of the monoamine transporters. In 1989 it was shown that 5-HT uptake in cultured endothelial cells decreases upon treatment with the PKC activator 4-\textit{β}-phorbol 12-myristate 13-acetate (\textit{β}-PMA), an effect that could be reversed in a concentration-dependent manner by
pretreatment of cells with the PKC inhibitor staurosporine\textsuperscript{146}. Since then, numerous studies on transfected cells as well as native cells or tissue have shown that PKC activation increases the phosphorylation level in SERT\textsuperscript{136,147-149}, NET\textsuperscript{138,150} and DAT\textsuperscript{135,141-143,151-154}. The general consequence of PKC activation is decreased transport capacity (reduced $V_{\text{max}}$) due to redistribution of the transporters from the surface to an intracellular compartment. Additionally, an initial rapid trafficking-independent inactivation of the transporters might occur at the plasma membrane as suggested for SERT\textsuperscript{148} and DAT\textsuperscript{155}.

In DAT, the N-terminal domain, which contains a cluster of five closely spaced Ser residues within the first 21 N-terminal residues, has been identified as a region for the majority of both basal and PKC-stimulated phosphorylation in native and recombinant cells\textsuperscript{140,142}. \textit{In vitro} phosphorylation experiments with the DAT N-terminus have also shown that this domain is a PKC\textsubscript{α} substrate\textsuperscript{144,145}. Although one study indicated loss of PKC-stimulated phosphorylation after mutation of only Ser7 in the N-terminal domain of DAT\textsuperscript{143}, other studies have found residual basal and PKC-stimulated phosphorylation following mutation of each of the residues in the Ser cluster\textsuperscript{156}. Surprisingly, despite the fact that PKC stimulation markedly increases DAT N-terminal phosphorylation at the same time as mediating endocytosis of DAT, the link between these two events has been questioned by the observation that truncation of the DAT N-terminus almost abolished detectable phosphorylation without blunting PKC-mediated down-regulation\textsuperscript{140,142}. These findings suggest that PKC-mediated down-regulation of DAT does not involve direct transporter phosphorylation. N-terminal phosphorylation of DAT appears instead to be essential for amphetamine-induced reverse transport (efflux) of DA\textsuperscript{157}. Another study has suggested Ser262 in IL2 and Ser586 and Thr613 in the C-terminus of DAT as PKC phosphoacceptor sites, as mutation of these residues abolished PKC-stimulated DAT phosphorylation\textsuperscript{135}. This study also suggested PKC-mediated DAT phosphorylation and endocytosis to be independent events. DAT Ser262 is part of a PKC consensus site\textsuperscript{158,159}, which is conserved across the monoamine transporters, and the corresponding residue in NET (Ser259) has been identified as a phosphoacceptor site for PKC-mediated phosphorylation\textsuperscript{138}. This study showed that β-PMA-induced phosphorylation of NET occurs on both Ser and Thr residues, and mutation of Thr258 and Ser259 to Ala significantly reduced transporter phosphorylation and prevented β-PMA-induced decrease in NE uptake and NET internalization. Although \textit{in vitro} phosphorylation with purified PKC\textsubscript{ε} on membrane preparations containing recombinantly expressed NET suggested Ser259 as the direct site of PKC-mediated phosphorylation, mutation of both Thr258 and Ser259 was required to impair functional down-regulation by the kinase\textsuperscript{138}.

The specific PKC phosphorylation sites in SERT are not well-characterized. \textit{In vitro} phosphorylation by PKC has been reported on both N- and C-termini of SERT\textsuperscript{160}, but no specific residues were identified. A study in platelets demonstrated that PKC-stimulated phosphorylation occurs at both Ser and Thr residues and suggested a biphasic effect of PKC on SERT encompassing an initial phase where decrease in 5-HT transport is accompanied by Ser phosphorylation and a subsequent phase where SERT internalization is accompanied by Thr phosphorylation\textsuperscript{148}. PKC-mediated phosphorylation of SERT appears to be
attenuated by substrates, which has been suggested to provide an adaptive feedback mechanism for maintaining the transporter at the cell surface during periods of high transport demand\textsuperscript{147}. This effect can be blocked by SERT antagonists such as cocaine and antidepressants\textsuperscript{147}. Although one study of SERT expressed in COS7 cells failed to show increased transporter phosphorylation after PKC stimulation\textsuperscript{161}, other studies of PKC-linked down-regulation of SERT activity using $^{32}$P incorporation in transfected cells, rat platelets and synaptosomes have demonstrated increased SERT phosphorylation after PKC activation\textsuperscript{136,147-149} that correlates with the decrease in 5-HT transport\textsuperscript{136}. However, a direct link between PKC-mediated SERT phosphorylation and transport down-regulation has not been demonstrated. In addition to the impact on SERT transport kinetics, PKC has been found to modulate protein-protein interactions between SERT and intracellular proteins, including the LIM domain adaptor protein Hic-5\textsuperscript{162}, protein phosphatase 2A (PP2A)\textsuperscript{149,163} and the vesicle fusion protein syntaxin1A\textsuperscript{149}. Similarly to DAT, amphetamine-induced substrate efflux through SERT is dependent on PKC activity\textsuperscript{164}.

1.3.1.b Non-PKC-mediated phosphorylation

While PKC activation results in reduced transport capacity of substrate, other kinases have opposite effects on the monoamine transporters. Numerous studies of SERT from native sources or transfected cells show increased 5-HT uptake upon protein kinase G (PKG) activation. The PKG-mediated effect is seen after activation of different pathways, including activation of the adenosine receptor A\textsubscript{3}\textsuperscript{134,165}, histamine receptors\textsuperscript{166}, nitric oxide (NO)\textsuperscript{167} as well as by treatment with cyclic GMP analogues\textsuperscript{134,137,165,168}. PKG\textsubscript{IIa} has been suggested as the responsible isoform as it co-localizes with SERT in rat serotonergic rape neurons and co-immunoprecipitates with SERT from transfected cells\textsuperscript{169}. The mechanism for PKG-mediated SERT stimulation remains controversial. While some studies report increased surface levels of SERT\textsuperscript{165,168}, others demonstrate trafficking-independent up-regulation of SERT by PKG\textsuperscript{134,137,167}. Only recently has evidence for direct PKG-mediated SERT phosphorylation been provided\textsuperscript{137}. It was shown that mutation of Thr276 to Ala abolished cGMP-mediated stimulation of 5-HT transport and SERT phosphorylation, which thereby provided the first indication of a specific phosphorylation site in SERT.

Both SERT and DAT are subject to regulation by mitogen-activated protein kinase (MAPK) pathways. Inhibition of p38MAPK decreases SERT phosphorylation and cell surface levels, while co-expression of SERT with the constitutively active MAP kinase kinase 3b(E) (M KK3b(E)) upstream of p38MAPK increases transport capacity\textsuperscript{149,165,170-172}. As for PKG, the degree to which p38MAPK regulates SERT surface expression is controversial and evidence exist for both trafficking-dependent\textsuperscript{149} and -independent\textsuperscript{165} mechanisms. A study suggested that p38MAPK activation occurs via PKG stimulation, resulting in intrinsic activation of SERT, whereas a separate PKG-mediated, p38MAPK-independent mechanism enhances SERT surface density\textsuperscript{165}. For DAT, increased DA transport was observed in cells co-expressing DAT and the ERK1/2 (MAPK) activator MEK\textsuperscript{173,174}, and decreased DAT phosphorylation and activity was correlated with inhibition of MEK\textsuperscript{143}. In support of direct phosphorylation of DAT by MAPKs, a recent in vitro phosphorylation study demonstrated that the MAPKs ERK1/2, JNK and
p38MAPK can phosphorylate Thr53 in the isolated DAT N-terminus. This residue in DAT has subsequently been identified as an in vivo phosphoacceptor in a proteomics study of whole mouse brain proteins. However, the role of Thr53 phosphorylation in ERK-mediated regulation of DAT activity is questionable, as mutation of Thr53 has no influence on the decrease in DAT activity observed upon ERK inhibition. Furthermore, DA receptor-mediated ERK1/2-dependent DAT up-regulation is maintained upon truncation of the first 55 N-terminal amino acids of DAT.

In addition to MAPK-mediated phosphorylation, an in vitro phosphorylation study also demonstrated significant Ser phosphorylation on the N-terminus of DAT by protein kinase A and B (PKA and Akt, respectively), PKG, Ca\(^{2+}\)/calmodulin-dependent protein kinase II \(\alpha\) (CaMKII\(\alpha\)), casein kinase II (CKII) and cyclin-dependent kinase 5 (cdk5). However, the identity of the specific phosphoacceptor residues within the N-terminus as well as potential functional effects of phosphorylation were not investigated further in this study. PKA-mediated in vitro phosphorylation has also been reported at the isolated N- and C-terminal domains of SERT and stimulation of PKA activity increases SERT phosphorylation in transfected cells, but with no effect on 5-HT uptake. In contrast, a study of 5-HT uptake in cortical synaptosomes showed increased transport activity upon PKA stimulation. Likewise, PKA-dependent up-regulation of striatal DAT has been reported without investigation of changes in transporter phosphorylation level.

CaMKII is proposed to be involved in phosphorylation of all three monoamine transporters, but with very different functional outcomes. For DAT, in vitro phosphorylation studies showed that CaMKII phosphorylates the transporter within the first 27 amino acids of the N-terminus, a crucial event during CaMKII-stimulated amphetamine-mediated DA efflux. For NET, CaMKII has been shown to phosphorylate a peptide corresponding to a segment of the NET C-terminus in vitro and stimulation of CaMKII activity in PC12 cells endogenously expressing NET correlates with enhanced NET transport activity. For SERT, CaMKII activity has been found to regulate the electrophysiological properties of the transporter by modulating the interaction of SERT with syntaxin1A. This effect could be abolished by mutating Ser13 located in the SERT N-terminus, and this residue was suggested as a CaMKII phosphorylation site, although no direct phosphorylation was established.

Little is known about Tyr phosphorylation of the monoamine transporters. A single study on SERT has suggested 5-HT uptake in platelets to be positively correlated with Src-mediated Tyr phosphorylation of the transporter.
Figure 1.8. Kinase-linked regulation of SERT
Schematic representation of possible elements of SERT regulation involving kinases and interacting proteins (based on previous reports). Transport activity can be regulated through trafficking-dependent and/or -independent mechanisms, i.e. involving changes in cell surface levels and/or transporter catalytic function (intrinsic activity). Syn1A, syntaxin1A; PP2A, protein phosphatase 2A; nNOS, neuronal nitric oxide synthase.

1.3.1.c Transporter dephosphorylation
Protein phosphatases generally play a significant role in regulation of monoamine transporter phosphorylation, and tonic phosphatase activity has been suggested to maintain the transporters in a relatively dephosphorylated state. Phosphorylation levels of all transporters increase upon inhibition of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) and phosphatase inhibition correlates with down-regulation of transport activity. The catalytic subunit of PP2A has been found to co-immunoprecipitate with all three transporters from native preparations. For SERT and NET, the association with PP2A can be reduced by treatment with PKC activators as well as PP1/2A inhibitors, which suggests that PP2A physically associates with SERT and NET to maintain the transporters in a dephosphorylated state, and that increased transporter phosphorylation occurs upon disruption of the complex. Inhibitors of p38MAPK can also reduce the association of SERT with PP2A. Combined with the observation that both cGMP- and p38MAPK-mediated up-regulation of SERT catalytic activity is PP1/2A-dependent, this indicates that PP2A is involved in a complex regulatory mechanism for SERT. In vitro dephosphorylation studies of DAT have demonstrated that
PP1 can dephosphorylate both PKC-stimulated metabolically phosphorylated rDAT and in vitro phosphorylated DAT N-terminus\textsuperscript{144,182}.

In conclusion, despite unequivocal evidence for the importance of kinase activity in regulatory processes of monoamine transporter activity, the number of identified specific phosphorylation sites in the monoamine transporters is limited, which hampers the understanding of the underlying molecular mechanisms. Detailed knowledge about the mechanisms for transporter regulation might advance the understanding of monoamine-related patophysiological conditions and potentially aid in development of new therapeutic regimens for these.

1.3.2 Protein-protein interactions

1.3.2.a Transporter oligomerization

SLC6 transporters are known to exist as constitutive oligomers at the plasma membrane, and oligomerization is a prerequisite for proper transporter trafficking\textsuperscript{185,186}. Different structural motifs for transporter oligomerization have been suggested. TM6 of SERT, NET and DAT contains a conserved GxxxxG sequence, which previously has been characterized as a dimerization motif\textsuperscript{187,188}, and mutation of either of the Gly residues within this sequence in DAT highly impaired expression of the transporter at the cell surface\textsuperscript{40}. Additionally, DAT could be crosslinked at the extracellular end of TM6, supporting the role of this TM in oligomer formation\textsuperscript{40,189}. A TM4-TM4 interface in DAT has also been suggested based on crosslinking studies supporting (at least) a tetrameric transporter arrangement\textsuperscript{189}. Another oligomerization motif, a so called Leu zipper consisting of four Leu residues periodically spaced by six amino acids\textsuperscript{190,191}, is found in TM2 of the monoamine transporters. Although the Leu zipper is incomplete in SERT and DAT with one of the Leu residues replaced by another amino acid, the motif was found to be required for DAT oligomerization and trafficking\textsuperscript{42}. Likewise, the closely related GAT1 was found unable to undergo normal intermolecular association upon mutation of the Leu zipper motif\textsuperscript{192}. For SERT, an intermolecular interaction site has been identified within TM11/TM12, which potentially depends on a GxxxxG motif in TM12 not conserved in the other monoamine transporters\textsuperscript{185,193}.

It has so far been difficult to put the obtained information about transporter oligomerization into a structural perspective. In the crystal structure, LeuT is found as a dimer with the dimeric interface formed by TM9 and TM12\textsuperscript{43}. Although this is in accordance with the role of TM11/TM12 in SERT oligomerization, it should be noted that the sequences of these TMs are completely divergent. TM2 and TM6 are found in the centre of the LeuT protein, which is difficult to reconcile with the suggested role of the Leu zipper and the GxxxxG motif in the oligomerization of mammalian transporters\textsuperscript{20,43}. Further experimental efforts are required to assess these apparent discrepancies.
1.3.2.b Interactions with other proteins

The monoamine transporters are known to interact with several proteins, such as intracellular scaffolding, cytoskeletal, anchoring and signaling proteins, including other membrane proteins. Many of these interactions seem critical for regulation of transporter function, including cellular trafficking, catalytic properties and the action of drugs targeting the transporters. Protein interaction partners for SERT, NET, and DAT are listed in Table 1.2 and partners with known interaction sites in the N- and C-termini of the transporters are displayed in Figure 1.9.

N-termini

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
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<td>SERT</td>
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</tr>
<tr>
<td>DAT</td>
<td>MLLAAMNPFVQPSNIGATDSFQHPLIRAKTETLVLKRTGLK75</td>
</tr>
<tr>
<td>NET</td>
<td>MLKHKANQPSNIGATDSFQHPLIRAKTETLVLKRTGLK71</td>
</tr>
</tbody>
</table>

C-termini

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERT</td>
<td>KFLSTQCSWLEMAYITPEHENHVLQDRISOPOQLQKAIPEG127</td>
</tr>
<tr>
<td>DAT</td>
<td>KFLSTQCSWLEMAYITPEHENHVLQDRISOPOQLQKAIPEG127</td>
</tr>
<tr>
<td>NET</td>
<td>KFLSTQCSWLEMAYITPEHENHVLQDRISOPOQLQKAIPEG127</td>
</tr>
</tbody>
</table>

Figure 1.9. N- and C-terminal sequences for the human monoamine transporters

Sequences for N- and C-termini of SERT, DAT and NET with indication of protein interaction partners for which the interaction motifs in the transporters are known. Numbering indicate the last amino acid of the N-terminus and the first amino acid of the C-terminus, respectively. Boxes indicate sites for protein interaction partners. UniProt-SwissProt human accession numbers: SERT, P31645; DAT, Q01959; NET, P23975. a Quick et al. 2003196, b Lee et al. 2007197, c Sucic et al. 2011198, d Chanrion et al. 2007199, e Carneiro et al. 2002200, f Fog et al. 2006145, g Torres et al. 2001201.

The vesicle fusion protein syntaxin1A interacts with SERT, NET and DAT, and it plays a role in regulating the level of transporters at the cell surface. For SERT and NET, syntaxin1A seems to support surface trafficking and/or aid in maintenance of transporter at the surface level, whereas interaction of syntaxin1A with DAT leads to reduction in transporter surface expression. Additionally, the interaction with syntaxin1A reduces the catalytic activity of NET. For DAT, the interaction site is contained within the first 33 residues of the N-terminus, whereas residues 11-30 in SERT, which harbours five negatively charged amino acids, are crucial for the binding of syntaxin1A. In addition to trafficking, the electrophysiological properties of SERT have been found to be influenced by syntaxin1A. The interaction was shown to abolish the transport-associated current that is a result of an electrogenic non-stoichiometric transport mode as well as the Na⁺ leak current observed in absence of substrate, resulting in an electroneutral transport mode in which substrate and ion transport is strictly coupled. The interaction between both SERT and DAT and syntaxin1A has been shown to be positively modulated by CaMKII. For SERT, mutation of Ser13 in the N-terminus impaired the effects of CaMKII, and it
was hypothesized that CaMKII phosphorylates SERT on Ser13, although no phosphorylation studies were undertaken. For DAT, the C-terminus interacts directly with CaMKII and the interaction was suggested to facilitate CaMKII-mediated phosphorylation of N-terminal Ser residues, which in turn stimulates amphetamine-induced DA efflux. This mechanism possibly involves interaction between DAT and syntaxin1A, which is strengthened by activation of CaMKII.

Despite the lack of sequence similarity in the N- and C-termini of the monoamine transporters, they all contain putative PDZ binding sequences in their extreme C-termini. A common role for PDZ domain-containing proteins is regulated targeting and trafficking of their interaction partners. The synaptic PDZ domain-containing protein PICK1 was initially identified as a binding partner to the PDZ recognition motif in the extreme C-termini of DAT and NET. Although the interaction was initially proposed to enhance DAT surface levels, this was later rendered improbable with identification of mutations that could disrupt PDZ domain interaction without affecting surface targeting and vice versa.

The PDZ domain-containing protein neuronal nitric oxide synthase (nNOS) interacts with the PDZ binding motif in the SERT C-terminus. Co-expression of SERT with nNOS decreases SERT cell surface expression, which can be abolished by removing the PDZ binding motif in SERT. Furthermore, 5-HT transport in synaptosomes from nNOS knock-out mice is enhanced compared to wild-type animals, and inhibition of the SERT/nNOS interaction in wild-type animals increases SERT activity in synaptosomes.

The focal adhesion protein Hic-5 is another scaffolding protein that is found to interact with the C-terminus of DAT, NET and SERT. It was suggested that the interaction of Hic-5 with SERT facilitates SERT internalization and it was linked to the PKC-mediated regulatory pathway, since stimulation of PKC positively modulated the Hic-5/SERT interaction. Likewise, the protein MacMARCKS (homologue of myristoylated alanine-rich C kinase substrate) has been proposed to be involved in PKC-mediated internalization of SERT via interaction with the SERT C-terminus.

The C-terminus of SERT interacts with two components of the COPII coat complex, SEC23A and SEC24C, which are involved in protein export from the endoplasmic reticulum. The RI motif in the C-terminus of SERT is important for the interaction, as mutation of this motif impairs SEC24C-dependent export of SERT. Although this motif is conserved across the SLC6 transporters, the interaction with the SEC24C isoform is exclusive for SERT, whereas DAT and NET are dependent on the SED24D isoform for proper export from the endoplasmatic reticulum, indicating that residues flanking this conserved motif are important for SEC24 isoform selectivity.

Two independent studies have shown that the D2 DA receptor short variant, which is the primary autoinhibitory receptor expressed in presynaptic dopaminergic neurons, interacts with DAT and causes an increase in DAT surface expression; an effect that is dependent on receptor activation as well as downstream ERK1 signaling. A potential coupling between D2 receptors and DAT activity seems
reasonable when considering the shared attenuating role of DAT and D2 receptors in control of dopaminergic transmission.

DAT, NET and SERT all interact with α-synuclein, which is a presynaptic protein that is implicated in the pathogenesis of Parkinson’s disease\textsuperscript{212-216}. While the interaction between both SERT and NET and α-synuclein leads to negative modulation of transporter cell surface availability\textsuperscript{212,213}, both up-regulation\textsuperscript{215,217} and down-regulation\textsuperscript{214} of DAT cell surface levels have been suggested.

Additional proteins interacting with SERT, NET and DAT are listed in Table 1.2. In general, the plethora of proteins so far found to interact with the monoamine transporters clearly implies that transporter function and subcellular distribution is tightly regulated by protein-protein interactions. However, the \textit{in vivo} significance and mechanistic role of most of these interactions is still far from being fully understood, including how the interactions might be regulated in response to cellular stimuli and by posttranslational modifications of the proteins.
Table 1.2. Protein-protein interaction partners for monoamine transporters

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Interaction partner</th>
<th>Site of interaction</th>
<th>References</th>
</tr>
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<td>SERT</td>
<td>SCAMP2</td>
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<td>218</td>
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<td></td>
<td>Syntaxin1A</td>
<td>N-terminus</td>
<td>196,202</td>
</tr>
<tr>
<td></td>
<td>Integrin αIIβ3</td>
<td>C-terminus</td>
<td>219</td>
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<td></td>
<td>Hic-5</td>
<td>C-terminus</td>
<td>162,200</td>
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<td></td>
<td>MacMARKS</td>
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<tr>
<td></td>
<td>nNOS</td>
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<tr>
<td></td>
<td>SEC23A</td>
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<td></td>
<td>SEC24C</td>
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<td>CIPP</td>
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1.4 Objectives

The overall aim of the present PhD study was to obtain further molecular insight into monoamine transporter regulation and interactions with antidepressants. Specifically, the studies aimed at:

a) Identifying specific phosphorylation sites for specific kinases in SERT by application of an \textit{in vitro} phosphorylation approach. To understand the mechanisms behind phosphorylation-dependent transporter regulation, we must first acquire knowledge of the specific residues that are substrate for phosphorylation by specific kinases. At present, such knowledge is scarce (Chapter 2).

b) Elucidating possible functional consequences of SERT phosphorylation at the residues identified \textit{in vitro}, focusing on key transport parameters determining SERT activity, such as $K_m$, $V_{max}$ and cell surface expression. This will potentially provide novel knowledge of phosphorylation-dependent SERT regulation and/or provide further molecular details to the current understanding of regulatory processes (Chapter 3).

c) Establishing a method for purification of SERT from a heterologous expression system and from native tissue, followed by conduction of mass spectrometry-based phosphorylation site analysis of the purified transporter protein. This offers the opportunity of identifying \textit{in vivo} phosphorylation sites in SERT (Chapter 4).

d) Gaining insight into molecular determinants for interactions of different classes of antidepressants with SERT and NET, focusing on effects on the inhibitory potency of mutations in the central substrate binding pocket. Such information will aid the general understanding of how antidepressants interact with the transporters at the molecular level and shed new light on determinants for SERT/NET selectivity of the different classes of compounds (Chapter 5).
2  *In vitro* phosphorylation of intracellular regions in hSERT

2.1 Introduction

The purpose of posttranslational modifications is to increase the diversity of functional groups beyond those in the side chains of the 20-22 proteinogenic amino acids. Posttranslational modifications of proteins by phosphorylation of amino acid side chains is highly evolved in eukaryotic organisms, and there are more than 500 predicted kinases in the human proteome, termed the human kinome. It is estimated that about one-third of the potential 30,000 proteins in the human proteome are substrates for phosphorylation. The side chains most commonly phosphorylated in eukaryotic proteins are Ser, Thr and Tyr. The phosphoryl donor is adenosine triphosphate (ATP), which transfers the $\gamma$-$PO_3^{2-}$ group to the alcohol side chain of the amino acid. Mg$^{2+}$ catalyses the reaction by forming bidentate chelates to two of the anionic phosphate oxygens in ATP, thereby lowering the energy barrier for the phosphoryl transfer (Figure 2.1).

![Figure 2.1. Phosphorylation of serine](image)

The OH-group of serine attacks the $\gamma$-$PO_3^{2-}$ of ATP to form phosphoserine. Mg$^{2+}$ acts as a catalyst.

Some protein kinases phosphorylate only one partner protein, whereas others, such as PKA can phosphorylate Ser and Thr in more than 100 proteins. Some proteins harbour a single phosphorylation site, whereas others, including many of the kinases themselves undergo multisite phosphorylation. Protein phosphorylation is a dynamic process. Although the phosphoserine (pSer/pS), phosphothreonine (pThr/pT) and phosphotyrosine (pTyr/pY) groups are chemically stable at physiological temperature and pH, there are about 150 human protein phosphatases counterbalancing the action of the kinases, altogether determining the level of phosphoprotein present at any time point within a cell. Protein phosphorylation dynamics are crucial in eukaryotic signalling pathways, where kinases acting in cascade ultimately can lead to dramatic amplification of signals, a prominent example being the MAPK cascade. Based on their substrate specificity, protein kinases are divided into two subfamilies: the Ser/Thr kinases that comprise approximately 80% of the mammalian kinase and the Tyr kinases making up the remaining 20%. Tyr phosphorylation is less abundant than phosphorylation of Ser and Thr and the relative abundance of pS:pT:pY has been listed to 1800:200:1. The determinants of kinase
specificity are not fully understood, although it is evident that both the amino acid sequence motif surrounding the Ser/Thr/Tyr residue and the three-dimensional structure of the substrate protein contribute to the specificity. Some kinases have been characterized for catalytic efficiency and consensus specificity\textsuperscript{159,233,234}, and computer based algorithms have been generated that predict phosphorylation sites based on observed phosphorylation events in other proteins\textsuperscript{158,235-239}.

The monoamine transporters SERT, NET and DAT are phosphoproteins and they are regulated by kinase-linked pathways, which change the intrinsic transport activity and/or trafficking of transporter to and from the cell membrane. However, the number of identified phosphorylation sites in these transporters is very limited. The aim of the work in this chapter was to identify phosphorylation sites in SERT.

2.2 Strategy

2.2.1 Strategies for detection of phosphorylation

Traditionally, protein phosphorylation has been detected by transfer of radioactive \[^{32}\text{P}\] from $\gamma$-\[^{32}\text{P}\]ATP to the protein substrate\textsuperscript{227}, and the majority of the phosphorylation studies of the monoamine transporters have been carried out using this technique. Immunoprecipitation with a protein-specific antibody followed by resolution by SDS-PAGE and autoradiographic detection of the metabolically labelled protein allows for detection of phosphorylation. Additionally, hydrolysis of the precipitated protein followed by chromatography and autoradiography allows separation of \[^{32}\text{P}\]pSer, \[^{32}\text{P}\]pThr and \[^{32}\text{P}\]pTyr and thereby identification of the type of residues that are phosphorylated\textsuperscript{229}. In this way, phosphorylation before and after a given stimulus, such as the activation of a specific kinase, can be investigated. A drawback to this technique is that it is difficult to locate phosphorylation at specific residues. Residue localization might be accomplished by subsequent mutagenesis studies; however, this is often hampered by the presence of multiple Ser/Thr/Tyr residues within the protein sequence, of which several might be phosphorylated at the same time or by a certain stimulus. Another limitation is that it is difficult to draw definitive conclusions regarding the identity of the kinase that performs the direct phosphorylation of the protein. This might be circumvented by performing \textit{in vitro} phosphorylation studies of the protein with the anticipated kinase, as has been done for studies of PKC-mediated phosphorylation of NET\textsuperscript{138} and CaMKII-mediated phosphorylation of DAT\textsuperscript{145}.

A recent and rapidly developing approach to phosphorylation site identification is by use of mass spectrometry-based phosphoproteomics\textsuperscript{232}. Typically, proteins to be analyzed (purified or in mixtures) are digested with trypsin and the resulting peptides are analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), which allows for identification of specific phosphorylation sites within a protein. The assignment process can be highly facilitated by automation of data processing with the use of computer algorithms, which also allows for analysis of rather complex protein mixtures\textsuperscript{240}. In recent years, phosphoproteomic studies have provided a plethora of novel phosphorylation sites in mammalian
proteins\textsuperscript{241-243}, including numerous sites in brain membrane proteins; among these a large number of ion channels and transporters\textsuperscript{175,244}. In this way Thr53 in mouse DAT was indentified as an \textit{in vivo} phosphorylation site\textsuperscript{175}, however, \textit{in vivo} sites have not yet been reported for SERT and NET. Although powerful, the mass spectrometry approach has drawbacks in addition to the requirement for highly specialized mass spectrometry instrumentation. Generally, the stoichiometry of phosphorylation is relatively low; only a small fraction of the available protein pool is phosphorylated at any given time, resulting in a low signal for the phosphopeptide compared to the dephosphopeptide\textsuperscript{232}. Accordingly, although major phosphorylation sites might be located easily, minor sites might be difficult to identify\textsuperscript{232}. Additionally, the proteomic analysis does not reveal the identity of the responsible kinases.

2.2.2 Choice of strategy and assay design

For this study, a strategy applying \textit{in vitro} phosphorylation followed by mass spectrometry was chosen to identify possible hSERT phosphorylation sites. This strategy offers the advantage of identifying both specific phosphorylation sites and responsible kinases. To our knowledge, this has not previously been applied to any of the monoamine transporters. The membrane topology of SERT has been extensively characterized and the intracellular regions are therefore well-defined\textsuperscript{56,245,246}; thus, segments harbouring potential phosphorylation sites are restricted to the short intracellular loop segments and the intracellular N- and C-termini (Figure 2.2).

![Figure 2.2. Membrane topology hSERT and boundaries for peptides investigated for \textit{in vitro} phosphorylation](image)

Transmembrane segments are shown as cylinders connected by intracellular and extracellular loops. Stipulated lines demarcate boundaries of the intracellular segments produced by solid-phase peptide synthesis and subjected to \textit{in vitro} phosphorylation assays: N1 to N5 in the N-terminus, IL1 to IL5 in the intracellular loops and C1 and C2 in the C-terminus. Ser, Thr and Tyr residues are represented as black (Ser/Thr) and grey (Tyr) spheres.
It was decided to apply solid-phase peptide synthesis to generate peptides corresponding to the entire intracellular regions of hSERT and to test the ability of these to serve as substrates for a selection of Ser/Thr and Tyr kinases that are suggested to phosphorylate mammalian SERTs. Analysis of the hSERT sequence reveals a total of 38 Ser/Thr/Tyr residues within the intracellular segments (13 Ser, 18 Thr, and 7 Tyr). Peptides were designed to cover all of the possible phosphorylation sites, including Ser/Thr/Tyr residues predicted to be within the first two residues of a predicted transmembrane segment 43,56, and all potential phosphorylation sites were flanked by at least 4 residues on either side. This resulted in a total of 12 peptides spanning in length from 11 to 35 amino acids (Figure 2.2 and Table 2.1).

<table>
<thead>
<tr>
<th>Domain</th>
<th>Peptide name</th>
<th>hSERT residues</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminus</td>
<td>N1</td>
<td>1-18</td>
<td>MTTPLNSQKQLSACEDG</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>27-49</td>
<td>LQKVVTQPGDKVESQGISNGYSA</td>
</tr>
<tr>
<td></td>
<td>N3</td>
<td>41-57</td>
<td>GQISNGYSAVPSPGAGD</td>
</tr>
<tr>
<td></td>
<td>N4</td>
<td>54-74</td>
<td>GAGDDTRHSIPATTTTLVAEL</td>
</tr>
<tr>
<td></td>
<td>N5</td>
<td>74-88</td>
<td>LHQGERETWGKKVDF</td>
</tr>
<tr>
<td>Intracellular loops</td>
<td>IL1</td>
<td>138-157</td>
<td>ALGQYHRNGCISIWRKICPI</td>
</tr>
<tr>
<td></td>
<td>IL2</td>
<td>271-281</td>
<td>WKGVKTSGKV</td>
</tr>
<tr>
<td></td>
<td>IL3</td>
<td>344-363</td>
<td>LLAFASYNKNMCYQDALV</td>
</tr>
<tr>
<td></td>
<td>IL4</td>
<td>443-463</td>
<td>LEGVITAHLDEFPHVWAKRE</td>
</tr>
<tr>
<td></td>
<td>IL5</td>
<td>508-538</td>
<td>EAVAVSWFYGITQFCRDVKEMLGFSPGWFWR</td>
</tr>
<tr>
<td>C-terminus</td>
<td>C1</td>
<td>586-602</td>
<td>FICIPTEAYRLIITPG</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>596-630</td>
<td>RLIITPGTFKRIKSTPETPETEIPCGDIRLNAV</td>
</tr>
</tbody>
</table>

These peptides were then to be subjected to a series of *in vitro* phosphorylation assays using a panel of recombinantly expressed and purified protein kinases. These were selected based on prior reports describing their potential involvement in SERT phosphorylation or from predictions from the kinase specific phospho-site prediction database NetPhosK 235 (Supplementary Figure 1). The insulin receptor (INSR), which is a tyrosine kinase, was included based on observations that SERT might be involved in a link between depression and abdominal obesity, which is associated with an increased risk of development of glucose intolerance and subsequently type 2 diabetes 247. The selected kinases are listed in Table 2.2.

Each peptide was tested in a separate *in vitro* phosphorylation assay with each of the kinases, except for the peptides not harbouring any Tyr residues, which were not tested as substrates for Src and INSR. Phosphorylation was detected by liquid chromatography-mass spectrometry (LC-MS) and identification
of phosphorylated residues was achieved by tandem mass spectrometry (MS/MS), for which our in-house MS equipment was adequate.

Table 2.2. List of selected kinases for the in vitro phosphorylation assay
Kinases were selected based on prior reports of involvement in SERT phosphorylation and predictions from the NetPhosK database.

<table>
<thead>
<tr>
<th>Type of kinase</th>
<th>Name</th>
<th>Abbreviation</th>
<th>References to involvement in SERT phosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser/Thr</td>
<td>Protein kinase A</td>
<td>PKA</td>
<td>136,178</td>
</tr>
<tr>
<td></td>
<td>Protein kinase C</td>
<td>PKC</td>
<td>136,148,248</td>
</tr>
<tr>
<td></td>
<td>Protein kinase G</td>
<td>PKG</td>
<td>137,249</td>
</tr>
<tr>
<td></td>
<td>p38 mitogen-activated protein kinase</td>
<td>p38MAPK</td>
<td>170,172</td>
</tr>
<tr>
<td></td>
<td>Ca(^{2+})/calmodulin-dependent protein kinase II</td>
<td>CaMKII</td>
<td>180,250</td>
</tr>
<tr>
<td></td>
<td>Glycogen synthase kinase 3</td>
<td>GSK3</td>
<td>NetPhosK</td>
</tr>
<tr>
<td></td>
<td>Cyclin-dependent kinase 5</td>
<td>cdk5</td>
<td>NetPhosK</td>
</tr>
<tr>
<td>Tyr</td>
<td>Sarcoma tyrosine kinase</td>
<td>Src</td>
<td>181</td>
</tr>
<tr>
<td></td>
<td>Insulin receptor</td>
<td>INSR</td>
<td>247</td>
</tr>
</tbody>
</table>

2.3 Results

2.3.1 Peptide synthesis and in vitro phosphorylation assay
All peptides were synthesized by Fmoc-based solid-phase peptide synthesis, purified to >98% by preparative high-performance liquid chromatography (HPLC), and the identity and purity was evaluated using liquid chromatography-mass spectrometry (LC-MS). In vitro phosphorylation assays were carried out in a total volume of 50 μl. In each reaction, the peptide was incubated with an amount of kinase calculated to achieve full conversion of a similar amount of control peptide in 1 h, based on the providers’ specification of activity. Furthermore, activity of all kinases was verified using known kinase-specific substrates (see Experimental Methods, Chapter 7 for details).

We identified and quantified peptide phosphorylation at different time points using analytical HPLC and LC-MS (Figure 2.3). Phosphorylation was initially detected as the occurrence of an additional peptide peak in the HPLC trace and identification of the ion corresponding to the phosphorylated peptide in the mass spectrum. The degree of phosphorylation was quantified by peak integration of the phosphorylated and non-phosphorylated peptide species from the analytical HPLC trace (Figure 2.3).
Figure 2.3. PKC-mediated in vitro phosphorylation of hSERT and a control peptide
Peptide samples were analyzed for phosphorylation using analytical HPLC and LC-MS. Shown are representative HPLC traces (left panels) and MS spectra (right panels) of peptides following 0, 1 (HPLC only) and 5 h incubation with PKC. Phosphorylation was detected as the occurrence of an additional peptide peak in the HPLC trace after incubation with the kinase, and the presence of ions in the mass spectrum corresponding to the phosphorylated peptide (addition of 80 Da). The degree of phosphorylation was quantified by peak integration of the phosphorylated and non-phosphorylated peptide species from the HPLC trace. A. Traces and spectra for the C2 peptide, [M+H]+ = 3892 Da. B. Traces and spectra for the control peptide (PKC substrate) QKRPSQRSKYL, [M+H]+ = 1391 Da.

Incubation with the kinases PKG, GSK3, cdk5 and Src promoted no detectable phosphorylation following incubation with any of the hSERT peptides for up to 24 h (Figure 2.4), while maintaining high catalytic activity at their respective control peptides. These low activities suggest that these kinases cannot phosphorylate the tested hSERT region in vitro and these kinases were not pursued further. As mentioned previously, Thr276 in IL2 has been suggested to be directly phosphorylated by PKG during cGMP-dependent up-regulation of SERT activity; however, we did not observe any direct phosphorylation by PKG in the in vitro assay. The kinases PKA and INSR each resulted in phosphorylation of three of the hSERT peptides: PKA phosphorylated the N1, N4 and C2 peptides and INSR phosphorylated the N3, IL1 and IL3 peptides (Figure 2.4). However, the degree of phosphorylation at these peptides was much less (<12% in 24 h) than for control peptides suggesting that the recognition sites in these peptides are poor substrates for PKA and INSR.
Figure 2.4. In vitro phosphorylation of hSERT segments

A. Summary of kinase activity at hSERT peptides. Each peptide was assayed in a separate experiment with each kinase. Fields in the matrix are color coded according to percent phosphorylation detected at different time points following kinase incubation. Dark red: >30% at 1 h and >90% at 5 h; medium red: >5% at 1 h and >20% at 5 h; light red: >5% at 24 h; green: <5% at 24 h; grey: not tested (Tyr not present in peptide).

B. Summary of the time course of kinase activity of all observed phosphorylation events. Kinase activity at control peptides are shown in red.

The kinases PKC, p38MAPK and CaMKII all displayed phosphorylation of several peptides (Figure 2.4). PKC phosphorylated IL1, IL2 and C2 to a degree of 98%, 21% and 90%, respectively, within 5 h. Phosphorylation of IL3 by PKC was also detected, but with lower activity (12% after 24 h). P38MAPK phosphorylated the C2 peptide and displayed the highest catalytic activity among any of the phosphorylated peptides. Specifically, the C2 phosphorylation level reached 90% within 1 h, which was substantially higher than the activity at the p38MAPK control peptide (30%) strongly indicating that a highly active p38MAPK phosphorylation site is contained in the C2 segment of hSERT. CaMKII
displayed robust phosphorylation of the N1 peptide (23% in 1 h) in addition to weak activity at IL1 (8% in 24 h) (Figure 2.4). Notably, the initial HPLC and LC-MS analysis unambiguously showed that all phosphorylated peptides only accepted one phosphate group. In summary, we observed the highest kinase activity at the C2 and IL1 peptides, which were phosphorylated by p38MAPK and PKC at catalytic rates comparable to those observed for their control peptides. N1 and IL2 were phosphorylated by CaMKII and PKC, respectively, at degrees somewhat lower than the control peptides. For the remaining peptides for which phosphorylation was detected, the phosphorylation rates were lower than 5% at 1 h. The peptides N2, N5, IL4, IL5 and C1 were not phosphorylated by any of the tested kinases. Thus, five major phosphorylation events were observed confined to N1 (CaMKII), IL1 (PKC), IL2 (PKC), and C2 (PKC and p38MAPK) (Figure 2.4).

2.3.2 Identification of phosphorylated residues within hSERT peptides

Only those peptides displaying more than 20% phosphorylation after 5 h incubation were further pursued (Figure 2.4). These five peptides were subjected to tryptic digestion and/or LC-MS/MS analysis to identify the phosphoacceptor residues. For the N1 and IL1 peptides, LC-MS/MS analysis was not required, as a single possible phosphorylation site was evident; in the case of N1 after tryptic digestion (Figure 2.5.A-B). Multiple possible phosphorylation sites existed for IL2 and the two C2 peptides, even after tryptic digestion of the C2 peptides, and therefore these were analyzed by LC-MS/MS. Using the phosphorylated peptide fragment as precursor ion, analysis of the fragmentation pattern in the mass spectrum allowed mapping of the phosphorylated residue within the amino acid sequence of the peptide (Figure 2.5.C-E). Thus, unambiguous identification of the specific phosphorylated residue within each phosphorylated peptide was accomplished.

Figure 2.5. MS and MS/MS spectra of phosphorylated hSERT peptides

Peptides that were subject to >20% phosphorylation following 5 h incubation with kinase were further analyzed to identify phosphorylated residues. A-B. Full-scan mass spectra of the phosphorylated N1 fragment after tryptic digestion (A) and phosphorylated IL1 (B), indicating the correspondence of ions observed to the molecular weight of the phosphorylated peptides. C-E. Product ion spectra from the MS/MS analyses showing ions resulting from fragmentation of the phosphorylated precursor peptides IL2 (C) and trypsin treated C2 (D-E). The resulting b- and y-ions are labelled. A. Trypsin treatment of the CaMKII phosphorylated N1 peptide cleaved the peptide after Lys10 (see Table. 2.1 for amino acid sequence) with the phosphorylated residue contained within the Gln11-Gly18 fragment, which contained only a single Ser/Thr residue (Ser13), thus identifying Ser13 as the phosphoacceptor. B. Ser149 was identified as the phospho acceptor in the PKC phosphorylated IL1 peptide by being the only Ser/Thr residue contained within this peptide. C. Ser277 was identified as the PKC phosphoacceptor in the IL2 peptide by analysis of the fragmentation pattern observed in the MS/MS spectrum of phosphorylated IL2, as the b6 ion appeared as a non-phosphorylated fragment. D. Thr603 was identified as the phosphoacceptor within the Leu597-Lys605 fragment of the trypsin digested, PKC phosphorylated C2 peptide, as the b4 ion appeared non-phosphorylated whereas the y5 and y7 ions appeared phosphorylated. E. Thr616 was identified as the phosphoacceptor within the Ser611-Arg626 fragment of the trypsin digested, p38MAPK phosphorylated C2 peptide, as the y10 ion appeared non-phosphorylated, whereas the y11 and y13 ions appeared phosphorylated.
Figure 2.6. Overview of location of the identified phosphoacceptor residues in hSERT
Shown are the surrounding amino acid sequences of the residues that were found to be subject to >20% phosphorylation after 5 h incubation with kinase.

Table 2.3. Consensus motifs for kinases found to phosphorylate hSERT *in vitro*
X denotes any amino acid. Brackets indicate a residue that is usually present and forms part of the consensus sequence, but is not invariant.

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Consensus sequence</th>
<th>Phosphorylated hSERT residues within consensus sequences</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKC</td>
<td>S/T-X-R/K</td>
<td>T-F-K (Thr603)</td>
<td>159,251</td>
</tr>
<tr>
<td></td>
<td>R/K-X-S/T</td>
<td></td>
<td>159</td>
</tr>
<tr>
<td></td>
<td>R/K-X-X-S/T</td>
<td></td>
<td>159</td>
</tr>
<tr>
<td>CaMKII</td>
<td>R-X-X-S/T</td>
<td></td>
<td>158,159,233,234</td>
</tr>
<tr>
<td></td>
<td>R/K-X-X-S/T</td>
<td>K-Q-L-S (Ser13)</td>
<td>158</td>
</tr>
<tr>
<td>Proline-directed MAP kinases (p38MAPK)</td>
<td>P-X-S/T-P</td>
<td>P-E-T-P (Thr616)</td>
<td>158,234</td>
</tr>
<tr>
<td></td>
<td>(P)-(L)-P-X-S/T-P-(P)</td>
<td></td>
<td>233</td>
</tr>
</tbody>
</table>

Phosphorylation of the N-terminal N1 peptide by CaMKII was found to occur at Ser13 (Figure 2.5.A) within the KQLS sequence motif, which is a typical CaMKII recognition site\(^{158}\) (Figure 2.6 and Table 2.3). Within each of the three peptides phosphorylated by PKC (IL1, IL2 and C2) we identified a single residue as the phosphoacceptor. In the highly phosphorylated IL1, the phosphoacceptor residue was Ser149 (Figure 2.5.B), which is contained within a GCISIW motif that does not constitute a typical PKC recognition sequence\(^{158,159}\) (Figure 2.6 and Table 2.3). This site in hSERT has to our knowledge never been reported as a potential recognition site for PKC or any other protein kinase. Ser277 was identified as the phosphoacceptor residue in IL2 (Figure 2.5.C) and is part of the PKC consensus sequence KTSGK\(^ {158,159}\) (Figure 2.6 and Table 2.3). As IL2 harbours an additional potential phosphorylation site (Thr276)
next to Ser277, the assignment from the MS/MS spectrum is based solely on one fragment ion (b₀, Figure 2.5.C). To provide additional evidence that phosphorylation indeed was located to Ser277, we therefore synthesized IL2 with incorporation of either phospho-Thr (IL2-pThr276) or phospho-Ser (IL2-pSer277) and co-injected each of these with PKC phosphorylated IL2 on analytical HPLC (Figure 2.7). This confirmed the MS/MS assignments, as IL2-pSer277 but not IL2-p276Thr co-eluted with the PKC phosphorylated IL2 peptide.

![Figure 2.7. HPLC elution profiles for PKC phosphorylated IL2 co-injected with IL2-pThr276 or IL2-pSer277](image)  

*In vitro* PKC phosphorylated IL2 co-injected on HPLC with IL2 where pSer (left panel) or pThr (right panel) was incorporated directly into the peptide sequence through solid-phase synthesis. **A.** Co-injection with IL2-pSer277 resulted in a single peak with the elution time of IL2 phosphorylated by PKC (marked with an asterisk), indicating that one single peptide species was present in the sample. **B.** Co-injection with IL2-pThr276 resulted in an additional peak with an earlier elution time than IL2 phosphorylated by PKC, demonstrating that two different peptide species were present in the sample.

In the highly PKC phosphorylated C2 peptide from the C-terminus, we found Thr603 as the phosphoacceptor (Figure 2.5.D). Thr603 is located within the PGTFK sequence motif, which forms a PKC recognition site (Figure 2.6 and Table 2.3). Phosphorylation of the C-terminal C2 peptide by p38MAPK was found to occur on Thr616 (Figure 2.5.E). This residue is located within the PETP sequence motif, which is a typical motif for the proline-directed kinases belonging to the MAPK family (Figure 2.6 and Table 2.3).

### 2.4 Discussion

Biochemical studies of rodent or human SERT have established that SERT is a phosphoprotein and that the function and number of neuronal transporters at the cell surface is linked to changes in SERT phosphorylation level. Understanding the role of SERT phosphorylation/dephosphorylation in regulatory processes requires identification of the residues in SERT that are substrate for phosphorylation by specific kinases. However, for all kinases that have been linked to SERT phosphorylation, affirmative assignment of their target phosphorylation sites SERT is lacking due to no or inconclusive experimental data supporting direct phosphorylation of specific residues in SERT. In an approach to localize sites in hSERT for phosphorylation by specific Ser/Thr and Tyr kinases, we decided to test the ability of the
intracellular regions of hSERT to serve as substrates for a selection kinases suggested to phosphorylate mammalian SERTs.

2.4.1 The in vitro phosphorylation approach
The study shows that peptide segments from the intracellular regions of hSERT are readily phosphorylated in vitro by PKC, CaMKII and p38MAPK. Using LC-MS/MS, we identified the specific phosphoacceptor sites contained within these hSERT segments leading to the identification of five potential in vivo phosphoacceptor sites. Kinase recognition is governed by up to ten residues surrounding the acceptor residue that determines kinase specificity and catalytic activity. In hSERT, the amino acid sequence surrounding four (Ser13, Ser277, Thr603 and Thr616) of the five identified phosphoacceptor residues indeed qualify these as putative recognition sites for the identified kinases (Table 2.3). However, only Ser13 has previously been reported as a potential phosphorylation site on basis of biochemical data\textsuperscript{180}. Previous analysis has proposed hSERT to contain additional kinase consensus sites, which we did not identify as phosphorylation sites in our in vitro studies\textsuperscript{134,235}. However, sites that display little or no in vitro kinase activity may be phosphorylated in vivo when embedded within a tertiary protein structure in a native cellular environment where key cellular factors are present. Specifically, in native cells SERT likely resides in protein-interaction complexes with scaffolding, adaptor and signalling proteins that may spatially and conformationally increase kinase activity at the transporter. Similarly, the ability of a specific sequence to serve as kinase substrate in vivo is affected by multiple parameters other than the linear sequence of a kinase recognition site such as the tertiary structure of the protein, which determine kinase accessibility to the site.

Despite these inherent limitations, in vitro phosphorylation strategies have proven powerful tools for initial identification of membrane protein phosphorylation sites. The methodology allows the screening of a large number of kinases, including those not previously predicted or known to phosphorylate the protein. For the monoamine transporters, in vitro phosphorylation of the isolated N-terminal segment of DAT expressed as a soluble construct has been used to identify a phosphorylation site for the proline-directed kinases ERK1, p38MAPK and JNK at Thr53\textsuperscript{144}. This residue in DAT has subsequently been identified as a native phosphoacceptor in a phosphoproteomic study of whole mouse brain proteins\textsuperscript{175}. This demonstrates the power of combining in vitro phosphorylation and proteomics for the identification and confirmation of both the specific phosphorylation site as well as the responsible kinase(s).

2.4.2 Identified in vitro phosphorylation sites
CaMKII was found to phosphorylate Ser13 in the N-terminus of hSERT. Little is known about possible involvement of CaMKII in SERT regulation; however, CaMKII phosphorylation of Ser13 has been proposed to regulate the interaction between SERT and syntaxin1A, which is involved in regulation of the conducting state of the transporter\textsuperscript{180}. Our findings support the suggestion that CaMKII phosphorylates
SERT directly at this site. Interestingly, DAT has also been found to contain a CaMKII site within this region of the N-terminal domain, whereas NET is phosphorylated by CaMKII within the C-terminal region.

The most rapid and extensive in vitro phosphorylation event that we observe is phosphorylation by p38MAPK of Thr616 located in the C-terminus. Support for SERT phosphorylation by p38MAPK has been provided in a previous study, where decreased basal SERT phosphorylation in rat synaptosomes was observed following p38MAPK inhibition, which correlated with decreased SERT membrane levels. However, no specific phosphorylation site in SERT has been proposed. Our results suggest that Thr616 within the C-terminal PETP motif is a potential phosphoacceptor site for p38MAPK.

It is well-described that PKC activation leads to increased phosphorylation levels of SERT, NET and DAT. In our phosphorylation studies, we find three PKC phosphorylation sites within the intracellular parts of the hSERT sequence; Ser149 in IL1, Ser277 in IL2 and Thr603 within the C-terminus. The existence of multiple PKC phosphorylation sites in SERT has previously been suggested and PKC-mediated phosphorylation has been found to occur at both Ser and Thr residues. To our knowledge, Ser149 has never been reported as a potential phosphorylation site for PKC or any other protein kinase and it is not a part of a typical PKC recognition motif. Such a phosphorylation site might not readily be discovered using conventional consensus-directed mutagenesis studies or phosphorylation site predictions; demonstrating the utility of in vitro phosphorylation studies in discovering unexpected phosphorylation sites. Ser277 in IL2 rests within a PKC recognition sequence, which is highly conserved between all SLC6 NT Ts. Within this motif, Thr276 has previously been suggested to be a specific phosphoacceptor during PKG-linked hSERT phosphorylation. Specifically, it was observed that the T276A mutation decreased the level of cGMP-stimulated Thr phosphorylation in SERT. Our results suggest that, at least for direct phosphorylation of hSERT by PKC, Ser277 may be the de facto phosphoacceptor residue within a potential PKC phosphorylation site in IL2. In support of this, the equivalent residue in IL2 in hNET (Ser259) has also been shown to be phosphorylated directly by PKC. Previous work has pointed to the existence of a PKC phosphorylation site in the SERT C-terminus, which potentially regulates a protein-protein interaction between the C-terminus of SERT and the scaffolding protein Hic-5. Our results suggest that Thr603 within the TFK motif is a potential phosphoacceptor site for PKC in the C-terminal domain of hSERT.

2.5 Summary

In summary, the in vitro phosphorylation study proposed five specific sites for direct hSERT phosphorylation mediated by the kinases CaMKII (Ser13), PKC (Ser149, Ser277 and Thr603) and p38MAPK (Thr616). These phosphorylation sites can guide future studies of direct links between SERT phosphorylation and regulatory processes.
3 Functional consequences of hSERT phosphorylation at the sites identified in vitro

3.1 Introduction

SERT is a phosphoprotein and SERT phosphorylation/dephosphorylation occurs during regulation by multiple pathways\(^{136,137,149,181}\). In particular, activation and/or inhibition of Ser/Thr kinases, including PKC, PKG, p38MAPK and CaMKII modulate SERT function and trafficking\(^{136,137,147-149,165,170,172,180}\). However, the molecular mechanisms by which kinase activity is linked to SERT regulatory processes are poorly understood, including the potential role of direct SERT phosphorylation and the identity of the specific phosphorylated SERT amino acid residues. Identification of specific phosphorylation sites by unguided mutagenesis studies is inherently difficult due to the presence of multiple Ser/Thr/Tyr residues in intracellular regions of SERT, together with the fact that a given kinase might phosphorylate the protein at more than one site\(^{148}\).

The aim of the work in this chapter was to study putative functional consequences of SERT phosphorylation at the residues identified in vitro as mediated by CaMKII (Ser13), PKC (Ser149, Ser277, Thr603) and p38MAPK (Thr616).

3.2 Results

3.2.1 Role of putative CaMKII phosphorylation of Ser13 for hSERT function and surface expression

As the first step towards identifying effects of possible phosphorylation of Ser13 on hSERT function and expression, we co-expressed hSERT bicistronically with a constitutively active variant of CaMKII in COS7 cells (Supplementary Figure 2) and determined \(K_m\) and \(V_{\text{max}}\) for cellular uptake of \(^{3}\text{H}\)5-HT and cell surface \(B_{\text{max}}\) for whole cell binding of \(^{125}\text{I}\)RTI-55 (Figure 3.1 and Table 3.1). We found no difference in \(K_m\), \(V_{\text{max}}\) or \(B_{\text{max}}\) in the presence or absence of constitutively active CaMKII (Figure 3.1.C). To ensure that expression of the CaMKII mutant indeed induces constitutive activity in COS7 cells, we co-expressed CaMKII with the ionotropic glutamate receptor GluA1, which contains a well-characterized CaMKII and PKC phosphorylation site\(^{252,253}\). Immunoblotting analysis using a GluA1 phospho-specific antibody of whole cell extracts of COS7 cells expressing GluA1 in the presence and absence of constitutively active CaMKII, showed that expression of CaMKII indeed increases phosphorylation in COS7 cells (Supplementary Figure 3). Since CaMKII activity in COS7 cells has no impact on the basal 5-HT transport function or cell surface expression of hSERT, a direct analysis of the functional role of potential CaMKII phosphorylation of Ser13 is prevented. Therefore, we introduced phospho-mimicking and phospho-blocking mutations at Ser13 by substituting this residue with Asp and Ala, respectively, and determined the transport kinetics and cell surface expression levels of the S13A and S13D mutants of hSERT (Table
3.1). For $K_m$, there was no significant difference between S13A and S13D compared to wild-type (WT) hSERT (Table 3.1), whereas a significant decrease in $V_{max}$ compared to hSERT WT was observed for both mutants (Table 3.1). To determine if the decrease in $V_{max}$ was due to decreased levels of transporter in the cell membrane, we determined cell surface $B_{max}$ of WT and mutant hSERT expressed under similar conditions. For both mutants, we found $B_{max}$ to be similarly decreased as $V_{max}$ relative to hSERT WT (Table 3.1); showing that the decreases in maximal transport capacity are caused by lower levels of transporter expressed at the surface of the cell and not by decrease in substrate turnover rate. The $K_d$ for binding of RTI-55 was not significantly different from hSERT WT for any of the mutants (Supplementary Table 2). Thus, Ser13 appears to be involved in cell surface expression of hSERT in COS7 cells, but the phosphorylation state of Ser13 appears to play only a subtle role in this effect as no significant difference in cell surface $B_{max}$ between phospho-mimicking (S13D) and phospho-blocking (S13A) mutations were observed.

![Figure 3.1](image-url)
Table 3.1. Summary of transport kinetics and surface expression of WT and mutant hSERT
All experiments were performed using transfected COS7 cells at RT. Values are mean ± SEM; n is the number of independent determinations. * Individual determinations of $V_{\text{max}}$ and $B_{\text{max}}$ for all mutants in the absence of kinases, and for hSERT WT in the presence of kinase, were performed in parallel with determinations for hSERT WT under similar conditions and are expressed as % of hSERT WT ($V_{\text{max}} = 48 \pm 4$ pmol/min/mg; $n = 30$; $B_{\text{max}} = 2.4 \pm 0.2$ pmol/mg, $n = 20$). # $p<0.05$, ## $p<0.01$ $K_m$ value significantly different from control (hSERT WT) (Student’s t-test). * $p<0.05$, ** $p<0.01$ $V_{\text{max}}$ or $B_{\text{max}}$ values significantly different from control (hSERT WT) (paired t-test). NA, not available (no detectable binding); ND, not determined.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$K_m$ (µM)</th>
<th>n</th>
<th>% of WT</th>
<th>$V_{\text{max}}$</th>
<th>n</th>
<th>% of WT</th>
<th>$B_{\text{max}}$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>hSERT</td>
<td>0.98 ± 0.08</td>
<td>27</td>
<td>100</td>
<td>--</td>
<td>--</td>
<td>100</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>hSERT + CaMKII</td>
<td>0.84 ± 0.08</td>
<td>6</td>
<td>106 ± 5</td>
<td>15</td>
<td>111 ± 4</td>
<td>6</td>
<td>106 ± 5</td>
<td>5</td>
</tr>
<tr>
<td>hSERT + p38MAPK</td>
<td>0.93 ± 0.10</td>
<td>6</td>
<td>96 ± 4</td>
<td>15</td>
<td>105 ± 6</td>
<td>5</td>
<td>106 ± 5</td>
<td>5</td>
</tr>
<tr>
<td>hSERT + PKC</td>
<td>0.72 ± 0.02</td>
<td>6</td>
<td>40 ± 2 **</td>
<td>7</td>
<td>28 ± 6 **</td>
<td>4</td>
<td>28 ± 6 **</td>
<td>4</td>
</tr>
<tr>
<td>hSERT S13A</td>
<td>1.03 ± 0.19</td>
<td>7</td>
<td>77 ± 6 *</td>
<td>4</td>
<td>66 ± 12</td>
<td>3</td>
<td>66 ± 12</td>
<td>3</td>
</tr>
<tr>
<td>hSERT S13D</td>
<td>0.76 ± 0.13</td>
<td>7</td>
<td>55 ± 5 **</td>
<td>4</td>
<td>51 ± 5 **</td>
<td>3</td>
<td>51 ± 5 **</td>
<td>3</td>
</tr>
<tr>
<td>hSERT S13E</td>
<td>0.95 ± 0.21</td>
<td>6</td>
<td>72 ± 10</td>
<td>4</td>
<td>57 ± 15</td>
<td>3</td>
<td>57 ± 15</td>
<td>3</td>
</tr>
<tr>
<td>hSERT S149A</td>
<td>0.38 ± 0.09 ##</td>
<td>5</td>
<td>26 ± 1 **</td>
<td>4</td>
<td>34 ± 5 **</td>
<td>3</td>
<td>34 ± 5 **</td>
<td>3</td>
</tr>
<tr>
<td>hSERT S149D</td>
<td>0.18 ± 0.06 ##</td>
<td>6</td>
<td>10 ± 1 **</td>
<td>4</td>
<td>10 ± 4 **</td>
<td>3</td>
<td>10 ± 4 **</td>
<td>3</td>
</tr>
<tr>
<td>hSERT S149E</td>
<td>0.32 ± 0.09 ##</td>
<td>7</td>
<td>21 ± 2 **</td>
<td>4</td>
<td>23 ± 9 *</td>
<td>3</td>
<td>23 ± 9 *</td>
<td>3</td>
</tr>
<tr>
<td>hSERT S277A</td>
<td>0.59 ± 0.10 #</td>
<td>6</td>
<td>41 ± 5 **</td>
<td>4</td>
<td>43 ± 7 *</td>
<td>3</td>
<td>43 ± 7 *</td>
<td>3</td>
</tr>
<tr>
<td>hSERT S277D</td>
<td>0.20 ± 0.09 ##</td>
<td>4</td>
<td>6 ± 2 **</td>
<td>4</td>
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<td></td>
<td>6 ± 2 **</td>
<td>ND</td>
</tr>
<tr>
<td>hSERT S277E</td>
<td>0.54 ± 0.07 #</td>
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<td>20 ± 4 **</td>
<td>4</td>
<td>42 ± 9 *</td>
<td>3</td>
<td>42 ± 9 *</td>
<td>3</td>
</tr>
<tr>
<td>hSERT T603A</td>
<td>1.26 ± 0.13</td>
<td>5</td>
<td>66 ± 11 *</td>
<td>4</td>
<td>62 ± 21</td>
<td>3</td>
<td>62 ± 21</td>
<td>3</td>
</tr>
<tr>
<td>hSERT T603D</td>
<td>1.02 ± 0.08</td>
<td>5</td>
<td>51 ± 8 **</td>
<td>4</td>
<td>48 ± 7 *</td>
<td>3</td>
<td>48 ± 7 *</td>
<td>3</td>
</tr>
<tr>
<td>hSERT T603E</td>
<td>1.02 ± 0.15</td>
<td>5</td>
<td>51 ± 8 **</td>
<td>4</td>
<td>43 ± 3 **</td>
<td>3</td>
<td>43 ± 3 **</td>
<td>3</td>
</tr>
<tr>
<td>hSERT T616A</td>
<td>1.25 ± 0.15</td>
<td>5</td>
<td>73 ± 5 **</td>
<td>4</td>
<td>71 ± 9</td>
<td>3</td>
<td>71 ± 9</td>
<td>3</td>
</tr>
<tr>
<td>hSERT T616D</td>
<td>1.18 ± 0.15</td>
<td>5</td>
<td>64 ± 9 *</td>
<td>4</td>
<td>63 ± 16</td>
<td>3</td>
<td>63 ± 16</td>
<td>3</td>
</tr>
<tr>
<td>hSERT T616E</td>
<td>1.20 ± 0.13</td>
<td>5</td>
<td>56 ± 6 **</td>
<td>4</td>
<td>57 ± 16</td>
<td>3</td>
<td>57 ± 16</td>
<td>3</td>
</tr>
<tr>
<td>hSERT S149A/S277A/T603A</td>
<td>0.30 ± 0.05 ##</td>
<td>4</td>
<td>36 ± 4 **</td>
<td>4</td>
<td>ND</td>
<td></td>
<td>36 ± 4 **</td>
<td>ND</td>
</tr>
<tr>
<td>hSERT T276A/S277A</td>
<td>0.31 ± 0.06 ##</td>
<td>3</td>
<td>63 ± 10 *</td>
<td>4</td>
<td>ND</td>
<td></td>
<td>63 ± 10 *</td>
<td>ND</td>
</tr>
</tbody>
</table>

3.2.2 Role of putative p38MAPK phosphorylation of Thr616 for hSERT function and surface expression
Previous studies have shown that p38MAPK activity has a stimulatory effect on SERT transport capacity, also when expressed in heterologous cell lines such as CHO and HEK293149,165,170,171,254. Although it has been shown that inhibition of p38MAPK in synaptosomes reduces basal SERT phosphorylation149, the role of direct transporter phosphorylation in the regulation process remains elusive and no specific SERT phosphorylation site for p38MAPK has been suggested. We therefore investigated the role of p38MAPK and Thr616 for hSERT transport kinetics and cell surface expression levels in COS7 cells. When hSERT was co-expressed with constitutively active p38MAPK, none of the kinetic parameters ($K_m$, $V_{\text{max}}$ or cell...
surface $B_{\text{max}}$) were significantly changed compared to hSERT WT in the absence of kinase (Figure 3.1C and Table 3.1). Introduction of phospho-mimicking (T616D) or phospho-blocking (T616A) mutations significantly decreased $V_{\text{max}}$ for both mutants by 36% and 27%, respectively with no significant change in $K_{m}$ (Table 3.1). The parallel determination of cell surface $B_{\text{max}}$ showed that the effects on $V_{\text{max}}$ were caused by decreased cell surface levels (Table 3.1); indicating that Thr616 influences hSERT cell surface expression in COS7 cells. However, the observed decrease in surface expression for the phospho-mimicking T616D mutant is not in agreement with previous observations of reduced surface levels of transporter after inhibition of p38MAPK in synaptosomes and HEK293 cells\textsuperscript{149}.

Previous studies have shown that transient activation or inhibition of endogenous p38MAPK in SERT-expressing cells affect 5-HT transport activity. p38MAPK activators, such as anisomycin cause an increase in 5-HT uptake\textsuperscript{170,172,254}, whereas p38MAPK specific inhibitors, such as PD169316 and SB203580 reduce transport activity\textsuperscript{149,165,170-172,254}. To study the role of the potential p38MAPK site in transient p38MAPK-mediated regulation of SERT, we therefore performed anisomycin and PD169316 experiments with hSERT WT and the phospho-blocking T616A mutant expressed in COS7 cells. Anisomycin-mediated stimulation of SERT transport levels occurs in a time- and concentration-dependent manner\textsuperscript{170}. Therefore, 5-HT uptake for hSERT WT was tested at different anisomycin concentrations (0.25-20 μM) and with pre-incubation periods of 5-60 min (Figure 3.2). In agreement with previous findings\textsuperscript{170}, a decrease in 5-HT uptake was observed at high anisomycin concentrations (5 μM and above) with a pre-incubation period of 15 min; however, no stimulation of 5-HT transport was observed at lower anisomycin concentrations (Figure 3.2.A). Furthermore, there was no influence of pre-incubation period with application of 1 μM anisomycin, which is the concentration that has typically been applied previously\textsuperscript{170,172,254} (Figure 3.2.B).

![Figure 3.2. Effect of p38MAPK activation on 5-HT uptake](image)

*Graphical summary of the effect of anisomycin treatment on $[^{1}\text{H}]$5-HT uptake for hSERT WT expressed in COS7 cells. Uptake experiments were carried out at 37 °C with a single 5-HT concentration. Data are presented as % of control (vehicle treated cells) and represent mean ± S.E.M. from 6 individual experiments with 16 wells for each condition. A. Cells were treated with varying concentrations of anisomycin (0.25-20 μM) for 15 min prior to transport assay. ** $p<0.01$ value significantly different from control (paired t-test). B. Cells were treated with 1 μM anisomycin for 5-60 min prior to transport assay.*
As we did not observe any stimulatory effect on SERT expressed in COS7 of p38MAPK activation, by either co-expression of constitutively active kinase or treatment with anisomycin, we instead turned our attention to p38MAPK inhibition. PD169316 treatment of COS7 cells transfected with hSERT WT decreased 5-HT uptake in a concentration-dependent manner (Figure 3.3.A). For further experiments, a concentration of 30 μM PD169316 (with 30 min pre-incubation) was chosen, which is comparable to conditions previously applied\textsuperscript{149}. Consistent with previous studies on transfected HEK293 cells and rat synaptosomes\textsuperscript{149}, we found that PD169319 treatment induced a ∼30% decrease in V\textsubscript{max} and a reduction in Km (from 1.40 ± 0.18 to 0.72 ± 0.08 μM) for hSERT WT (Figure 3.3.B-C). However, the phospho-blocking mutant T616A was affected by the treatment to the same extent as WT, thus, potential direct phosphorylation of Thr616 may not be involved in PD169316-mediated SERT down-regulation in COS7 cells.

![Graphical summary of the effect of PD169316 treatment on 5-HT uptake for hSERT WT expressed in COS7 cells.](image1)

**Figure 3.3. Effect of p38MAPK inhibition on 5-HT uptake**

A. Graphical summary of the effect of PD169316 treatment on [3H]5-HT uptake for hSERT WT expressed in COS7 cells. Uptake experiments were carried out at 37 °C with a single 5-HT concentration. Cells were pre-incubated with PD169316 or vehicle for 30 min. Data are presented as % of control (vehicle treated cells) and represent mean ± S.E.M. from 6 individual experiments with 16 wells for each condition. * p<0.05 value significantly different from control (paired t-test).

B. Values were determined from saturation experiments of [3H]5-HT uptake carried out at 37 °C with COS7 cells expressing hSERT WT or T616A. Cells were pre-incubated with PD169316 (30 μM) or vehicle for 30 min prior to uptake. Km values for hSERT WT and T616A. * p<0.05 value significantly different from control (vehicle treated cells) (paired t-test).

C. Graphical summary of the PD169316-induced decrease in V\textsubscript{max}, calculated as the difference between V\textsubscript{max} for vehicle treated cells and V\textsubscript{max} for PD169316 treated cells expressed as % of V\textsubscript{max} for vehicle treated cells. Bars represent mean ± S.E.M.; n = 8 experiments.

### 3.2.3 Role of putative PKC phosphorylation of Ser149, Ser277 and Thr603 for hSERT function and surface expression

Regulation of SERT surface expression and transport kinetics by PKC-linked mechanisms are well-described. In heterologous expression systems, pharmacological activation of endogenous PKC has been shown to acutely decrease SERT activity by lowering transporter surface levels, which correlate with increase in SERT phosphorylation\textsuperscript{136,255}. To investigate the role of Ser149, Ser277 and Thr603 in PKC-mediated down-regulation of SERT, we first expressed hSERT with a constitutively active variant of PKC in COS7 cells. This lead to a ~60% reduction in V\textsubscript{max} compared to hSERT WT expressed alone.
(Figure 3.1.C and Table 3.1). The reduction in $V_{\text{max}}$ was accompanied by a reduction in cell surface $B_{\text{max}}$, demonstrating that decreased transport capacity is due to decreased cell surface levels of hSERT. The turnover rate of the transporter for 5-HT was not changed significantly ($0.33 \pm 0.05 \text{ s}^{-1}$ vs. $0.49 \pm 0.12 \text{ s}^{-1}$, for hSERT WT in the absence and presence of PKC, respectively). These results are consistent with the previous findings of increased SERT internalization upon PKC activation with little or no effect on intrinsic transport function\textsuperscript{147,148,255}.

To study the role of the potential PKC phosphoacceptors Ser149, Ser277 and Thr603 for these effects in COS7 cells, we introduced phospho-mimicking and phospho-blocking mutations (Asp and Ala, respectively) at these positions and determined transport kinetics (Figure 3.4 and Table 3.1). All phosphosite mutants displayed a decrease in $V_{\text{max}}$ compared to hSERT WT. In addition, significant decreases in $K_m$ compared to hSERT WT were observed for all mutants; except for mutations at Thr603. Interestingly, phospho-mimicking Asp mutations at Ser149 and Ser277 had a much larger effect; producing 10- and 17-fold decreases in $V_{\text{max}}$, whereas the phospho-blocking Ala mutations produced 2- and 3-fold decreases, respectively (Figure 3.4.A and Table 3.1). A similar pattern of significantly larger effects by the Asp mutations compared to the Ala mutations was observed for $K_m$ ($0.38 \pm 0.09 \mu\text{M}$ for S149A vs. $0.18 \pm 0.06 \mu\text{M}$ for S149D and $0.59 \pm 0.08$ for S277A vs. $0.20 \pm 0.09$ for S277D). The observed changes in $V_{\text{max}}$ were all found to be caused by lower cell surface expression levels of the mutant transporters; observed as decreases in cell surface $B_{\text{max}}$ (Figure 3.4.A and Table 3.1) without any significant change in $K_d$ for binding of RTI-55 (Supplementary Table 2). For hSERT S277D, there was no detectable binding of RTI-55 and hence, we could not obtain any $K_d$ value for this mutant. Together, these results demonstrate that manipulation of Ser149 and Ser277, but not of Thr603, influences hSERT-mediated 5-HT uptake in COS7 cells by decreasing cell surface levels, similarly to the effects observed when hSERT is co-expressed with constitutively active PKC.

To test if the observed effects of constitutively active PKC on hSERT activity in COS7 cells could be blocked by phospho-blocking Ala mutations in the three potential PKC sites, we co-expressed the single phospho-blocking hSERT mutants S149A, S277A and T603A and the triple hSERT mutant S149A/S277A/T603A together with constitutively active PKC, and determined the transport kinetics. For all mutants, PKC induced a ~60% decrease in $V_{\text{max}}$ similar to the effect on hSERT WT (Figure 3.4.B and Table 3.2). These findings indicate that the mechanism by which constitutive PKC activity down-regulates SERT cell surface expression in COS7 cells is independent of direct phosphorylation of potential PKC phosphoacceptor residues, at least of the residues identified \textit{in vitro}.
Figure 3.4. Effect of mutation of Ser149, Ser277 and Thr603 on transport kinetics, surface expression and PKC-mediated down-regulation of hSERT

A. Graphical summary of the effect on $V_{\text{max}}$ (left panel), $B_{\text{max}}$ (middle panel) and $K_m$ (right panel) of phospho-mimicking (D) and phospho-blocking (A) mutations of Ser149, Ser277 and Thr603. All experiments were performed with transfected COS7 cells and were carried out at RT. Bars represent mean ± S.E.M.; n=3–7 experiments. For $V_{\text{max}}$ and $B_{\text{max}}$, values are expressed as % of values for hSERT WT tested in parallel. For $K_m$, the dotted line indicates value for hSERT WT (0.98 ± 0.08 µM). ** $p<0.01$ $V_{\text{max}}$ or $B_{\text{max}}$ value significantly different from value for hSERT WT (paired $t$-test). ## $p<0.01$ $K_m$ value significantly different from value for hSERT WT (Student’s $t$-test).

B. Graphical summary of effect of co-expression of constitutively active PKC on $V_{\text{max}}$ for hSERT WT and phospho-blocking hSERT mutants. Decrease in $V_{\text{max}}$ is calculated as the difference between $V_{\text{max}}$ in the absence and presence of kinase expressed as % of $V_{\text{max}}$ in the absence of kinase. Bars represent mean ± S.E.M.; n = 4–5 experiments.

Table 3.2. Effect of constitutive PKC activity on transport kinetics for WT and mutant hSERT

All experiments were performed with transfected COS7 cells and were carried out at RT. Values are mean ± SEM; n is the number of independent determinations. a Individual determinations of $V_{\text{max}}$ for hSERT WT and all mutants in the presence of PKC were performed in parallel with determinations for hSERT WT and the respective mutant in the absence of PKC under similar conditions and are expressed as % of hSERT WT or the respective mutants in the absence of PKC. The $K_m$ values for WT and mutant hSERT co-expressed with PKC were not significantly different from corresponding values in the absence of PKC (Student’s $t$-test); listed in Table 3.1. ** $p<0.01$ value significantly different from control (hSERT WT or mutant expressed in the absence of PKC) (paired $t$-test).

<table>
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<th>Mutant</th>
<th>$K_m$ (µM)</th>
<th>$n$</th>
<th>$V_{\text{max}}$ % of control</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>hSERT + PKC</td>
<td>WT</td>
<td>0.72 ± 0.02</td>
<td>6</td>
<td>40 ± 2 **</td>
</tr>
<tr>
<td>hSERT + PKC</td>
<td>S149A</td>
<td>0.54 ± 0.14</td>
<td>4</td>
<td>40 ± 4 **</td>
</tr>
<tr>
<td>hSERT + PKC</td>
<td>S277A</td>
<td>0.53 ± 0.11</td>
<td>4</td>
<td>45 ± 2 **</td>
</tr>
<tr>
<td>hSERT + PKC</td>
<td>T603A</td>
<td>0.99 ± 0.08</td>
<td>3</td>
<td>45 ± 2 **</td>
</tr>
<tr>
<td>hSERT + PKC</td>
<td>S149A/S277A/T603A</td>
<td>0.35 ± 0.07</td>
<td>3</td>
<td>37 ± 8 **</td>
</tr>
<tr>
<td>hSERT + PKC</td>
<td>T276A/S277A</td>
<td>0.36 ± 0.13</td>
<td>4</td>
<td>38 ± 8 **</td>
</tr>
</tbody>
</table>
3.2.4 Phospho-blocking mutants of potential PKC site inhibit short-term down-regulation of hSERT

Transient activation of endogenous PKC in SERT-expressing cells by the PKC activator β-PMA triggers a rapid down-regulation of 5-HT uptake capacity by lowering transporter cell surface levels. To study the role of the potential PKC sites in transient PKC-mediated down-regulation of SERT, we therefore performed β-PMA experiments with hSERT WT and phospho-blocking Ala mutants expressed in COS7 cells and determined V_max and K_m following 30 min β-PMA treatment (Figure 3.5.A). Consistent with previous studies, we found that β-PMA treatment induced a ~30% decrease in V_max of hSERT WT, which was abolished by co-incubation with the PKC inhibitor staurosporine (Figure 3.5.A). There was no significant effect on K_m (Table 3.3). All single phospho-blocking Ala mutants of Ser149, Ser277 and Thr603 as well as the triple combination S149A/S277A/T603A were found to exhibit similar decreases in V_max (Figure 3.5.B and Table 3.3). Thus, potential direct phosphorylation of these residues appears to not be involved in this type of PKC-linked hSERT down-regulation in COS7 cells.

Figure 3.5. Transport activity of hSERT WT and phospho-blocking mutants treated with β-PMA for 30 min

A. Concentration-response curves from representative experiments of saturation [3H]5-HT uptake in COS7 cells expressing hSERT WT. Cells were pre-incubated with 1 μM β-PMA, 1 μM β-PMA + 1 μM staurosporine or vehicle (control) for 30 min at 37 °C prior to the uptake assay. Data points represent mean ± S.E.M. from triplicate determinations. B. Graphical summary of decrease in V_max induced by 30 min β-PMA treatment for hSERT WT and the phospho-blocking mutants S149A, S277A, T603A and S149A/S277A/T603A expressed in COS7 cells (upper panel) or hSERT WT and S149A/S277A/T603A expressed in HEK293 cells (lower panel). The β-PMA-induced decrease in V_max is calculated as the difference between V_max in vehicle treated cells and V_max in β-PMA treated cells expressed as % of V_max in vehicle treated cells. Bars represent mean ± S.E.M.; n = 3–5 experiments.

Previous studies have suggested that β-PMA treatment does not promote SERT phosphorylation in COS7 cells. To ensure that possible effects of the phospho-blocking mutations were not overlooked due to cell line-dependent differences in β-PMA-induced down-regulation, we repeated the experiment with the triple Ala mutant in the HEK293 cell line, for which it has previously been shown that β-PMA treatment induces Ser and Thr phosphorylation of SERT. Similar 30 min treatment of HEK293 cells...
expressing WT and the triple Ala mutant of hSERT with β-PMA showed that $V_{\text{max}}$ for both mutants was decreased by ∼25%, which is consistent with the results from COS7 cells (Figure 3.5.B).

β-PMA-induced down-regulation of SERT activity proceeds in a biphasic manner with an initial phase (5 min) where SERT transport function changes, resulting in increased $K_m$ and decreased $V_{\text{max}}$, which correlates with an increase in SERT Ser phosphorylation. Prolonged β-PMA treatment (≥30 min) induces a second phase where cell surface levels of SERT decrease and Thr phosphorylation increases. To test the role of the putative PKC sites in short-term hSERT down-regulation, we expressed hSERT WT and the single and triple Ala mutants in HEK293 cells and subjected these to β-PMA treatment for 5 min. For hSERT WT and all of the phospho-blocking mutants, we observed a similar ∼25% decrease in $V_{\text{max}}$ compared to vehicle treated controls (Figure 3.6.A and Table 3.3). Thus, direct phosphorylation of Ser149, Ser277, and Thr603 alone or in combination is not responsible for the short-term PKC-linked down-regulation of hSERT.

![Figure 3.6. Effect of phospho-blocking mutants on β-PMA-mediated short-term regulation of hSERT](image_url)

**A.** Graphical summary of decrease in $V_{\text{max}}$ induced by 5 min pre-incubation with β-PMA for hSERT WT and the phospho-blocking mutants S149A, S277A, T603A and S149A/S277A/T603A expressed in HEK293 cells. Values are calculated as described in legend for Figure 3.5.B. Bars represent mean ± S.E.M.; n = 3–5 experiments. **B.** Graphical summary of the effect of 5 min (upper panel) or 30 min (lower panel) pre-incubation with β-PMA on $V_{\text{max}}$ for hSERT WT and phospho-blocking mutants T276A, S277A and T276A/S277A expressed in HEK293 cells. Values are calculated as described in legend for Figure 3.5.B. Bars represent mean ± S.E.M; n = 4–8 experiments. **p<0.01 value significantly different from hSERT WT (paired t-test).
Table 3.3. Effect of β-PMA on transport kinetics for WT and mutant hSERT

All experiments were carried out at 37 °C. Values are mean ± S.E.M.; n is the number of independent determinations. a Individual determinations of $V_{\text{max}}$ for hSERT WT and all mutants following treatment of cells with β-PMA were performed in parallel with determinations for vehicle treated cells and are expressed as % of vehicle treated cells. ** $p<0.01$ $V_{\text{max}}$ value significantly different from corresponding vehicle treated control (paired t-test). ## $p<0.01$ $V_{\text{max}}$ value significantly different from β-PMA treated hSERT WT, S149A, T276A, S277A, T603A and S149A/S277A/T603A (two-way ANOVA with Bonferroni’s post hoc test).

<table>
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<th>30 min preincubation</th>
<th>5 min preincubation</th>
</tr>
</thead>
<tbody>
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<td>$K_m$ (μM)</td>
<td>$V_{\text{max}}$ (μM)</td>
</tr>
<tr>
<td>WT</td>
<td>2.25 ± 0.17</td>
<td>2.03 ± 0.22</td>
</tr>
<tr>
<td>S149A</td>
<td>1.07 ± 0.03</td>
<td>0.90 ± 0.11</td>
</tr>
<tr>
<td>T276A</td>
<td>1.73 ± 0.06</td>
<td>1.60 ± 0.05</td>
</tr>
<tr>
<td>S277A</td>
<td>0.96 ± 0.08</td>
<td>0.88 ± 0.09</td>
</tr>
<tr>
<td>T603A</td>
<td>2.16 ± 0.18</td>
<td>1.68 ± 0.04</td>
</tr>
<tr>
<td>S149A/S277A/T603A</td>
<td>0.60 ± 0.04</td>
<td>0.55 ± 0.03</td>
</tr>
<tr>
<td>T276A/S277A</td>
<td>1.10 ± 0.13</td>
<td>1.10 ± 0.11</td>
</tr>
</tbody>
</table>

The T276A mutant of SERT has previously been shown to abolish cGMP-mediated regulation of SERT transport function via activation of PKG\textsuperscript{137}. As Thr276 is located within the putative PKC site in IL2 next to Ser277, we speculated whether this residue could assume a compensatory role upon phospho-blocking mutation of Ser277. We therefore generated the T276A and the double T276A/S277A mutants of hSERT and expressed these in parallel with S277A and hSERT WT in HEK293 cells and measured $V_{\text{max}}$ and $K_m$ following 5 min β-PMA treatment (Figure 3.6.B, upper panel and Table 3.3). Interestingly, the T276A/S277A mutant displayed significantly less reduction of $V_{\text{max}}$ compared to hSERT WT and the individual T276A and S277A mutants, having a $V_{\text{max}}$ value of 90% of vehicle treated cells with no change in $K_m$ (Table 3.3). In contrast, 30 min β-PMA treatment produced the same decrease in $V_{\text{max}}$ (∼25%) for T276A/S277A as for hSERT WT (Figure 3.6.B, lower panel and Table 3.3). Interestingly, the IL2 sequence that contains the Thr276-Ser277 motif is conserved in both NET and DAT. In NET, the equivalent T258A/S259A mutation has been found to inhibit PKC-mediated NET internalization, where Ser259 was proposed as the de facto site of kinase action\textsuperscript{138}. Our results support that the Thr276-Ser277 motif in IL2 is involved in an initial phase of PKC-mediated down-regulation of SERT.

3.2.5 Role of putative CaMKII phosphorylation of Ser13 for the interaction with syntaxin1A

As described in Chapter 1, all monoamine transporters interact with the synaptic vesicle fusion protein syntaxin1A\textsuperscript{202-204}. Using the *Xenopus* oocyte expression system combined with an *in vitro* binding assay, it was demonstrated that the interaction between SERT and syntaxin1A could be regulated by CaMKII,
and SERT Ser13 was suggested as a possible phosphorylation site, although investigation of transporter phosphorylation state was not undertaken. As our in vitro studies indeed identified SERT Ser13 as phosphorylation site for CaMKII, we wanted to investigate how possible phosphorylation at this site influences the interaction between SERT and syntaxin1A in our mammalian cell line. We decided to apply a bioluminescence resonance energy transfer (BRET) assay to investigate the interaction, as this has proven a powerful technique for studying protein-protein interactions in living cells. To our knowledge, BRET studies have not previously been applied to monoamine transporters.

Studies of protein-protein interactions by the BRET methodology relies on nonradiative (dipole–dipole) energy transfer between a luminescent donor enzyme and a fluorescent acceptor protein that are genetically fused to the two proteins of interest. The most frequently applied luminescent donor protein is Renilla luciferase (Rluc), which, upon binding and subsequent oxidation of the cell-permeable substrate coelenterazine, releases energy and emits light. This energy can be captured by the fluorescent acceptor protein if the two proteins are sufficiently close and their excitation and emission spectra overlap. The distance range at which energy transfer can occur is in the same order of magnitude as conventional protein dimensions (<100 Å), rendering BRET particularly suitable for studying protein-protein interactions. The most commonly applied BRET version is termed BRET2, which uses a coelenterazine analogue (DeepBlueC) as substrate and a genetically modified version of green fluorescent protein termed GFP2 as acceptor protein. Following oxidation of the substrate, Rluc emits light with a wavelength of ~400 nm. However, if in close proximity, GFP2 will absorb energy and subsequently emit light at a longer wavelength (maximum around 510 nm), and the ratio between emission at the two different wavelengths is a measure of the distance between the two proteins (with the efficiency of energy transfer being correlated to the sixth power of the inter-protein distance). The BRET signal intensity is dependent on both the distance between the donor and acceptor protein and their relative orientation. To study the interaction between SERT and syntaxin1A we therefore generated a series of Rluc and GFP2 fusion proteins with different positions of the donor and acceptor (Figure 3.7.C). Syntaxin1A possesses a single transmembrane helix with the C-terminus located on the extracellular side, and therefore only N-terminal fusion proteins of syntaxin1A were generated (Figure 3.7.C). For hSERT, we generated fusion proteins at both N- and C-termini as this has previously been shown to be possible with variants of GFP without compromising transporter function and regulation. This resulted in two donor and two acceptor constructs for hSERT and an additional construct with both GFP2 and Rluc fused to the transporter, which was used as a positive control (Figure 3.7.C). All hSERT constructs displayed activities similar to WT as assessed by 5-HT uptake in transfected COS7 cells (Supplementary Figure 4). No functional studies were undertaken for the syntaxin1A fusion proteins; however, using fluorescent microscopy it was verified that GFP2-syntaxin1A as well as all the GFP2-tagged hSERT constructs indeed were expressed at the cell surface of transfected COS7 cells (data not shown).
Figure 3.7. Outline of the BRET\(^2\) assay used to study the hSERT/syntaxin1A interaction

A-B. Schematic illustration of the BRET principle. A. When Rluc (displayed as hSERT fusion protein) oxidizes the substrate DeepBlueC (presented as a yellow star), energy is released and light with a wavelength of \(~400\) nm is emitted. B. When Rluc is sufficiently close to the acceptor protein GFP\(^2\) (displayed as syntaxin1A fusion protein), resonance energy transfer (RET) will occur resulting in excitation of GFP\(^2\) and emission of light with a wavelength of \(~510\) nm. The ratio between emissions at the two different wavelengths is a measure of the distance between the two proteins.

C. Outline of the various BRET\(^2\) constructs generated. Donor and acceptor constructs as well as the positive control were generated by fusion of Rluc or GFP\(^2\) to syntaxin1A (N-terminus) and hSERT (N- or C-terminus).

D. BRET\(^2\) saturation experiments with relevant donor-acceptor pairs of GFP\(^2\) and Rluc fusion proteins of hSERT and syntaxin1A co-expressed in COS7 cells. BRET values in mBRET are plotted as a function of the DNA ratio of GFP\(^2\) to Rluc. Data points represent mean ± S.E.M from 16 wells.

Combining the generated donor and acceptor constructs resulted in four different BRET pairs (Figure 3.7.D). Each pair was assayed in saturation BRET\(^2\) experiments, where increasing amounts of GFP\(^2\)-tagged construct and decreasing amounts of Rluc-tagged construct were co-transfected in COS7 cells. The BRET ratio is usually plotted as a function of the ratio of expressed GFP\(^2\) to expressed Rluc, where expression levels are measured as total fluorescence after direct excitation of GFP\(^2\) and total luminescence after addition of an Rluc substrate, respectively\(^{256}\). Our assay, however, did not reach a sufficient detection level for total GFP\(^2\) fluorescence and therefore the DNA ratio of GFP\(^2\) to Rluc was used as the abscissa instead. For all four hSERT/syntaxin1A BRET\(^2\) pairs, plotting the measured BRET levels as a function of the GFP\(^2\)/Rluc DNA ratio resulted in hyperbolic curves where the signal reached a saturation level at high concentration of GFP\(^2\)-tagged protein (Figure 3.7.D). This is characteristic for
specific protein interactions, whereas nonspecific interactions result in BRET signals that increase with increasing acceptor concentration in a near-linear fashion^{256,257}. There was substantial variation in the maximal BRET signal for the four pairs, ranging from \(~10\) mBRET for GFP^{2}-hSERT/Rluc-Syn1A to \(~100\) mBRET for GFP^{2}-Syn1A/Rluc-hSERT. The maximal BRET signal for GFP^{2}-Syn1A/Rluc-hSERT is comparable to BRET signals previously measured for membrane proteins and this pair was chosen for further experiments^{261-264}.

To test the influence on the hSERT/syntaxin1A interaction of potential phosphorylation at hSERT Ser13, Ala and Asp mutations were generated at this position in the Rluc-hSERT construct and saturation BRET\(^2\) experiments with GFP^{2}-Syn1A were carried out (Figure 3.8.A). There was no significant difference between hSERT WT and the S13A or S13D mutants in maximal BRET value or the BRET\(_{50}\) value, which represents the relative amount of acceptor giving 50% of the maximal energy transfer and reflects the relative affinity of the donor and acceptor fusion proteins. This suggests that mutation at hSERT Ser13 does not affect the interaction with syntaxin1A. Further saturation assays were carried out with GFP^{2}-Syn1A and Rluc-hSERT WT or the S13A mutant in the presence and absence of constitutively active CaMKII, where a constant level of kinase or empty vector DNA was co-transfected in COS7 cells with the donor/acceptor pair (Figure 3.8.B). No difference in maximal BRET value or BRET\(_{50}\) value was observed for hSERT WT or S13A expressed in the absence or presence of CaMKII. This suggests that in this assay, CaMKII has no influence on the interaction between hSERT and syntaxin1A.

![Figure 3.8. BRET\(^2\) saturation experiments with syntaxin1A and hSERT Ser13 mutants](image)

Representative curves from BRET\(^2\) saturation assays in transfected COS7 cells. At least three independent experiments were carried out for each BRET\(^2\) pair. BRET values in mBRET are plotted as a function of the DNA ratio of GFP^{2} to Rluc. Data points represent mean ± S.E.M from 16 wells. A. Saturation assay for GFP^{2}-Syn1A/Rluc-hSERT WT or the phospho-blocking (S13A) or phospho-mimicking (S13D) hSERT mutants. B. Saturation assay for GFP^{2}-Syn1A/Rluc-hSERT WT or the phospho-blocking (S13A) hSERT mutant in the absence or presence of co-expressed constitutively active CaMKII.

Oligomerization of monoamine transporters is a prerequisite for proper trafficking, and the transporters are known to exist as constitutive oligomers in the plasma membrane\(^^{185,186}\). Previously, oligomerization
has been studied by crosslinking or co-immunoprecipitation, but several studies have also demonstrated the use of fluorescence resonance energy transfer (FRET) to study oligomerization of SERT, DAT and GAT. Having the Rluc- and GFP-tagged hSERT constructs in hand, it was therefore obvious to investigate whether we could study hSERT oligomerization using the BRET technique. Indeed, BRET saturation experiments with all four hSERT donor/acceptor pairs expressed in COS7 cells resulted in hyperbolic curves with the BRET signal reaching a saturation level at high concentration of GFP-tagged transporter (Supplementary Figure 5). This suggests a specific interaction between the donor- and acceptor-tagged transporters, and thereby demonstrates that the BRET technique is suitable for studying the oligomerization process for monoamine transporters.

3.3 Discussion

Understanding the molecular mechanisms underlying SERT regulation by phosphorylation has so far been hampered by lack of knowledge of specific phosphorylation sites. As an initial approach to identify possible regulatory roles of the phosphorylation sites identified in vitro, we employed site-directed mutagenesis to manipulate these potential phosphorylation sites in the presence and absence of constitutively active or pharmacologically induced kinases, and evaluated potential effects on the primary functional parameters determining SERT activity: $V_{\text{max}}$, $K_m$ and cell surface expression ($B_{\text{max}}$).

For CaMKII, no effects on these parameters have been reported for SERT expressed in heterologous cells such as HEK293 or COS7 upon CaMKII activation or inhibition. In this study, we do not observe any effect on hSERT activity of co-expression with constitutively active CaMKII; however, we do observe a decrease in $V_{\text{max}}$ and cell surface density of hSERT when mutating the in vitro identified CaMKII phosphorylation site Ser13 to either Ala or Asp. CaMKII phosphorylation of Ser13 has been proposed to regulate the interaction between SERT and syntaxin1A. In thalamocortical neurons, interaction of syntaxin1A with endogenous SERT positively affects surface expression of the transporter and when SERT is expressed in oocytes, syntaxin1A is involved in regulation of transporter conducting states. The interaction between SERT and syntaxin1A has previously been studied using co-immunoprecipitation and pull-down assays. Here we demonstrate that the interaction can be studied in living cells using a BRET-based assay. In this assay, potential CaMKII-mediated phosphorylation of hSERT at Ser13 seems not to influence the interaction with syntaxin1A. In agreement with previous reports, we find that mutation of SERT Ser13 to Ala does not affect the affinity to syntaxin1A. In contrast we do not see any positive modulatory effect on the interaction of the presence of constitutively active CaMKII and neither do we see any effect of the hSERT S13D mutation, which mimics constitutive phosphorylation. The discrepancy between the previous study and this study remains unclear. Many factors could contribute to the observed differences, including the use of different expression systems and different assays for studying the interaction.
The most rapid and extensive in vitro phosphorylation event that we observed was phosphorylation by p38MAPK of Thr616 located in the hSERT C-terminus. Support for SERT phosphorylation by p38MAPK has previously been provided, where decreased basal SERT phosphorylation in rat synaptosomes was observed following p38MAPK inhibition that correlated with decreased SERT cell surface levels\textsuperscript{149}. Additionally, co-expression in HEK293 cells of SERT and MKK3b, an upstream kinase of p38MAPK, increased transport capacity due to elevated surface levels of the transporter\textsuperscript{149}. Stimulation of intrinsic SERT activity in transfected cells, RBL-2H3 cells and mouse synaptosomes upon activation of p38MAPK has also been reported\textsuperscript{165,170,171}. In the present study, we did not observe any difference in activity or cell surface expression of hSERT when the transporter was expressed in COS7 cells in the presence or absence of constitutively active p38MAPK, and we did not observe any stimulation of SERT activity upon treatment with the p38MAPK activator anisomycin. We do, however, observe a decrease in $V_{\text{max}}$ when inhibiting p38MAPK, suggesting that p38MAPK has a positive effect on basal activity of SERT expressed in COS7 cells. To our knowledge, p38MAPK-mediated changes in phosphorylation status for recombinantly expressed SERT has not been investigated, and therefore it remains elusive whether the observed modulations are caused by direct transporter phosphorylation or due to indirect effects. Our results, demonstrating that hSERT WT and the phospho-blocking mutant T616A is affected to the same extent by p38MAPK inhibition, support the idea that direct transporter phosphorylation is not required for p38MAPK-mediated regulation of recombinantly expressed SERT, at least not at Thr616, which we identified as the sole p38MAPK phosphorylation site in vitro. However, our experimentation focused on COS7 cells only, where important cellular factors for regulation in native SERT expressing cells might be absent. These include potential protein-protein interaction partners as Thr616 is located within the C-terminus, which is known to form interactions with intracellular proteins. These might be regulated by putative p38MAPK phosphorylation of Thr616. Further work is required to clarify this.

Regulation of the monoamine transporters, SERT, DAT and NET by PKC has been extensively studied. For these, the general consequence of PKC activation is decreased transport capacity due to redistribution of the transporter from the surface to an intracellular compartment\textsuperscript{132}. It is suggested that PKC activation leads to increased phosphorylation levels of the three transporters\textsuperscript{135,136,138,141,142,148-150,152}. In our in vitro phosphorylation studies, we found three PKC phosphorylation sites within the intracellular parts of the hSERT sequence: Ser149 in IL1, Ser277 in IL2 and Thr603 within the C-terminus, which is in agreement with the previously suggested existence of multiple PKC phosphorylation sites in SERT\textsuperscript{148}. We find that introduction of phospho-mimicking mutations at Ser149 and Ser277 indeed causes a substantial decrease in $V_{\text{max}}$; however, phospho-blocking mutations at these positions do not alleviate the decrease in $V_{\text{max}}$ caused by constitutive PKC activity. This suggests that phosphorylation of Ser149 and Ser277 may cause SERT down-regulation, but that the negative effect of constitutive PKC activity on transport activity for heterologously expressed SERT may not be dependent on direct transporter phosphorylation. Previous work has pointed towards a biphasic effect of $\beta$-PMA-mediated down-regulation of SERT with an initial
phase accompanied by phosphorylation of Ser residue(s) and a subsequent phase where SERT internalization is accompanied by Thr phosphorylation\textsuperscript{148}. However, the role of direct PKC-mediated phosphorylation in these processes remains enigmatic. Previous mutational analysis has found that no single phospho-blocking Ala mutation of predicted PKC phosphorylation sites can inhibit β-PMA-induced down-regulation of SERT\textsuperscript{248}. Our findings for Ser149, Ser277 and Thr603 corroborate these observations and suggest a complex mechanism for PKC-mediated regulation of SERT. We find that disruption of the Thr276-Ser277 motif can inhibit down-regulation induced by transient short-term, but not constitutive or long-term PKC activation. This suggests that phosphorylation of one or both residues is involved in the short-term phase of down-regulation, but not in the long-term phase that involves SERT internalization. Similarly, for PKC-mediated down-regulation of DAT, it has been demonstrated that removal of the N-terminal Ser cluster abolished β-PMA-mediated DAT phosphorylation without affecting internalization of the transporter, indicating that the down-regulation is independent on direct transporter phosphorylation\textsuperscript{142}. On the contrary, PKC-mediated phosphorylation of the DAT N-terminal Ser cluster has proven essential for amphetamine-induced reverse transport (efflux) of DA\textsuperscript{157}. Likewise, amphetamine-induced substrate efflux via SERT is dependent on PKC activity\textsuperscript{164} and it can be speculated that this involves direct transporter phosphorylation, perhaps at one or more of the PKC sites identified in vitro. Lastly, PKC has been found to modulate protein-protein interactions between SERT and intracellular proteins including Hic-5\textsuperscript{162}, PP2A\textsuperscript{149,163} and syntaxin1A\textsuperscript{149} and the potential roles of Ser149, Ser277 and Thr603 for these interactions remain to be investigated.

3.4 Summary

We have investigated possible functional consequences of phosphorylation of hSERT at the five phosphorylation sites identified in vitro, focusing on the main functional parameters $V_{\text{max}}$, $K_m$ and cell surface expression using a heterologous expression system.

A marked reduction in $V_{\text{max}}$ and $B_{\text{max}}$ was seen when phospho-mimicking mutations were introduced at two of the identified PKC sites (Ser149 or Ser277), in agreement with a negative influence of PKC on SERT activity and cell surface expression. However, mutation of the three identified PKC sites in hSERT did not alleviate the decrease in $V_{\text{max}}$ and $B_{\text{max}}$ caused by co-expression of constitutively active PKC. We found evidence, however, that the Thr276-Ser277 motif in IL2 is involved in a transient short-term PKC-dependent down-regulation of hSERT mediated by β-PMA. Constitutive activity of co-expressed CaMKII or p38MAPK did not affect hSERT transport activity, but pharmacological inhibition of endogenous p38MAPK caused a reduction in 5-HT uptake. This effect seemed independent on phosphorylation at Thr616, which was the only identified phosphorylation site for p38MAPK in vitro. Furthermore, we found that the interaction between hSERT and syntaxin1A expressed in COS7 cells could be studied by the use of the BRET technique, but that the interaction was not affected by phosphorylation of hSERT Ser13 nor regulated by CaMKII.
Future work might be directed at investigating the role of the identified phosphorylation sites in additional protein-protein interactions for SERT and in the action of transporter modulators such as amphetamines. Additionally, the functional consequences of phosphorylation at the identified sites could be studied in cellular systems expressing SERT natively.
4 Purification and phosphoproteomic analysis of SERT

4.1 Introduction

Global and site-specific analysis of in vivo phosphorylation sites by mass spectrometry is a rapidly developing and powerful technique, and phosphoproteomic analyses have already identified thousands of novel phosphorylation sites in a wide range of proteins\textsuperscript{232,244,268-271}. Highly pure protein is not an absolute prerequisite for mass spectrometry based phosphorylation site analysis, and indeed numerous phosphorylation sites have been identified in proteins from complex mixtures. For thorough analysis, however, the protein of interest needs to be present at a certain level in the mixture, as all but the most abundant proteins are typically covered only by a few sequenced peptides\textsuperscript{272}. In a recent phosphoproteomic study of mouse brain, phosphorylation sites were identified in the SLC6 transporters DAT, GAT1-3 and GLYT1-2, and in numerous receptors and ion channels\textsuperscript{175}. SERT phosphorylation was not detected in this study, which might be caused by too low abundance of the protein or the fact that no major phosphorylation sites were present.

We wanted to undertake a phosphoproteomic analysis of SERT to identify possible in vivo phosphorylation sites. We reasoned that purification of the transporter was necessary to accomplish this. Accordingly, the aim of the work in this chapter was to establish a method to purify full-length SERT in order to obtain sufficient protein material for subjection to LC-MS/MS-based phospho-site analysis.

4.2 Strategy

It was decided to apply affinity chromatography for purification of SERT. Generally, affinity chromatography is a well-established method for selective purification of a protein or group of proteins from complex mixtures based on highly specific biological interactions between the protein and a selective ligand. Typically, the ligand is immobilized on a chromatographic matrix, while the target protein is in a mobile phase as part of a mixture, and the interaction is most often reversible\textsuperscript{273,274}. An initial binding phase is generally followed by washing and elution, resulting in recovery of purified protein (Figure 4.1).

Nickel affinity chromatography and (S)-citalopram-based affinity chromatography were applied for the purpose of purifying SERT from a heterologous expression system and native tissue, respectively.
4.3 Results

4.3.1 Purification of His-tagged hSERT expressed in COS7 cells

As a first approach, we wanted to establish a system for purification of hSERT from a heterologous expression system, by which we could generate transporter protein applicable for LC-MS/MS-based phospho-site analysis. Utilization of a heterologous expression system allows incorporation of purification tags into the protein of interest, which in theory enables separation from native proteins. Furthermore, heterologous expression is a readily accessible source of protein. We chose the commonly applied 6-histidine tag (His-tag), which we incorporated into hSERT at either the N- or C-terminus (His-hSERT and hSERT-His, respectively). The His-tag has previously been shown not to compromise transporter function\(^{169,275}\), and accordingly we observed WT transport behavior of hSERT tagged at either terminus (Supplementary Figure 6). The tagged transporter was expressed in COS7 cells and purified by nickel affinity chromatography. 1% Triton X-100 was used as detergent as it was efficient in solubilizing the transporter and compatible with the chromatography system. It is well-known that Triton X-100 disrupts SERT structure/function\(^{276}\); however, this was not an issue for our purposes as we were not dependent on functional transporter. The N- and C-terminally tagged versions of hSERT performed equally well in pilot experiments, and His-hSERT was chosen for larger scale purifications of the transporter. Application of the solubilized COS7 membranes to the nickel column led to retention of His-hSERT, which was detected on Western blot as a broad band around 90 kDa, in agreement with previous reports for heterologously expressed hSERT\(^{277}\) (Figure 4.2.A). Subsequently, His-hSERT was eluted using a 5-500 mM imidazole gradient. Although the majority of contaminating proteins were washed through the column prior to the elution steps, a significant amount of unwanted protein was present in the eluted fractions containing His-hSERT (as judged from a silver-stained gel, Figure 4.2.A). There was, however, a distinct band at ~90 kDa, which was selectively retained by the column, presumably containing His-hSERT (Figure 4.2.A). For the phosphoproteomic analysis, a protein band from a
Coomassie-stained gel was required and therefore the His-hSERT-containing eluted fractions required concentration, which was achieved by acetone precipitation of the protein. Subsequently, the area on the Coomassie-stained gel corresponding to the mass of His-hSERT (Figure 4.2.B) was excised and subjected to phosphoproteomic analysis.

**Figure 4.2. Purification of His-hSERT using nickel affinity chromatography**

A. Western blot (left panel) and silver-stained gel (right panel) displaying the nickel affinity chromatography purification process for His-hSERT from COS7 membranes. His-hSERT was solubilized in binding buffer at a protein concentration of ~5 mg/ml. ~16 mg solubilized protein (equal to approximately 40-45 pmol hSERT based on \[^{125}\text{I}\]RTI-55 binding to COS7 membranes) in 4 ml binding buffer was applied to a 1 ml nickel charged sepharose column (having a maximum theoretical binding capacity of around 15 \(\mu\)mol) connected to an ÄKTAprime chromatography system. The column was washed with 15-20 ml of binding buffer followed by elution with a linear gradient of 5-500 mM imidazole in binding buffer. 1 ml fractions were collected during elution. The eluates were analyzed by dot blot and the fractions containing His-hSERT further analyzed by SDS-PAGE followed by Western blotting or silver-staining. The fractions displayed on the blot and gel are: (from left) the solubilizate, the wash and the three eluate fractions containing the majority of His-hSERT activity. A distinct band around 90 kDa (presumably containing His-hSERT) was selectively retained by the column (indicated on the silver-stained gel by an arrow).

B. Coomassie-stained gel of the combined eluted fractions containing His-hSERT after concentration. The gel area corresponding to the mass of His-hSERT (encircled in red) was excised for subjection to LC-MS/MS analysis.

### 4.3.2 Phosphoproteomic analysis of His-tagged hSERT expressed in COS7 cells

The excised SDS gel band from purification of His-hSERT from COS7 cells was treated with trypsin (in-gel protein digestion) and the resultant tryptic peptides were analyzed by LC-MS/MS using a LTQ-Orbitrap Velos instrument\(^1\). Mass spectrometry clearly identified hSERT in the sample, although a number of contaminating proteins were also identified. hSERT was “ranked” 16 in the analyzed sample, which means that there were 15 proteins, for which more tryptic peptides were identified. Although the number of identified peptides is not a quantitative measure of protein amount in the sample, the LC-MS/MS analysis showed that purification of His-hSERT was only partial. A total of 16 hSERT-specific peptides were retrieved from the analysis, covering 59% of the intracellular regions (21 out of 38

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1. LC-MS/MS analysis (including sample preparation) of hSERT purified from COS7 cells was carried out by post doc Alicia Lundby, Center for Protein Research, University of Copenhagen.
intracellular Ser/Thr/Tyr residues) and 21% of the entire hSERT amino acid sequence (Figure 4.3 and Supplementary Table 3). The uncovered regions were mainly hydrophobic segments of the transmembrane core, which are inherently difficult to cover\textsuperscript{278,279}, and segments harbouring tryptic peptides that were either too short (a few amino acids) or too long (>30 amino acids) to be within the detection range of the MS instrument\textsuperscript{272}. Of the previously identified \textit{in vitro} phosphorylation sites (Chapter 2), Ser13, Ser149, Thr603 and Thr616 were covered in the retrieved peptides. Ser277 was not covered, as it resides within a tryptic fragment of only four amino acids (TSGK) that was too small to be detected in the MS/MS analysis.

Phosphorylation status was investigated by MS/MS analysis of tryptic peptides with or without enrichment of phosphopeptides. No phosphorylation sites were identified within the hSERT peptides retrieved from the sample. This was somewhat surprising, as SERT has previously been shown to be a phosphoprotein when expressed in a heterologous system, even under basal conditions\textsuperscript{136,137,149}. However, not all intracellular Ser/Thr/Tyr residues were covered by the MS/MS analysis, and accordingly, phosphorylation might be present at some of the non-covered residues. Additionally, the lack of identification of a phosphorylation site does not definitively exclude its possible existence, as phosphorylation might be present only at low stoichiometry resulting in suppression of the signal by non-phosphorylated species\textsuperscript{232,240,272,280}. This phenomenon is reinforced by the fact that phosphorylation changes the character of the peptide to be more acidic and therefore, these peptides are often reluctant positive charge acceptors during ionization in the MS instrument\textsuperscript{232}.

Figure 4.3. Areas covered by the LC-MS/MS analysis of His-hSERT purified from COS7 cells by nickel affinity chromatography
Two-dimensional schematic representation of SERT based on the topology of LeuT. Intracellular Ser, Thr and Tyr residues are colored in blue, green and yellow, respectively. The residues covered by the LC-MS/MS analysis are colored in black. Numbers denote the first and last residues of the covered segments.
4.3.3 (S)-Citalopram-based affinity purification of hSERT expressed in HEK293 cells and synaptosomal rSERT

As a second approach, we wanted to generate a system for purification of SERT from native tissue, which clearly excludes use of genetically encoded purification tags. For this purpose, we therefore generated an (S)-citalopram-derived affinity resin.

4.3.3.a Generation of an (S)-citalopram-derived affinity resin

Purification of SERT from native tissue has previously been accomplished by use of affinity chromatography with either 5-HT analogues or antagonists, such as citalopram and imipramine as immobilized ligand. We chose to apply a citalopram-based affinity system, as this has been found superior to systems with other immobilized ligands. We employed the pure (S)-enantiomer due to its improved affinity and selectivity towards SERT compared to the racemic mixture. The synthetic scheme for preparation of the affinity resin is outlined in Figure 4.4 ii. The cyano group of (S)-citalopram was reduced and the resulting primary amine utilized as anchoring point to the solid support, as this has previously proven successful. Two different solid supports were tested; tosyl-activated magnetic beads (Dynabeads MyOne, Invitrogen, Carlsbad, CA, USA) and N-hydroxysuccinimide (NHS) -activated sepharose. In pilot experiments, the former gave variable results in terms of coupling of the ligand and/or retention of SERT (data not shown), whereas the sepharose-based material showed reproducible protein retention. Therefore, the sepharose-based material was chosen for further experiments.

Figure 4.4. Scheme for generation of the (S)-citalopram-derived affinity resin

(S)-Citalopram was treated with LiAlH₄, generating an aminomethyl group in the 5-position, which was subsequently coupled under mildly basic conditions to N-hydroxysuccinimide (NHS) -activated sepharose containing a 10 atom spacer arm.

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ii Preparation of aminomethyl (S)-citalopram and initial coupling and purification trials were performed by MSc student Troels Eskildsen.
4.3.3.b Affinity purification of hSERT expressed in HEK293 cells

The evaluation of the generated affinity system as well as optimization of the purification procedure was done with heterologously expressed hSERT. HEK293 cells were chosen as the expression system for these experiments, as opposed to COS7 cells, as we contemplated that there might be cell-specific differences in phosphorylation patterns. Furthermore, hSERT was co-expressed with constitutively active p38MAPK in an attempt to promote phosphorylation. For the (S)-citalopram-based affinity system to be successful, SERT must interact specifically with the immobilized ligand, which requires protein in the correct fold and limits the choice of suitable detergents. Digitonin has previously been found to be superior to other detergents in solubilizing SERT in an active form\(^{275,276}\) and it has been extensively utilized in SERT purification\(^{275,281-284}\). Therefore, digitonin was chosen as solubilizing agent and near-complete solubilization of hSERT from HEK293 membranes was achieved with a 1% detergent concentration (Figure 4.5.A). hSERT was detected on Western blot as a broad band around 75-80 kDa. The slightly smaller mass compared to that observed when expressed in COS7 cells is probably due to different glycosylation patterns in different cell types\(^{277}\). Incubation of the solubilized protein with the (S)-citalopram-derived affinity resin led to almost complete absorption and selective retention of hSERT, whereas the majority of other present proteins were not retained by the resin (Figure 4.5.B). Subsequently, hSERT was eluted with 5 mM (S)-citalopram. Concentration of the combined eluates were attempted by precipitation with acetone as described above; however, this was unsuccessful as the resulting precipitated material was impossible to re-dissolve (even in SDS loading buffer), presumably due to the presence of digitonin. Instead, the eluates were concentrated by spin filtration (Figure 4.5.C), although this inevitably resulted in loss of protein (based on Western blot intensities in proportion to sample volume before and after the concentration procedure). Although the majority of interfering proteins were removed during the washing steps, a number of contaminating proteins were present in the eluates, as evident from the Coomassie-stained gels of the eluted fractions and especially the concentrated eluates (Figure 4.5.B-C). We attempted to circumvent this by the employment of different washing buffers with variable ionic strengths and organic additives such as ethylene glycol\(^{282}\); however, this did not afford any notable improvement (data not shown).

The overall purification factor was estimated to be around 250-fold (based on Western blot intensities of the solubilizate and the combined eluates compared to the respective protein concentrations), which is consistent with previous reports\(^{282}\). The gel area corresponding to the mass of hSERT (Figure 4.5.C.) was excised and subjected to phosphoproteomic analysis.
Near-full solubilization of hSERT from HEK293 membranes was achieved using a physiological buffer containing 1% digitonin (protein concentration of ~6 mg/ml). Western blot (upper panel) and Coomassie-stained gel (lower panel) displaying the (S)-citalopram-based affinity purification process for hSERT from HEK293 membranes. ~10 mg solubilized protein (equal to approximately 25-30 pmol hSERT based on [125I]RTI-55 binding to HEK293 membranes) in 2 ml solubilization buffer was incubated with an equal volume of (S)-citalopram-derived affinity resin (having a maximum theoretical binding capacity of around 40 μmol) for 2 h at 4 °C. The resin was washed with two different washing buffers (10-25 column volumes of each) followed by elution with 5 mM (S)-citalopram. 2 ml fractions were collected during wash and 1 ml fraction collected during elution. The fractions displayed on the blot and gel are: (from left) solubilizate, the first three fractions of washing buffer 1, the first two fractions of washing buffer 2 and the first five fractions of the elution buffer.

Western blot (left panel) and Coomassie-stained gel (right panel) of the eluted fractions containing hSERT before and after concentration. The gel area corresponding to the mass of hSERT (encircled in red) was excised for subjection to LC-MS/MS analysis.

**Affinity purification of rSERT from rat brain**

Having established a purification system for WT SERT, we next sought to apply this for purification of SERT from rat cerebral cortex. Solubilization of SERT was initially attempted from a crude membrane preparation (prepared as described previously287). Western blotting of the homogenate revealed two sharp bands at 75-80 kDa in agreement with previous findings277; however, only negligible solubilization was observed when applying the same solubilization conditions as for solubilization of hSERT from HEK293 membranes (Figure 4.6.A). Various efforts were made to improve solubilization: increasing digitonin concentration, increasing solubilization time, sonication during solubilization, varying salt concentration, varying protein concentration, addition of NaSCN282,288, as well as the employment of different detergents (decyl maltoside or dodecyl maltoside), which have recently been used for purification of membrane
proteins for crystallization purposes\textsuperscript{43,289}. However, none of the additional measures resulted in satisfactory solubilization (data not shown). Instead, it was decided to employ a different tissue preparation procedure, consisting of the preparation of synaptosomes. This was achieved using a Percoll gradient procedure as previously described\textsuperscript{290}. The presence of SERT in the synaptosomal preparation was confirmed by Western blotting (showing two bands at 75-80 kDa) and \([^{125}\text{I}]-\text{RTI-55}\) binding, which showed a \(B_{\text{max}}\) value of \(~0.5\) pmol/mg protein in agreement with previous reports\textsuperscript{291}, and a \(K_d\) of \(~4\) nM, which is consistent with values from the literature for binding of RTI-55 to SERT\textsuperscript{292} (Figure 4.6). It should be noted that RTI-55 also binds to DAT and NET, which presumably were also present in the synaptosomal preparation. Complete solubilization of SERT was achieved with the solubilization conditions applied for HEK293 membranes (Figure 4.6.A).

**Figure 4.6. Solubilization of SERT from rat cerebral cortical preparations and synaptosomal binding of RTI-55**

A. No appreciable solubilization of rSERT from a crude cerebral cortical membrane preparation was achieved using a physiological buffer containing 1% digitonin (protein concentration of \(~5\) mg/ml) (left panel), whereas complete solubilization of rSERT was achieved from a synaptosomal preparation under the same solubilization conditions (right panel). B. Representative concentration-response experiment of saturation \([^{125}\text{I}]-\text{RTI-55}\) binding to rat cortical synaptosomes. Non-specific binding was assessed in parallel in the presence of 0.1 mM fluoxetine. Data points are values from single determinations. Three independent experiments were carried out.

Synaptosomes from one rat brain were applied to the affinity resin and subjected to the purification procedure (Figure 4.7.A). There were no detectable Western blot signals in neither the wash fractions nor the eluates making it impossible to assess the course of the purification by this means. However, the Coomassie-stained gel showed that the majority of present proteins were not retained by the resin (Figure 4.7.A). The lack of Western blot signal was probably due to a lower amount of SERT (5-8-fold lower than for purification of hSERT from HEK293 based on RTI-55 binding activity) and the markedly lower signals obtained with the antibody used for rSERT compared to the one used for hSERT.
Figure 4.7. (S)-Citalopram-based affinity purification of rSERT from rat cortical synaptosomes

A. Western blot (left panel) and Coomassie-stained gel (right panel) displaying the (S)-citalopram-based affinity purification process for rSERT from rat cortical synaptosomes. ~10 mg solubilized protein (equal to approximately 4.5 pmol [125I]RTI-55-binding activity) in 2 ml solubilization buffer was incubated with an equal volume of (S)-citalopram-derived affinity resin (having a maximum theoretical binding capacity of around 40 μmol) for 2 h at 4 °C. Washing and elution was carried out as described in the legend to Figure 4.5. The fractions displayed on the blot and gel are: (from left) the first three fractions of washing buffer 1, the first two fractions of washing buffer 2 and the first five fractions of the elution buffer. B. Left panel: Western blot of the solubilizate and the concentrated wash fractions and eluates. Right panel: Coomassie-stained gel of the combined eluates after concentration.

Due to the lack of signals in the collected purification fractions, the combined wash fractions and combined eluates were both concentrated by spin filtration. After this procedure, Western blot signal was clearly evident in the concentrated eluate, whereas no signal was present in the concentrated wash fractions, demonstrating that SERT had been retained by the column (Figure 4.7.B). In addition to the two bands around 75-80 kDa and a band at 45 kDa observed in the solubilizate, the concentrated eluate showed an additional band at ~65 kDa (Figure 4.7.B). This might originate from partial degradation and/or deglycosylation of the transporter during the purification. From the Coomassie-stained gel of the concentrated eluate it was evident that a number of contaminating proteins were present in the purified sample, as was also seen for purification of hSERT from HEK293 cells (Figure 4.7.B). The Western blot was used as template for excising the Coomassie-stained SDS gel bands that were to be subjected to LC-MS/MS analysis.
4.3.4 Phosphoproteomic analysis of hSERT expressed in HEK293 cells

The excised SDS gel band from the (S)-citalopram-based affinity purification of hSERT co-expressed with p38MAPK in HEK293 cells was subjected to LC-MS/MS analysis in a similar manner to that described above for His-hSERT\textsuperscript{iii}. Mass spectrometry identified hSERT in the sample with 6 specific peptides covering 23\% of the intracellular regions (8 out of 38 intracellular Ser/Thr/Tyr residues) and 10\% of the entire SERT amino acid sequence (Figure 4.8 and Supplementary Table 4). Of the identified \textit{in vitro} phosphorylation sites, Thr603 and Thr616 were covered. The sequence coverage was markedly lower compared to the analyses described above of His-hSERT expressed in COS7 cells. This might be caused by a lower relative abundance in the sample of hSERT, which was “ranked” 29 in the present analysis. It might also be caused by less successful preparation, handling and processing of the sample, as the analysis was carried out by a different laboratory.

No phosphorylation sites were identified within the hSERT-specific peptides, indicating a lack of appreciable basal phosphorylation at the covered residues in conformity with the results from hSERT expressed in COS7 cells. Furthermore, this indicates that p38MAPK does not promote detectable phosphorylation of hSERT expressed in HEK293 cells at the residues covered by the analysis, including Thr616. LC-MS/MS-analyses of the purified synaptosomal rSERT cannot be presented here, as these experiments are still ongoing.

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\textsuperscript{iii} The analysis was carried out by Proteome Factory (Berlin, Germany).
4.4 Discussion

In this study, we aimed at undertaking a phosphoproteomic analysis of SERT both from a heterologous expression system and from native tissue. For this purpose, we established two methods for purification of SERT: nickel affinity chromatography for purification of His-tagged SERT and (S)-citalopram-based affinity chromatography for purification of WT SERT.

We were able, by both methods, to generate purified SERT material applicable for phospho-site analysis by LC-MS/MS. The course of the purifications was assessed by SDS-PAGE and Western blotting. Although this does not allow for precise quantitation, the purification factor for (S)-citalopram-based affinity purification of hSERT was estimated to be around 250-fold. Based on the ranking of SERT in the LC-MS/MS analyses of His-hSERT from COS7 cells and WT hSERT from HEK293 cells, the nickel affinity chromatography systems seems to perform somewhat better than the (S)-citalopram-based system for purification of heterologously expressed SERT. However, care should be taken when comparing the results from the two LC-MS/MS analyses, as they were carried out by different laboratories. The progress of the purifications could have been characterized in greater detail by techniques such as radioligand binding with the purified transporter as described previously.

SDS-PAGE as well as the LC-MS/MS analyses of the purified SERT samples revealed the presence of contaminating proteins, indicating that the purification protocols adopted provided only partial purification. Although attainment of entirely pure protein was not a prerequisite for phosphoproteomic analysis, it could have been advantageous, especially in combination with the (S)-citalopram-based chromatography, to apply a second purification step such as concavalin A or wheat germ-based glycoprotein purification. A higher degree of purity could hereby have been obtained, which might have led to better sequence coverage in the LC-MS/MS analysis.

Investigation of the phosphorylation status of the purified SERT did not identify any phosphorylation sites in His-hSERT expressed in COS7 cells or hSERT WT expressed in HEK293 cells in the presence of p38MAPK. The lack of identified phosphorylation sites indicates that the transporter is not highly phosphorylated at the covered sites under basal conditions. Although, as described above, the lack of identification of a phosphorylation site does not definitively exclude its existence. Furthermore, p38MAPK does not seem to promote phosphorylation of SERT expressed in HEK293 cells at the covered residues, including Thr616, which is in agreement with the lack of functional effect on transport of co-expressing the kinase (Chapter 3). However, it should be noted that we cannot rule out the possibility that the activity level of the kinase was simply to low to promote any observable phosphorylation. As no phosphorylation sites were identified in the purified SERT samples, it was an obvious concern that extensive dephosphorylation had taken place during the purification procedures leading to undetectable phosphorylation levels. However, phosphatase inhibitors were included throughout all purifications, and
moreover, phosphorylation sites were identified in some of the contaminating proteins from purification of His-hSERT from COS7 cells, thus rendering this concern improbable.

Obviously, it would be of utmost interest to obtain MS/MS results for SERT covering the intracellular Ser/Thr/Tyr residues not covered in the present analyses. This might be achieved by using a different protease for digestion of the purified protein, at this would generate a new set of peptides with different masses\(^{272,278}\). Additionally, peptides that were too small for detection in the MS instrument (as was the case for the tryptic TSGK fragment harbouring Ser277) could be chemically modified in order to increase their mass prior to LC-MS/MS analysis\(^{272}\). However, 100% sequence coverage is extremely difficult to obtain, as peptides from a given protein, although present at the same level will result in very different signal intensities due to unknown factors \(^{272,293}\). Thus, some of the peptides within a protein will be clearly visible, while some will never be apparent.

The phosphoproteomic approach could also have been used for studying SERT phosphorylation status after pharmacological kinase induction. Such phosphorylation dynamics studies have been undertaken for the ionotrophic glutamate receptor GluA1, which led to the identification of a novel activity-regulated phosphorylation site for CaMKII\(^{269}\). LC-MS/MS analysis of heterologously expressed SERT after treatment with \(\beta\)-PMA might have revealed specific PKC phosphorylation sites in the full-length transporter. Of particular interest, the phosphorylation status of the Thr276-Ser277 motif in IL2 could have been assessed after treatment with \(\beta\)-PMA, provided that a method had been established for detection of a peptide covering this area in the MS/MS analysis. Such disclosures await future investigations.

### 4.5 Summary

In summary, we have established two procedures: nickel affinity chromatography and \((S)\)-citalopram-based affinity chromatography, for purification of His-tagged and WT SERT, respectively. Although not yielding highly pure SERT protein, the methods were adequate for attainment of protein material applicable for LC-MS/MS based phospho-site analysis of full-length SERT.

Accordingly, phosphoproteomic analysis of purified hSERT was undertaken. The results indicate that the transporter is not highly phosphorylated at the covered (21 out of 38) intracellular Ser/Thr/Tyr residues when expressed in COS7 cells. Furthermore, co-expression of hSERT with constitutively active p38MAPK in HEK293 cells did not result in identification of phosphorylation sites for this kinases at the covered (8 out of 31) intracellular Ser/Thr residues.
5 Interactions of antidepressants with SERT and NET: mutational studies of the S1 substrate binding pocket

5.1 Introduction
Inhibitors of SERT and NET form the cornerstone in the treatment of major depressive disorder with more than 30 drugs in current clinical use\(^2\)\(^1\). Also, widely used psychostimulants such as amphetamine and “ecstasy” (3,4-methylenedioxymethamphetamine, MDMA) have SERT and NET as primary targets\(^2\)\(^5\),\(^2\)\(^9\)\(^4\). Despite the vast clinical importance of inhibitors of SERT and NET, key aspects of their molecular pharmacology have remained largely unknown; including location and structure of inhibitor binding sites, inhibitor binding modes and the mechanism by which inhibitor binding antagonizes transporter function. Several residues in SERT and NET have been indentified that influence binding of inhibitors\(^2\)\(^1\), but the lack of X-ray crystal structures of SERT and NET has limited the development of such information into structural models of inhibitor binding. However, a major step forward was provided with the crystallization of LeuT, a bacterial homologue of the mammalian SLC6 transporters\(^4\)\(^3\). LeuT has proven an excellent model for understanding structural aspects of SLC6 transporters, including the binding sites for inhibitors. Specifically, LeuT structures have provided evidence for the existence of binding sites for inhibitors in two major regions: the central substrate-binding pocket, denoted the S1 site, and the S2 site located in the extracellular vestibule\(^4\)\(^9\)-\(^5\)\(^2\) (Figure 5.2.A). The equivalent regions in the mammalian transporters are likely candidates for harbouring ligand binding sites, and indeed, several LeuT-guided studies of the molecular basis for SERT and NET inhibition have suggested that inhibitors bind in the S1 pocket\(^1\)\(^2\),\(^5\)\(^7\),\(^8\)\(^1\)\(^1\),\(^1\)\(^1\),\(^1\)\(^8\),\(^2\)\(^9\)\(^5\), the S2 pocket\(^5\)\(^1\),\(^5\)\(^2\),\(^6\),\(^2\)\(^9\)\(^6\) or overlapping sites in both the S1 and S2 pockets\(^1\)\(^1\)\(^9\).

The aim of the studies in this chapter was to get a better understanding of the role of the S1 pocket for the inhibitory potency of antidepressant drugs and to get further insight into the structural basis of drug inhibition and SERT/NET selectivity. Specifically, we studied the effect of six single-point mutations of key residues surrounding the S1 pocket of SERT and NET on the inhibitory potency of 15 structurally diverse inhibitors.

5.2 Results

5.2.1 Selection of antidepressants drugs
We selected a panel of 15 representative SERT and NET inhibitors that are either widely used pharmacological tool compounds (MADAM and nisoxetine) or in clinical use as antidepressants (the remaining 13 compounds). These included inhibitors selective for SERT (SRIs), inhibitors selective for NET (NRIs) and dual SERT/NET inhibitors (SNRIs) (Figure 5.1). The selection of compounds aimed to cover structurally diverse inhibitors with distinct selectivity profiles.
Figure 5.1. Overview of the SERT and NET inhibitors evaluated at WT hSERT and hNET and their S1 pocket mutants

Shown are the chemical structures of the 15 compounds tested for inhibitory potency at hSERT and hNET mutants. Compounds are classified according to their hSERT/hNET selectivity profile determined at WT hSERT and hNET (Table 5.2 and Table 5.3): serotonin reuptake inhibitors (SRIs) are defined by $K_i$(WT hNET)/$K_i$(WT hSERT) > 10; norepinephrine reuptake inhibitors (NRIs) are defined by $K_i$(WT hNET)/$K_i$(WT hSERT) < 0.10 and dual serotonin/norepinephrine reuptake inhibitors (SNRIs) are defined by $K_i$(WT hNET)/$K_i$(WT hSERT) in the range 0.1 to 10.

5.2.2 Selection of S1 residues in hSERT and hNET and generation mutant library

To explore the role of specific regions within the S1 pocket as potential determinants for inhibitor activity at SERT and NET, we determined the inhibitory potency ($K_i$) of each of the 15 inhibitors at WT hSERT and WT hNET as well as two sets of hSERT and hNET mutants; each containing six single-point mutations of S1 residues proposed to be key determinants for inhibitor binding (Figure 5.2, Table 5.2 and Table 5.3). The selection of residues for mutation as well as the amino acid substitutions were guided by results from previous mutational studies as well as transporter homology models (Figure 2.5.B). Several LeuT-based homology models of SERT and NET that is formed by residues located in TMs 1, 3, 6 and 8. The S1 pocket is overall a non-polar, hydrophobic cavity except for the region that is believed to accommodate the polar aminoalkyl chain that is present in both SERT/NET substrates and inhibitors, here denoted subsite A. The polar surroundings in subsite A are formed by side chains from TM1 and TM6 residues, and a break in the helical structure of these two TMs further expose backbone carbonyl oxygen and nitrogen atoms as potential hydrogen bonding partners for ligands. Previous mutational analysis of subsite A in SERT have shown that Y95A, D98E and S438T greatly perturb inhibitor binding and that these mutations

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iv The majority of $K_i$ values were determined by MSc students Mette Thomsen and Stinna M. R. Hansen.
confer differential effects on inhibitors\textsuperscript{12,57,67}. Thus, these mutations are excellent for probing potential inhibitor interactions with the subsite A region. The effects of these mutations on the basal 5-HT transport function of hSERT expressed in heterologous cells are well tolerated (Table 5.1) and allow robust determination of inhibitor $K_i$ in 5-HT uptake assays. In contrast to SERT, less mutational data is available for subsite A residues in NET. Initial mutational analysis of the hNET residues Phe72, Asp75 and Ser419, corresponding to the selected subsite A residues in hSERT, showed that transport function of hNET expressed in COS7 cells was highly sensitive to mutation at all three residues (Figure 5.2.D). Specifically, transport activity of the F72A, D75E and D75A mutations could not be measured (Figure 5.2.D); leading to inclusion of only the transport active mutants F72Y and S419T as probes for the equivalent subsite A in hNET. The regions that are located opposite to subsite A within the central S1 pocket (here denoted subsite B and C; Figure 5.2.B) are overall non-polar and formed by hydrophobic side chains from residues in TM3 and TM8 (Figure 5.2.C). To modify subsite B, we selected the hSERT N177S mutant that previously has been shown to perturb inhibitor binding\textsuperscript{57,116}, and the corresponding hNET mutant N153S. For subsite B in hNET, we also included the G149A mutant, which we previously have found to affect inhibitor binding in hNET (unpublished data). For subsite C, we selected the hSERT mutants I172M and F341Y, which previously have been found to greatly affect inhibitor potencies\textsuperscript{57,116,298}. The corresponding mutants in hNET (V148M and F323Y) were generated, but as found previously, the V148M mutant is non-functional\textsuperscript{299}. Two additional mutants in this position (V148F and V148I) were subsequently generated. Whereas the V148F mutant was non-functional, V148I retained functional activity and was included in the mutant library as probe for subsite C in hNET (Figure 5.2.D).

Table 5.1. Uptake kinetics for hSERT and hNET mutants

Transport activities and substrate $K_m$ values were determined in a [$^3$H]5-HT (hSERT) or [$^3$H]DA (hNET) uptake assay. Results are presented as mean ± S.E.M. from at least three independent experiments each performed in triplicate.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$K_m$ (μM)</th>
<th>n</th>
<th>% of WT</th>
<th>n</th>
<th>Transport activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hSERT WT</td>
<td>0.98 ± 0.09</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Y95A</td>
<td>0.22 ± 0.04\textsuperscript{a}</td>
<td>3</td>
<td>66 ± 3</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>D98E</td>
<td>0.22 ± 0.06\textsuperscript{a}</td>
<td>3</td>
<td>47 ± 2</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>I172M</td>
<td>0.77 ± 0.05</td>
<td>4</td>
<td>86 ± 3</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>N177S</td>
<td>4.24 ± 0.12\textsuperscript{a}</td>
<td>3</td>
<td>66 ± 3</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>F341Y</td>
<td>0.08 ± 0.02\textsuperscript{a}</td>
<td>3</td>
<td>48 ± 3</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>S438T</td>
<td>0.14 ± 0.02\textsuperscript{a}</td>
<td>3</td>
<td>32 ± 2</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>hNET WT</td>
<td>0.52 ± 0.11</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>14</td>
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<tr>
<td>F72Y</td>
<td>0.26 ± 0.16</td>
<td>4</td>
<td>50 ± 2</td>
<td>48</td>
<td></td>
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<tr>
<td>V148I</td>
<td>0.35 ± 0.05</td>
<td>3</td>
<td>29 ± 3</td>
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<tr>
<td>G149A</td>
<td>0.39 ± 0.21</td>
<td>3</td>
<td>48 ± 3</td>
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<tr>
<td>N153S</td>
<td>1.67 ± 0.20</td>
<td>4</td>
<td>19 ± 2</td>
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</tr>
<tr>
<td>F323Y</td>
<td>1.35 ± 0.18</td>
<td>4</td>
<td>10 ± 1</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>S419T</td>
<td>2.52 ± 0.40</td>
<td>4</td>
<td>11 ± 1</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Values from Andersen et al. 2010\textsuperscript{57}.
Figure 5.2. Overview of the position of hSERT and hNET mutations and their effects on transport activity

A. Cross-sectional view of hSERT showing the position of the S1 and S2 pockets. The shown hSERT homology model\textsuperscript{57} is based on a crystal structure of LeuT in an extracellular-facing substrate-occluded conformation\textsuperscript{43}. B. Cross-sectional top view into the S1 pockets in the homology model of hSERT (left) and a model of hNET (right). The hNET model was made by introduction of hNET residues in non-conserved positions surrounding the substrate binding pocket in hSERT. The amino acid residues, which were subjected to mutagenesis are shown as stick representations and the surface contour is coloured according to physical properties. Subsite A is defined as the polar region surrounding Asp98 (hSERT) and Asp75 (hNET), which co-ordinates the charged primary amine of the monoamine substrates, whereas subsite B and C are largely hydrophobic regions located opposite each other. C. Alignment of the residues in hSERT and hNET, which contribute side chains to regions surrounding subsite A, B and C. Residues examined in this study are highlighted in yellow. D. Graphical summary of the transport activity of point-mutants of the S1 residues shown in panel B. Bars represent mean ± S.E.M. expressed as % of transport activity for WT from at least three independent experiments each performed in triplicate.

5.2.3 Inhibitory potency of antidepressants at hSERT and hNET mutants

To determine the impact of selective perturbations in subsite A, B and C of the S1 pocket in hSERT and hNET (Figure 5.2), we determined $K_i$ for inhibition by each of the 15 compounds of [\textsuperscript{3}H]-labeled substrate uptake by the hSERT and hNET mutants expressed in COS7 cells (Table 5.2 and Table 5.3). $K_i$ values were determined from concentration-inhibition curves constructed from measurements of [\textsuperscript{3}H]5-HT (SERT) and [\textsuperscript{3}H]DA (NET) uptake with increasing concentrations of inhibitor (Figure 5.3). We used the resulting data set to construct a sensitivity profile for each compound towards the 6 mutants in hSERT and hNET. Specifically, for each drug and mutant combination, we calculated the fold-change in $K_i$ from paired experiments of mutant and WT as $K_i$(mutant)/$K_i$(WT) or $-K_i$(WT)/$K_i$(mutant) for mutants decreasing or increasing inhibitory potency, respectively. These values are displayed in Figure 5.4 as a heat map.
Figure 5.3. Analysis of inhibitor potencies at WT and mutant hSERT and hNET
Dose-response curves from representative experiments of inhibition by paroxetine and milnacipran of $[^3]$H5-HT uptake and inhibition by nisoxetine and milnacipran of $[^3]$HDA uptake in COS7 cells transfected with hSERT and hNET cDNA, respectively, carrying point mutations at the selected amino acid positions in S1: Y95A, D98E, I172M, N177S, F341Y and S438T for hSERT and F72Y, V148I, G149A, N153S, F323Y and S419T for hNET. Data points represent mean ± S.E.M. from triplicate determinations of accumulated radioactivity in cells incubated with $[^3]$H5-HT or $[^3]$HDA for SERT and NET, respectively, in the presence of increasing concentrations of inhibitor. Uptake has been normalized to % uptake of cells incubated in absence of inhibitor. The normalized data were plotted versus log of the molar concentration of inhibitor and fitted to a non-linear one-site competition curve.
Table 5.2. Impact of hSERT mutations on the inhibitory potencies of the 15 compounds
The compounds are grouped according to their SERT/NET selectivity. Ki values were determined in a [3H]5-HT uptake inhibition assay. Results are presented as mean ± S.E.M.; n = 5-12 experiments each performed in triplicate. * p<0.05 Ki value significantly different from hSERT WT (Student’s t-test).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>WT</th>
<th>Y95A</th>
<th>D98E</th>
<th>I172M</th>
<th>N177S</th>
<th>F341Y</th>
<th>S438T</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SRIs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>167 ± 22</td>
<td>1397 ± 165 *</td>
<td>59 ± 15 *</td>
<td>1418 ± 41 *</td>
<td>659 ± 131 *</td>
<td>214 ± 33</td>
<td>36 ± 9 *</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>255 ± 61</td>
<td>2830 ± 177 *</td>
<td>857 ± 113 *</td>
<td>4304 ± 929 *</td>
<td>2238 ± 244 *</td>
<td>487 ± 67 *</td>
<td>438 ± 102</td>
</tr>
<tr>
<td>MADAM</td>
<td>9 ± 3</td>
<td>905 ± 188 *</td>
<td>12 ± 2</td>
<td>299 ± 39 *</td>
<td>54 ± 16 *</td>
<td>13 ± 4</td>
<td>67 ± 27 *</td>
</tr>
<tr>
<td>Paroxetine</td>
<td>24 ± 6</td>
<td>1759 ± 306 *</td>
<td>195 ± 37 *</td>
<td>61 ± 12 *</td>
<td>143 ± 31 *</td>
<td>741 ± 73 *</td>
<td>2885 ± 559 *</td>
</tr>
<tr>
<td>Sertraline</td>
<td>242 ± 33</td>
<td>1455 ± 331 *</td>
<td>472 ± 110 *</td>
<td>1918 ± 295 *</td>
<td>712 ± 146 *</td>
<td>342 ± 78</td>
<td>6551 ± 1435 *</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>147 ± 48</td>
<td>10862 ± 1668 *</td>
<td>775 ± 104 *</td>
<td>14740 ± 3182 *</td>
<td>1454 ± 208 *</td>
<td>863 ± 158 *</td>
<td>88 ± 19</td>
</tr>
<tr>
<td>(S)-Citalopram</td>
<td>32 ± 1a</td>
<td>408 ± 54 **</td>
<td>856 ± 117 **</td>
<td>13946 ± 3167 *</td>
<td>332 ± 25 *</td>
<td>1691 ± 208 *</td>
<td>7693 ± 874 **</td>
</tr>
<tr>
<td><strong>SNRIs</strong></td>
<td></td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>Amitriptyline</td>
<td>188 ± 36</td>
<td>227 ± 36</td>
<td>712 ± 100 *</td>
<td>1705 ± 215 *</td>
<td>929 ± 190 *</td>
<td>1137 ± 277 *</td>
<td>2655 ± 425 *</td>
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<tr>
<td>Clomipramine</td>
<td>91 ± 9</td>
<td>324 ± 40 *</td>
<td>158 ± 28 *</td>
<td>1519 ± 182 *</td>
<td>394 ± 27 *</td>
<td>303 ± 21 *</td>
<td>641 ± 127 *</td>
</tr>
<tr>
<td>Duloxetine</td>
<td>62 ± 4</td>
<td>1488 ± 90 *</td>
<td>161 ± 36 *</td>
<td>1541 ± 155 *</td>
<td>314 ± 39 *</td>
<td>147 ± 21 *</td>
<td>28 ± 5 *</td>
</tr>
<tr>
<td>Imipramine</td>
<td>163 ± 24</td>
<td>1310 ± 151 *</td>
<td>530 ± 129 *</td>
<td>3227 ± 617 *</td>
<td>850 ± 140 *</td>
<td>785 ± 149 *</td>
<td>1241 ± 106 *</td>
</tr>
<tr>
<td>Milnacipran</td>
<td>68 ± 8</td>
<td>6989 ± 1452 *</td>
<td>87 ± 6</td>
<td>2071 ± 342 *</td>
<td>917 ± 90 *</td>
<td>470 ± 58 *</td>
<td>570 ± 48 *</td>
</tr>
<tr>
<td><strong>NRIs</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Atomoxetine</td>
<td>329 ± 47</td>
<td>498 ± 122</td>
<td>1335 ± 431 *</td>
<td>7694 ± 1269 *</td>
<td>1242 ± 185 *</td>
<td>694 ± 111 *</td>
<td>1535 ± 292 *</td>
</tr>
<tr>
<td>Nisoxetine</td>
<td>977 ± 210</td>
<td>4790 ± 645 *</td>
<td>2915 ± 567 *</td>
<td>16815 ± 3362 *</td>
<td>2662 ± 273 *</td>
<td>1921 ± 213 *</td>
<td>1959 ± 222 *</td>
</tr>
<tr>
<td>Maprotiline</td>
<td>10682 ± 1363</td>
<td>8044 ± 1901</td>
<td>7258 ± 1334</td>
<td>10878 ± 1605</td>
<td>11837 ± 2809</td>
<td>11731 ± 2392</td>
<td>4477 ± 1098 *</td>
</tr>
</tbody>
</table>

* Values from Andersen et al. 201057.
Table 5.3. Impact of hNET mutations on the inhibitory potencies of the 15 compounds
The compounds are grouped according to their SERT/NET selectivity. \( K_i \) values were determined in a \( [\text{3H}] \) DA uptake inhibition assay. Results are presented as mean ± S.E.M.; \( n = 4-11 \) experiments each performed in triplicate. * \( p<0.05 \) \( K_i \) value significantly different from hNET WT (Student’s \( t \)-test).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>WT</th>
<th>F72Y</th>
<th>V148I</th>
<th>G149A</th>
<th>N153S</th>
<th>F323Y</th>
<th>S419T</th>
</tr>
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<tr>
<td><strong>SERTs</strong></td>
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<tr>
<td>Fluvoxamine</td>
<td>3332 ± 522</td>
<td>6212 ± 1217 *</td>
<td>379 ± 90 *</td>
<td>579 ± 74 *</td>
<td>4512 ± 632</td>
<td>1702 ± 142 *</td>
<td>286 ± 43 *</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>5813 ± 520</td>
<td>8975 ± 831 *</td>
<td>1257 ± 109 *</td>
<td>1974 ± 243 *</td>
<td>8655 ± 1104 *</td>
<td>7255 ± 649</td>
<td>10769 ± 610 *</td>
</tr>
<tr>
<td>MADAM</td>
<td>364 ± 65</td>
<td>287 ±44</td>
<td>38 ± 10 *</td>
<td>60 ± 13</td>
<td>1541 ± 369 *</td>
<td>842 ± 202 *</td>
<td>101 ± 13 *</td>
</tr>
<tr>
<td>Paroxetine</td>
<td>385 ± 89</td>
<td>587 ± 100</td>
<td>418 ± 103</td>
<td>378 ± 86</td>
<td>641 ± 164</td>
<td>1406 ± 243 *</td>
<td>1866 ± 262 *</td>
</tr>
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<td>Sertraline</td>
<td>8556 ± 1384</td>
<td>4972 ± 825</td>
<td>2453 ± 664 *</td>
<td>5694 ± 1466</td>
<td>4343 ± 866 *</td>
<td>4499 ± 996 *</td>
<td>15200 ± 3192 *</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>1830 ± 64</td>
<td>1770 ± 141</td>
<td>100 ± 19 *</td>
<td>1163 ± 78 *</td>
<td>2044 ± 534</td>
<td>2753 ± 413 *</td>
<td>4747 ± 750 *</td>
</tr>
<tr>
<td>(S)-Citalopram</td>
<td>23421 ± 2102</td>
<td>5649 ± 1356 *</td>
<td>3534 ± 737 *</td>
<td>19357 ± 2773</td>
<td>21717 ± 3528</td>
<td>28447 ± 5443</td>
<td>53842 ± 7845 *</td>
</tr>
<tr>
<td><strong>SNRIs</strong></td>
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<tr>
<td>Amitriptyline</td>
<td>477 ± 95</td>
<td>480 ± 80</td>
<td>575 ± 77</td>
<td>515 ± 58</td>
<td>1269 ± 145 *</td>
<td>2330 ± 346 *</td>
<td>1593 ± 312 *</td>
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<tr>
<td>Clomipramine</td>
<td>420 ± 27</td>
<td>462 ± 55</td>
<td>275 ± 43 *</td>
<td>326 ± 58</td>
<td>939 ± 123 *</td>
<td>826 ± 205 *</td>
<td>747 ± 67</td>
</tr>
<tr>
<td>Duloxetine</td>
<td>81 ± 19</td>
<td>100 ± 26</td>
<td>152 ± 2 *</td>
<td>211 ± 27 *</td>
<td>256 ± 51 *</td>
<td>253 ± 61 *</td>
<td>65 ± 7</td>
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<tr>
<td>Imipramine</td>
<td>130 ± 31</td>
<td>230 ± 54</td>
<td>230 ± 116</td>
<td>316 ± 60 *</td>
<td>348 ± 60 *</td>
<td>448 ± 47 *</td>
<td>558 ± 324 *</td>
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<tr>
<td>Milnacipran</td>
<td>52 ± 9</td>
<td>5 ± 1 *</td>
<td>3 ± 1 *</td>
<td>118 ± 22 *</td>
<td>76 ± 18</td>
<td>586 ± 195 *</td>
<td>2072 ± 298 *</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Atomoxetine</td>
<td>9 ± 2</td>
<td>7 ± 1</td>
<td>2 ± 1 *</td>
<td>31 ± 5 *</td>
<td>21 ± 2 *</td>
<td>35 ± 8 *</td>
<td>114 ± 18 *</td>
</tr>
<tr>
<td>Nisoxetine</td>
<td>5 ± 1</td>
<td>2 ± 1 *</td>
<td>2 ± 1 *</td>
<td>30 ± 5 *</td>
<td>11 ± 1 *</td>
<td>47 ± 12 *</td>
<td>139 ± 58 *</td>
</tr>
<tr>
<td>Maprotiline</td>
<td>153 ± 34</td>
<td>91 ± 27</td>
<td>756 ± 237 *</td>
<td>275 ± 77</td>
<td>358 ± 88</td>
<td>761 ± 161 *</td>
<td>899 ± 223 *</td>
</tr>
</tbody>
</table>
5.2.4 Impact of subsite A mutations on inhibitor potency

We probed the role of subsite A for compound potency by the Y95A, D98E and S438T mutants in hSERT, and the F72Y and S419T mutants were used to probe the same region in hNET (Figure 5.2.B-C). In hSERT, the Y95A mutation removes a potentially hydrogen bonding hydroxyl group from the S1 pocket and likely alters the size and shape of the pocket within subsite A. Notably, Y95A promoted 10- to 100-fold loss of potency for the majority of the tested SRIs and SNRIs with exception of sertraline (6-fold loss), fluvoxamine (8-fold loss) and the structurally similar tricyclic antidepressants (TCAs) imipramine (8-fold loss), clomipramine (4-fold loss) and amitriptyline (no significant change) (Figure 5.4). The NRIs were the least affected class of inhibitors, displaying no significant change in potency except for a 6-fold loss of potency for nisoxetine. Thus, these results corroborate the suggested key role of Tyr95 for high-affinity inhibitor binding of SRIs and SNRIs in hSERT.

The lack of transport activity for the equivalent mutant in hNET (F72A) prevents similar analysis in hNET. Alternative perturbation of this residue in the form of the F72Y mutation did not significantly decrease potency of any compound, suggesting that this mutation does not significantly perturb the steric or physiochemical properties of this part of the S1 pocket. A possible explanation for this observation is that the aromatic part of Phe72, which is retained in the F72Y mutant, is important for accommodation of compounds in this part of the S1 pocket. Combined, our mutational analysis of Phe72 does therefore not provide conclusive information regarding the general role of Phe72 for inhibitor binding in hNET. However, F72Y did display 18- and 6-fold gain of potency for the SNRI milnacipran and the SRI (S)-citalopram, respectively (Figure 5.4). As F72Y introduces only a hydrogen bond donor hydroxyl group into the S1 pocket, the highly selective gain of potency may suggest that the hydroxyl group enables hydrogen bond interaction to these two inhibitors. Furthermore, this idea is supported by the observation that removal of the WT hydroxyl group in hSERT by the Y95A mutation promotes a dramatic 114-fold loss of potency for milnacipran; indicating that milnacipran is in close proximity to this residue in both transporters. However, further mutational analysis of Tyr95 and Phe72 in hSERT and hNET, respectively, is required to verify this idea. As mentioned further below, milnacipran also displayed 10- and 43-fold loss of potency at the equivalent hSERT and hNET mutations S438T and S419T, respectively, which furthermore suggests that milnacipran occupies and adopts similar binding modes within subsite A of the S1 pocket in hSERT and hNET (Figure 5.4).

The impact of the D98E and S438T mutants in subsite A in hSERT on the putative accommodation of the aminoalkyl chain of the substrate 5-HT and the TCAs imipramine, clomipramine, and amitriptyline has been extensively characterized previously. Specifically, Asp98 has been suggested to be an important contact point in the S1 pocket of SERT by forming a direct ionic interaction with the charged amino group of 5-HT and SERT inhibitors. However, we found that the D98E mutation only had substantial (≥10-fold) impact on the potency of (S)-citalopram and paroxetine.
This result is surprising, since all 15 tested inhibitors contain an amino group that potentially can coordinate the acidic side chain of Asp98. The general weak effect of D98E on inhibitor potencies suggests that the proposed role of the acidic side chain of Asp98 as a common key contact point for inhibitors containing an amine may be overestimated. However, as D98E does not remove the negative charge at this position, it may also be that this mutation simply is a poor probe for assessing potential ligand interactions with Asp98.

We have previously described the effect of S438T on the potency of the inhibitors included in this study except for fluvoxamine, milnacipran, atomoxetine and maprotiline. From these previous studies, it was suggested that the additional methyl group that is introduced into the S1 pocket by the S438T mutation promotes a steric clash with selected SRIs and SNRIs. The additional examination of fluvoxamine, milnacipran, atomoxetine and maprotiline at S438T corroborates our previous findings: introduction of a methyl group into the protein at this position in subsite A has highly selective effects on inhibitor potency that seem to correlate with the structure of aminoalkyl chain present on the inhibitor compounds. Specifically, the potency of milnacipran is decreased by this mutation, whereas fluvoxamine, atomoxetine and maprotiline experience no significant change or minor increases in potency. Like other S438T-insensitive compounds such as fluoxetine, duloxetine and nisoxetine, the insensitive compounds contain aminoethyl or aminopropyl chains with no or a single methyl group at the amine, making these groups less bulky and prone to experiencing a steric clash with the methyl group introduced by S438T if these are orientated towards subsite A. Milnacipran with its bulkier cyclopropyl-containing aminoalkyl chain may thus experience a steric clash by S438T, which clearly suggests that milnacipran occupies subsite A of the S1 pocket.

In hNET, the S419T mutation (equivalent to S438T in hSERT) also showed highly selective effects across the tested inhibitors by inducing large decreases in potency for paroxetine (11-fold), milnacipran (43-fold), atomoxetine (10-fold) and nisoxetine (22-fold), whereas a 14-fold increase in potency was observed for the SRI fluvoxamine. Interestingly, atomoxetine and nisoxetine, which were insensitive to S438T in hSERT, are highly affected by S419T in hNET, suggesting that the alkyl amine side chains of these NRIs is in close proximity to Thr419 thus occupying subsite A in hNET.
In subsite B, Asn177 in hSERT has been suggested to contribute to accommodation of polar ligand moieties. This residue is conserved in hNET. To probe for inhibitor interactions in subsite B of the S1 pocket, we employed the N177S mutation in hSERT and the equivalent N153S mutation in hNET; hereby changing the potential hydrogen bonding properties of the side chain, which previously has been shown to perturb inhibitor binding in hSERT. In hSERT, N177S decreased the inhibitory potency of the majority of the tested SRIs and SNRIs (5- to 15-fold), while minor changes were observed for the NRIs (Figure 5.4). These results suggest that subsite B could be an important region for accommodation of inhibitors.

In hNET, the N153S mutant did not significantly affect any of the inhibitors tested (Figure 5.4). We therefore also included the G149A mutation, which we recently have found to be an important determinant for high-affinity binding of the (S)-citalopram analog talopram in hNET (Andersen et al., in press). This mutation likely promotes a subtle increase in hydrophobicity and bulk size of the protein in subsite B; thus potentially decreasing the ability of ligands to occupy this region of the S1 pocket in hNET. We find that the inhibitory potency of all SRIs and SNRIs at hNET is not significantly decreased by the G149A mutation (Figure 5.4). However, the high-affinity NRIs atomoxetine and...
nisoxetine display 4- and 6-fold decrease in potency (Figure 5.4). Combined with the observation of significant 6- and 8-fold increase in potency at hNET for the SRIs fluvoxamine and MADAM induced by G149A, these data suggest that subsite B is important for accommodation of these inhibitors in the hNET S1 pocket and furthermore suggest Gly149 as a determinant for SERT/NET selectivity as the equivalent residue in hSERT is Ala173.

5.2.6 Impact of subsite C mutations on inhibitor potency

Previous mutational analyses of SERT and NET have identified the residues Ile172 and Phe341 in hSERT and the NET residue Val148 (equivalent to Ile172 in hSERT) as key residues in subsite C for inhibitor binding. In models of hSERT, the side chain of Ile172 forms a hydrophobic ridge (Figure 5.2.B) and has been suggested to be a key determinant for the shape of the S1 pocket. Previous studies have shown that the I172M mutation has marked selectivity in terms of its impact on the affinity of SERT substrates and inhibitors: I172M produces more than 700-fold loss of affinity for (R,S)-citalopram, whereas high-affinity binding of other SRIs and SNRIs, such as paroxetine and amitriptyline is not affected. Characterization of the 15 inhibitors included in the present study across I172M showed that all compounds except for paroxetine and maprotiline suffered a marked decrease in potency (9- to 127-fold). 11 of the tested compounds have previously been tested at I172M and the observed changes in potencies correspond well with the previously reported changes. Our results expand the previous findings of Ile172 as a major determinant for the inhibitory potency of structurally diverse SERT and NET inhibitors, and strongly support the role of the side chain of Ile172 as key for accommodation of the affected compounds within the S1 pocket in hSERT.

In hNET, we probe the role of Val148 (equivalent to Ile172 in hSERT) with the V148I mutation. In contrast to the consistent loss of potency observed for all compounds except for paroxetine and maprotiline by the I172M mutation in hSERT, we found no significant loss of potency for any compound by the V148I mutation in hNET except for a 5-fold decrease for the NRI maprotiline (Figure 5.4). This might be the result of the conservative nature of the V148I mutation in NET. Indeed, previous binding studies have shown 14-18-fold decrease in affinity of nisoxetine for NET V148M compared to WT. However, V148I produced marked gain of potencies for several compounds (Figure 5.4). Specifically, V148I induced 4- to 18-fold gain of potency for all tested SRIs with the exception of paroxetine; hereby identifying Val148 as a major determinant for SERT/NET selectivity for these compounds as Ile172 is the equivalent residue in hSERT. Gain of potency was also observed for the SNRIs duloxetine (6-fold) and milnacipran (25-fold) and the NRI atomoxetine (6-fold). Taken together, these results add further support for a key role of Val148 in hNET; potentially similar to Ile172 in hSERT, as a major determinant for inhibitor accommodation in the S1 pocket in hNET.

Introduction of a hydroxyl group at Phe341 (F341Y) in hSERT has been proposed to perturb the shape of the hydrophobic ridge formed by Ile172 and Phe341 as well as introducing polarity to the region, hereby
potentially altering the accessibility to the hydrophobic crevice in subsite C of the S1 pocket \(^{57,116,118}\) (Figure 5.2.B). Notably, we observe very selective effects on compound potency by F341Y in hSERT and for the equivalent F323Y mutation in hNET. The majority of the tested compounds are not markedly affected by these mutations in hSERT and hNET (Figure 5.4). However, marked decreases in potency are found for (S)-citalopram (56-fold) and paroxetine (42-fold) at hSERT, and milnacipran (13-fold) at hNET (Figure 5.4), whereas 6- to 8-fold decreases are observed for the SRI venlafaxine and the SNRIs amitriptyline and milnacipran at hSERT and the NRIs nisoxetine and maprotiline as well as the SNRI amitriptyline at hNET. Interestingly, all of the SNRIs experience similar decreases in potency at hSERT F341Y and hNET F323Y. This may indicate that these compounds have common orientations in hSERT and hNET relative to subsite C in the S1 pockets. For hSERT, it is only (S)-citalopram among the evaluated SRIs for which the loss of potency produced by F341Y correlates with the loss of potency observed for I172M. Generally, this might indicate that subsite C contributes differentially to accommodation of the tested SRIs in the S1 pocket in hSERT. In accordance with this idea, striking differences are also observed in hNET between the effects of the V148I and F323Y mutations on the potencies of the NRIs atomoxetine and nisoxetine and the SNRI milnacipran in particular, for which V148I produced a 25-fold gain of potency and F323Y produced 13-fold loss of potency (Figure 5.4).

### 5.3 Discussion

The arrival of LeuT crystal structures and their validation as excellent templates for comparative modeling of mammalian SLC6 transporters have provided a unique opportunity to translate mutational data, compound structure-activity relationship studies and pharmacophore data into atomic-level transporter models. This gives an unprecedented insight into the molecular basis for recognition of inhibitors by the transporters. Recently, such studies have established the binding modes and molecular mechanisms of prominent SLC6 neurotransmitter transporter inhibitors such as cocaine at DAT\(^{59}\) and the prototypical antidepressants (S)-citalopram\(^{57,116}\) and imipramine\(^{111,119}\) at hSERT. Although these studies utilize extensive functional experimentation to guide modeling and ligand-docking approaches and to validate the resulting molecular models, they also benefit from a large body of existing data made available previously for these important inhibitors. A similar level of information is not presently available for the vast majority of clinically relevant SERT and NET inhibitors. We here report the characterization of 15 prototypical SERT and NET inhibitors, most of which are in clinical use as antidepressants, at S1 binding pocket mutants in hSERT and hNET. This comprehensive data set, describing specific molecular determinants within SERT and NET for recognition of a range of antidepressants, represents the first molecular data on the interactions with the transporters for many of the included inhibitors. The data set represents a valuable platform for performing molecular modeling of the individual compounds at SERT and NET needed for understanding the inhibitory mechanism of the different antidepressants at the molecular level and is of importance for future structure-based design of novel therapeutics.
In the present study, we do not apply ligand docking of any of the investigated compounds into molecular models of SERT and NET to further analyze the implications of the mutational data. However, analysis of the data set within the framework of previously established homology models of SERT and NET, allows us to derive several notable features regarding SERT and NET inhibition by the tested compounds. First, our data set is useful for assessing probabilities of the S1 pocket as the primary site for high-affinity binding of the various antidepressants. As the S1 pocket harbours the central substrate binding site, classical interpretation of the pharmacologically well-established competitive mode of inhibition of most antidepressants\(^{112}\) is that their binding sites overlap with the substrate binding site; thus indicating that the central S1 pocket holds the primary binding site. However, recent findings from structural and biophysical studies of LeuT have suggested that the S2 pocket harbours a functionally important substrate binding site\(^{55,91,93}\) that can also be occupied by inhibitors\(^{50-53}\). Furthermore, studies of the role of the equivalent S2 pocket in mammalian SLC6 transporters, including SERT and NET\(^{51,52}\), have identified S2 mutations that can perturb high-affinity inhibitor binding; possibly by removing direct ligand interactions with S2 residues.

It is therefore important to establish antidepressant binding site locations in hSERT and hNET as a first step towards modeling their interactions with the transporters. Figure 5.5 summarizes compound sensitivity towards the mutations that we here employ to probe the role of the S1 pocket for binding of antidepressants at hSERT. Four compounds, three SRIs and one SNRI, are found to display profound and consistent sensitivity towards perturbation in all three subsites of the S1 pocket (here defined as >5-fold loss of potency induced by at least five of the six S1 mutations) (Figure 5.5, left panel). For compounds with this level of sensitivity, it seems clear that the S1 pocket must harbour their high-affinity binding site. (\(S\))-Citalopram has previously been extensively studied by us and others\(^{57,116}\) and it was established that (\(S\))-citalopram most likely assumes a binding mode, where it is deeply buried within the S1 pocket and occupies all three sub sites of the S1 pocket. The other three highly sensitive compounds (paroxetine, venlafaxine and milnacipran) have not yet been subjected to similar mutational analysis and modeling studies. However, on basis of their similar sensitivity profile to (\(S\))-citalopram, we here propose that these compounds bind exclusively within the S1 pocket.

We find six compounds, three SRIs and three SNRIs, that display medium sensitivity (here defined as >5-fold loss of potency induced by three to four mutations) towards the six S1 mutations in hSERT (Figure 5.5, middle panel). Fluoxetine, imipramine and sertraline are among the compounds with medium sensitivity. Binding modes of these inhibitors have recently been proposed from ligand docking studies supported by extensive biostructural, mutational and kinetic analyses to guide and validate inhibitor binding models\(^{51,52,111,118,119}\). On basis of mutational analysis of S2 residues in hSERT, Zhou \textit{et al.}\(^{51,52}\) have proposed that the binding sites for sertraline, fluoxetine and imipramine in hSERT are located in the lower part of the S2 pocket, similarly to what has been found for LeuT. Recently, Sarker \textit{et al.}\(^{119}\) proposed a model for imipramine binding in hSERT, where the binding site overlaps both the S2 and the
S1 pocket, such that only the dimethylaminopropyl chain of imipramine is anchored in the S1 pocket. In contrast, Sinning et al.\textsuperscript{111} arrived at a binding mode for imipramine in which the inhibitor molecule is completely buried within the S1 pocket. In relation to these models, it is noteworthy that we find sertraline, fluoxetine and imipramine to display medium sensitivity towards S1 perturbation; suggesting that these compounds might not occupy the entire S1 pocket.

![Figure 5.5. Summary of compound sensitivity towards S1 mutants in hSERT](image)

**Figure 5.5. Summary of compound sensitivity towards S1 mutants in hSERT**

Schematic overview of the S1 pocket and the mutated residues with compounds grouped according to their sensitivity. The class of highly sensitive compounds (left panel) is defined by displaying more than 5-fold loss of potency at five mutants or more, whereas medium sensitivity (middle panel) is defined by displaying more than 5-fold loss of potency at three to four mutants. Low or no sensitivity (right panel) is defined by displaying more than 5-fold loss of potency at two mutants or less. Residue color coding illustrates the average impact of mutation on the compound class: dark red indicates that mutation of the residue affects 80% to 100% of the compounds, light red indicates 33% to 80% of the compounds and gray indicates 0% to 33% of the compounds.

We find five compounds, the NRIs maprotiline, nisoxetine and atomoxetine, the SNRI clomipramine and the SRI fluvoxamine that display no or very limited sensitivity towards the S1 mutations (Figure 5.5, right panel). This suggests that these inhibitors only bind partly or not at all in the S1 pocket in hSERT, or that they adopt different binding modes than the inhibitors displaying high and medium sensitivity towards the six evaluated mutations, which allow them to better tolerate modifications at the three evaluated subsites.

In general, we observe large differences between the level of compound sensitivity toward the mutations at hSERT and hNET (Figure 5.4). Specifically, whereas all of the hSERT mutations produce marked perturbations of potency across the majority of SRIs and SNRIs, only the SRI paroxetine, the SNRI milnacipran and the high affinity NRIs atomoxetine and nisoxetine display marked loss of potency...
towards the employed S1 mutations in hNET (Figure 5.4). This limits our ability to conclude on the role of the S1 pocket for inhibitor binding in hNET. A likely explanation for the generally much lower impact of the hNET mutations to perturb compound potency may lay in the fact that the selection of the employed hNET mutations was based predominantly on previous mutational analysis of SERT that focused on characterizing only a small subset of inhibitors (e.g. the SRIs citalopram and fluoxetine and the SNRI imipramine and close analogs of these). Thus, we may likely have biased our mutant library towards S1 features that are important for high-affinity inhibitor binding in hSERT, but not in hNET.

An inherent caveat of the use of site-directed mutagenesis to probe potential ligand-protein interactions in allosteric proteins is that effects on apparent ligand affinity can be caused indirectly by long-range perturbations of the ligand binding site, or by shifting of equilibriums between different conformational states. However, with the possible exception of Y95A in hSERT, all the employed mutations introduce conservative changes that are entirely confined within the S1 pocket and well-separated from the gating region and the S2 site (Figure 5.6). Therefore, consistent changes in potencies produced by two or more of these mutations seem highly likely to represent localized direct effects on compound accommodation in the S1 pocket, and not by long-range allosteric effects on a different binding pocket. Thus, the data set provided by the current work represents a valuable expansion of the knowledge of the role of key S1 residues for potential antidepressant accommodation in this region of hSERT and hNET, and identifies several novel structural determinants important for high-affinity inhibition of the transporters. Combined with previous data, this can serve as a platform for future modeling of the molecular mechanisms of antidepressants at these transporters and aid development of novel inhibitors.

Figure 5.6. Position of mutated S1 residues in hSERT and hNET relative to the S2 pocket
Cross-sectional side view into the S1 pockets in the models of hSERT (left) and hNET (right) (see legend to Figure 5.2.B) with indication of the relative position of the S2 pocket.
5.4 Summary

We have examined the inhibitory potency (\(K_i\)) of 15 SERT/NET inhibitors from different drug classes at six hSERT and hNET single-point mutants of key residues in the central S1 binding pocket. In general, the majority of compounds were more sensitive to mutations introduced in hSERT compared to those introduced in hNET; as well in terms of the number of mutated residues affecting the \(K_i\) as the degree to which \(K_i\) was affected at the individual residues. At hSERT, three SRIs and one SNRI were highly sensitive to mutation at at least five of the six residues, strongly suggesting that the S1 pocket harbours their high-affinity binding site. Another set of compounds, three SRIs and three SNRIs, displayed medium sensitivity towards the mutations in hSERT with substantial loss of potency at half of the introduced mutants, suggesting a binding site overlapping but possibly not entirely occupying the S1 pocket. The last group of compounds, one SRI, one SNRI and the three NRIs, displayed limited sensitivity towards S1 mutations in hSERT, suggesting that these inhibitors bind only partly or not at all in the S1 pocket in hSERT, or that they adopt different binding modes than the inhibitors displaying high and medium sensitivity. In hNET, only a few compounds displayed marked loss of potency at the employed mutations, limiting the ability to draw final conclusions on the role of the S1 pocket for inhibitor binding in hNET. However, the majority of compounds showed significant gain of potency at the hNET V148I mutant. This suggests an important role of this residue for accommodation of inhibitors in hNET, potentially similar to the role of Ile172 in hSERT, and furthermore highlights this position in the S1 pocket as a major determinant for SERT/NET selectivity.

Generally, the data set provided by the current work represents a valuable expansion of the knowledge of the role of key S1 residues for potential antidepressant accommodation in this region of hSERT and hNET, and identifies several novel structural determinants important for high-affinity inhibition of the transporters.
6 Concluding remarks and perspectives

Phosphorylation is one of the most important reversible posttranslational protein modifications in nature; being involved in multiple cellular processes, including metabolic pathways, homeostasis, cellular signalling and communication and cell survival. The monoamine neurotransmitter transporters are extensively regulated by kinase-linked pathways; however, without knowledge of specific phosphorylation sites, the underlying molecular mechanisms remain elusive. This study aimed at identifying and characterizing phosphorylation sites in SERT. Initially, we conducted an in vitro phosphorylation assay, by which we identified five residues to be substrate for phosphorylation by CaMKII (Ser13), PKC (Ser149, Ser277 and Thr603) and p38MAPK (Thr616). Despite the fact that the nature of the in vitro phosphorylation assay is far from conditions found in vivo, the approach offers the possibility of identifying sequences in the protein that can act as substrate for phosphorylation, which leads to identification of specific phosphorylation sites for specific kinases; a task that is not easily performed by other methods. We studied possible functional consequences of phosphorylation at the identified sites for transport kinetics and surface expression of hSERT expressed in a heterologous expression system. We found evidence that the Thr276-Ser277 motif in IL2 is involved in a transient PKC-dependent down-regulation of SERT, whereas the persistent down-regulation caused by activation of PKC was found independent of potential phosphorylation at the three identified PKC sites. The identified site for p38MAPK, Thr616, seemed not to be important for p38MAPK-dependent regulation of SERT transport capacity. In this perspective, our initial analysis of the identified phosphorylation sites offered only modest novel insight into the involvement of phosphorylation of specific residues in kinase-dependent regulation of SERT activity and surface expression. Although one interpretation of our results is that phosphorylation sites identified in vitro cannot readily be correlated to phosphorylation events in vivo, another possible explanation of our observations is that the regulatory processes for SERT in the host cell lines used in our study, although dependent on kinase activity, are independent on direct transporter phosphorylation. Although previous studies have found correlation between transporter phosphorylation and transport capacity for both PKC- and p38MAPK-dependent regulation of SERT, it has not been demonstrated that the regulatory processes actually require direct transporter phosphorylation. Another explanation, however, is that our functional studies were carried out using COS7 and HEK293 cells only, where important cellular factors for regulation in native SERT expressing cells might be absent. It would therefore be interesting in future studies to investigate the role of phosphorylation at the identified sites for regulation of SERT activity in cells native to the transporter, such as cultured neurons or neuronal cell lines. Furthermore, future studies could be directed at investigating the role of the identified in vitro phosphorylation sites in other aspects of SERT function and regulation, such as the influence on the action of amphetamines and on interactions with other proteins, some of which have been demonstrated to be regulated by kinases. Notably, studies of the role...
of SERT phosphorylation for protein-protein interactions could be investigated using the BRET technique, which we found applicable for studying the interaction between SERT and syntaxin1A.

To identify possible in vivo phosphorylation sites in SERT, various protein purifications strategies were pursued to obtain preparations of the full-length transporter from native or heterologous cells for subjection to phosphoproteomic analysis. For hSERT expressed in COS7 cells, the MS/MS analysis did not detect phosphorylation at the covered intracellular Ser/Thr/Tyr residues (21 out of 38), including four of the five phosphorylation sites identified in vitro. This result is suggestive of either a low phosphorylation level under basal conditions, or a transient nature of prospective phosphorylation events that prevent us from capturing sufficient amounts of the phosphorylated form. Preparations of native SERT isolated from rat cortical synaptosomes still awaits phosphoproteomic analysis, potentially revealing whether phosphorylation sites are present under basal conditions in vivo. The fact that SERT phosphorylation sites have not previously been identified in phosphoproteomic studies of brain proteins suggest the lack of the existence of major phosphorylation sites; however, minor sites might be present, which can potentially be identified by analysis of purified protein. Additional future studies could be directed at obtaining coverage of the intracellular regions of SERT not covered in the present LC-MS/MS analysis, possibly by optimisation of the purification procedures and pre-analysis sample treatment. Furthermore, the phosphoproteomic approach could be used for studying SERT phosphorylation dynamics, potentially discovering phosphorylation sites arising from activation of specific kinases.

Inhibitors of SERT and NET form the cornerstone in the treatment of depression, but for the majority of compounds, detailed information about their interaction with the transporters is lacking. We studied the effect of six single-point mutations of key residues surrounding the S1 binding pocket in SERT and NET on the inhibitory potency of 15 structurally diverse inhibitors. Comparison of the drug activity profiles across each set of transporter mutants revealed distinct sets of drugs that have similar perturbation patterns; suggesting that these share similar binding modes within the S1 pocket. Hereby, we provide novel information aiding the general understanding of how antidepressants interact with and distinguish between the transporters at the molecular level. Future studies could be directed at assessing potential ligand interactions with other residues of the S1 pocket of hNET, as the majority of the mutants employed here affected the potency of only few compounds. Furthermore, the role of the S2 pocket in SERT and NET for the inhibitory function of the examined compounds could be probed by undertaking a similar mutational analysis of this region in the transporters. In combination with the present data set, this could serve as a platform for performing molecular modelling of the individual compounds at SERT and NET, which is needed for understanding the inhibitory mechanism of the different antidepressants and can guide future structure-based design of novel therapeutics.
7 Experimental methods

7.1 Materials
Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum, trypsin and penicillin–streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Cell culture dishes and 96-well plates were from Nunc (Roskilde, Denmark). [³H]5-HT, [³H]DA, [¹²⁵I]RTI-55 and MicroScint-20 scintillation cocktail were obtained from PerkinElmer (Waltham, MA, USA). ATP was purchased from Invitrogen (Carlsbad, CA, USA) and all other reagents and buffer components were purchased from Sigma Aldrich (Copenhagen, Denmark). In vitro phosphorylation assays were conducted using the following recombinant or purified kinases: PKAα catalytic subunit, PKCα, p38MAPKα, Src (Biaffin, Kassel, Germany); PKGIα catalytic subunit (Merck Chemicals, Nottingham, UK); CaMKIIα, GSK3α, cdk5, INSR catalytic domain (Invitrogen, Carlsbad, CA, USA). Digitonin was obtained from Gold Biotechnology (St. Louis, MO, USA). (S)-Citalopram (oxalate), fluoxetine (HCl), fluvoxamine (maleate), MADAM, paroxetine (HCl), sertraline (HCl), nisoxetine (HCl), atomoxetine (oxalate), duloxetine (oxalate), milnacipran (HCl), venlafaxine (HCl), amitriptyline (HCl), clomipramine (HCl), imipramine (HCl) and maprotiline (HCl) were kindly provided by H. Lundbeck A/S (Copenhagen, Denmark).

7.2 High-performance liquid chromatography (HPLC)
Analytical HPLC was performed on an Agilent 1100 system using the following C18 reverse phase columns: Zorbax 300 SB-C18, 5 micron, 4.6 x 150 mm (analytical) and Zorbax 300 SB-C18, 21.2 × 250 mm, 7 u cartridge (preparative). All HPLC runs were performed with a linear gradient of the binary solvent system of H₂O/acetonitrile/trifluoroacetic acid (TFA) (A: 95/5/0.1 and B: 5/95/0.086) with a flow rate of 1 ml/min (analytical) and 20 ml/min (preparative) and UV detection at 230 nm.

7.3 Liquid chromatography-mass spectrometry or -tandem mass spectrometry (LC-MS or LC-MS/MS)
Mass spectra for analysis of synthetic peptides and aminomethyl (S)-citalopram were obtained with an Agilent 6410 Triple Quadrupole Mass Spectrometer instrument using electron spray coupled to an Agilent 1200 HPLC system (ESI-LC/MS) with autosampler and diode-array detector, using a linear gradient of the binary solvent system of H₂O/acetonitrile/TFA (A: 95/5/0.1 and B: 5/95/0.086) with a flow rate of 1 ml/min. A Zorbax C18 reverse phase column, 4.6 x 50 mm, was used.

7.4 Peptide synthesis
Peptides were synthesized by Fmoc-based solid-phase peptide synthesis on a 0.25 mmol scale, either manually or using a Liberty microwave peptide synthesizer (CEM, Matthews, NC, USA). All peptides were synthesized from pre-loaded Wang resins (Novabiochem, Darmstadt, Germany). Side-chain
protection for amino acids was as follows: Arg(Pbf), Asn(Trt), Asp(OtBu), Cys(Trt), Gln(Trt), Glu(OtBu), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu), Trp(tBu), Tyr(tBu), Ser(PO(OBzl)OH), Thr(PO(OBzl)OH). Fmoc deprotection was performed with 20% piperidine in DMF (2×10 min) and coupling of the consecutive amino acid was carried out with O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU) and diisopropylethylamine (DIPEA) (resin:amino acid:HBTU:DIPEA, 1:4:4:4) and monitored by the ninhydrin test. For incorporation of phosphoamino acids, a 1:6:6:12 ratio of resin:amino acid:HBTU:DIPEA was used. The final peptide was cleaved from the resin by treatment with 5% H₂O and 5% triisopropylsilane in TFA for 2 h. Crude peptides were analyzed by LC-MS and were purified to >98% using preparative HPLC. Purified peptides were lyophilized and analyzed by LC-MS.

7.5 In vitro phosphorylation assay

Phosphorylation reactions were carried out in a reaction volume of 50 μl in thin-walled PCR tubes containing 12.5 nmol peptide. The reaction buffers all contained 25 mM Tris-HCl, pH 7.5, 15 mM MgCl₂, 10 mM NaF, 0.5 mM Na₃VO₄ and 0.3 mM ATP. Additional buffer components were added for certain kinases (Supplementary Table 1). 17 units of kinase were used per nmol peptide in all reactions. For peptides with low solubility, dimethyl sulfoxide (DMSO) was added to a final concentration of maximum 0.5%. Reactions were incubated at 30 °C and 5 μl samples were collected at 0 min, 30 min, 1 h, 2 h, 5 h and 24 h. Kinase activity was terminated by addition of an equal volume of HPLC solvent A and samples were stored at -80 °C until further analysis. Enzymatic activity all kinases under assay conditions, including presence of 0.5% DMSO, was assessed using control peptide substrates: PKA, LRRASLG; PKC, QKRPSQRSKYL; PKG, RKRSRAE; Src, KVEKIGEGTYGVVK (all synthesized by solid-phase peptide synthesis as described above); p38MAPK, RRRLVEPLTPS.getAllHAPNQK (Jena Bioscience, Jena, Germany); CaMKII, KKLRRQETVDAL-amide; GSK3, YRRAAVPSPLSRHSSPHQ(pS)EDEEE; INSR, KKSREGYMTMQIG (Signalchem, Richmond, Canada); cdk5, PKTPKKAKKL (Anaspec, San Jose, CA, USA).

Peptide phosphorylation was initially investigated and quantified using LC-MS and analytical HPLC. Briefly, the samples from the in vitro phosphorylation assay were analyzed for the presence of a phosphorylated peptide species by LC-MS and the degree of phosphorylation was assessed by peak integration of the phosphorylated and non-phosphorylated species using analytical HPLC. In case of multiple possible phosphorylation sites within a phospho-positive peptide, the specific identification of the phosphorylated residues was performed by LC-MS/MS. Where applicable, the purified phosphorylated peptide was treated with trypsin (peptide:trypsin 1:50 (w/w) in 50 μl 0.1 M Tris-HCl, pH 7.5, 37 °C, 1 h) prior to LC-MS or LC-MS/MS analysis.
7.6 Molecular biology

The mammalian expression plasmids pcDNA3.1 and pCI-IRES-neo containing human SERT and human NET cDNA has been described previously. Generation of point mutations in hSERT and hNET was performed by site-directed mutagenesis using the QuickChange mutagenesis kit (Stratagene, Carlsbad, CA, USA), followed by sequencing of the entire gene (MWG Biotech, Martinsried, Germany). For bicistronic expression of hSERT with various kinases, the neomycin resistance gene in pCI-hSERT-IRES-neo was excised by digestion with the restriction enzymes MluI and BstBI and replaced with PCR-generated inserts with compatible MluI and BstBI 5’ and 3’ overhangs, respectively, and containing the coding sequence for constitutively active versions of p38MAPKα, PKCα and CaMKIIα (kindly provided by D. Mousseau, University of Saskatchewan, Saskatoon, Canada), PKCα and CaMKIIα (kindly provided by P. Parker, Cancer Research UK, London, UK) and CaMKIIα (kindly provided by T. Soderling, Vollum Institute, Portland, OR, USA). The sequence integrity of the resulting bicistronic hSERT/kinase expression vectors (Supplementary Figure 2), named pCI-hSERT-IRES-p38MAPK, pCI-hSERT-IRES-PKC, and pCI-hSERT-IRES-CaMKII was verified by sequencing of the entire hSERT and kinase coding regions. For generation of N- or C-terminally His-tagged hSERT, the hSERT gene in pCI-hSERT-IRES-neo was excised by digestion with the restriction enzymes XhoI and XbaI, and replaced with PCR-generated inserts with compatible XhoI and BstBI 5’ and 3’ overhangs, respectively, and containing the coding sequence for hSERT carrying a 6 x histidine tag at either the N- or C-terminus. The sequence integrity of the resulting vectors was verified by sequencing of the entire hSERT coding regions. For generation of GFP and Rluc fusion proteins of syntaxin1A and hSERT, pGFP-C2 and pRluc-C3 (for N-terminal fusion proteins) and pGFP-N1 and pRluc-N1 (for C-terminal fusion proteins) were digested with the restriction enzymes HindIII and ApaI. PCR-generated fragments with compatible HindIII and ApaI 5’ and 3’ overhangs, respectively containing the coding region of syntaxin1A (pcDNA3.1-hsyntaxin1A kindly provided by Dr. Kasper Hansen, Emory University School of Medicine, Atlanta, GA, USA) or hSERT were inserted. The sequence integrity of the resulting vectors was verified by sequencing of the entire coding regions.

7.7 Cell culturing and expression

COS7 and HEK293 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in DMEM supplemented with 10% foetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified 5% CO2 environment. Cells were transfected using TransIT DNA transfection reagent following the protocol supplied by the manufacturer (Mirus, Madison, WI, USA). Briefly, prior to transfection, confluent cells were trypsinated and re-suspended in DMEM at a concentration of 1 to 1.5 × 10^6 cells/ml. Per 96-well plate, 6 µg DNA and 18 µl transfection reagent was mixed in 0.6 ml DMEM and incubated at room temperature for 20 min. Subsequently, this mixture was added to 5 ml cell suspension and immediately plated into white 96-well plates with 50 µl per well.
7.8 \[^3H\]5-HT and \[^3H\]DA transport measurement

Uptake assays were performed 48 h after transfection and carried out in triplicate. Wells were washed twice with phosphate buffered saline (in mM: NaCl, 137; KCl, 2.7; Na\(_2\)HPO\(_4\), 4.3; and KH\(_2\)PO\(_4\), 1.4, pH 7.3) containing 0.5 mM CaCl\(_2\) and 0.5 mM MgCl\(_2\) (PBSCM) and pre-incubated in this buffer at RT for 20 min prior to uptake experiments. For determination of \(V_{\text{max}}\) and \(K_m\) values for hSERT phospho-site mutants and tagged hSERT, uptake saturation assay were carried out, where cells were incubated with increasing concentration of \[^3H\]5-HT diluted 1+50 with unlabelled 5-HT (total concentrations of 0.02-60 \(\mu\)M) in PBSCM at RT for 5 min. For single concentration 5-HT uptake experiments, cells were incubated with 100 nM \[^3H\]5-HT in PBSCM for 5 min. In experiments involving pharmacological induction or inhibition of PKC or p38MAPK, glucose (10 mM) was added to the PBSCM buffer. Cells were pre-incubated in PBSCM + glucose (vehicle) at 37 °C for 20 min followed by treatment with vehicle, β-PMA (1 \(\mu\)M), staurosporine (1 \(\mu\)M), β-PMA + staurosporine (1 \(\mu\)M + 1 \(\mu\)M), PD169316 (1-50 \(\mu\)M) or anisomycin (0.25-20 \(\mu\)M) at 37 °C for the desired time period. Uptake was allowed to proceed for 5 min at 37 °C in the same manner as described above. For inhibition assays, cells were incubated with increasing concentrations of inhibitors for 30 min at RT followed by addition a fixed concentration of radioligand (50 nM \[^3H\]5-HT for hSERT mutants and 20-50 nM \[^3H\]DA for hNET mutants). Uptake was allowed to proceed for 5 min for hSERT mutants and 5-20 min for hNET mutants. For determination of transport activities and \(K_m\) values for hSERT and hNET substrate binding pocket mutants, cells were incubated with increasing concentrations of cold substrate (5-HT in hSERT assays and DA in hNET assays) and a fixed concentration of hot substrate (50 nM). Uptake was allowed to proceed as described above for inhibition assays. All uptake experiments were terminated by washing three times with PBSCM. The amount of accumulated radioligand was determined by solubilizing cells in scintillant (MicroScint-20) with counting of plates in a Packard TopCounter (Packard Inc., Prospect, CT, USA). Non-specific uptake was determined in parallel by measuring uptake in non-transfected cells.

7.9 \[^{125}\text{I}]\text{RTI}-55 binding assays

Whole cell binding: For assessment of cell surface expression levels of hSERT in transfected cells, an intact cell radioligand binding assay was used\(^\text{15}\). Briefly, 48 h post-transfection, cells were rinsed twice with PBSCM and incubated 20 min on ice prior to binding experiments. For saturation binding studies, increasing concentrations of the \(^{125}\text{I}\)-labelled cocaine analogue RTI-55 diluted 1+10 with unlabelled RTI-55 (total concentrations of 0.2-25 nM) were added to cells and binding was allowed to proceed for 2 h on ice with gentle rocking. Subsequently, cells were transferred to 96-well filter plates (Unifilter C, PerkinElmer, Waltham, MA, USA) pre-incubated with 0.1% polyethyleneimine using a Packard Bell cell harvester (PerkinElmer, Waltham, MA, USA) and washed four times with H\(_2\)O. Under these conditions, unspecific binding of \(^{125}\text{I}]\text{RTI}-55 to a potential pool of intracellular hSERT in COS7 cells does not appear\(^\text{15}\). Non-specific binding was determined in parallel using non-transfected cells. Filter plates were
dried and soaked in scintillant followed by counting in a Packard Topcounter (PerkinElmer, Waltham, MA, USA).

BINDING TO RAT CORtical SYNAPTosomes OR HEK293 MEMBRANES: 35 μg protein per sample in a total volume of 80 μl PBSCM was incubated 2 h on ice with increasing amounts of [125I]RTI-55 diluted 1+2 with unlabelled RTI-55 (total concentrations of 0.2-22.5 nM). Synaptosomes/membranes were transferred to 96-well filter plates and counted as described above. Non-specific binding was determined in parallel in the presence of 0.1 mM fluoxetine.

7.10 DATA ANALYSIS
Data analysis from uptake and binding experiments was performed using Prism 4.0 software (GraphPad Inc., San Diego, CA, USA). For determination of $V_{max}$ and $K_m$ values, dose-response data from functional [3H]5-HT uptake assays were fitted by the equation:

$$V = V_{max} \times [5-HT]/(K_m + [5-HT]),$$

where [5-HT] is the total concentration of 5-HT (labelled and unlabelled).

For determination of IC50 values for inhibitors, dose-response data from functional uptake inhibition assays were fitted by the equation:

$$\% \text{ specific uptake} = 100/(1 + 10^{(\log IC50 - \log[\text{inhibitor}]) \times \text{Hill slope}}),$$

where IC50 is the concentration of inhibitor that produces a half-maximal inhibition of uptake.

For determination of $K_m$ values for hSERT and hNET mutants, dose-response data from functional uptake inhibition assays with increasing concentration of unlabelled substrate were fitted by the equation:

$$\% \text{ specific uptake} = 100/(1 + 10^{(\log K_m - \log[\text{substrate}]) \times \text{Hill slope}})$$

IC50 values were converted to $K_i$ values using the Cheng-Prusoff approximation:

$$K_i = IC50/(1 + [L]/K_m),$$

where [L] is the concentration of radioligand.

For determination of $B_{max}$ and $K_d$ values, concentration-response data from [125I]RTI-55 binding assays were fitted by the equation:

$$B = B_{max} \times [\text{RTI-55}]/(K_d + [\text{RTI-55}]),$$

where [RTI-55] is the total concentration of RTI-55.
7.11 Bioluminescence resonance energy transfer (BRET) assay

Transfected cells growing in 96-well plates were 48 h post-transfection washed twice with PBSCM and pre-incubated in 50 μl/well of this buffer at RT for 20 min prior to BRET measurements. Immediately before measurement, 5 μl of DeepBlueC coelenterazine (Gold Biotechnology, St. Louis, MO, USA) from a freshly prepared stock solution of 55 μM in PBSCM was added to each well to give a final concentration of 5 μM. Measurements of Rluc-mediated luminescence and GFP²-mediated emission from each well were performed using a Mithras LB 940 plate reader (Berthold Technologies, Bad Wildbad, Germany) by counting the luminescence and emission using 410 nm (80 nm bandwidth) and 515 nm (40 nm bandwidth) emission filters, respectively (Berthold Technologies, Germany). Background emission of luminescence and fluorescence was determined from wells containing mock-transfected cells (transfected with pcDNA3.1). For each well, the BRET ratio was calculated as: (E515 – background515)/(E410 – background410), where E515 is the emission, E410 is the luminescence, background515 is the background emission and background410 is the background luminescence. For each tested combination of donor and acceptor, the obtained BRET signal was corrected for the baseline BRET signal, caused by crosstalk of the strong luminescence signal into the fluorescence channel, by subtracting the BRET ratio obtained in parallel from COS7 cells expressing only the donor protein. The BRET signal is reported as mBRET (10³ x BRET ratio). For BRET saturation curve experiments, cells were transfected with decreasing amount of plasmid with the Rluc-tagged construct and increasing amounts of plasmid with the GFP²-tagged construct. The total amount of plasmid DNA was kept constant at 0.06 μg/well. For saturation assays involving CaMKII, a constant amount of plasmid DNA (0.04 μg/well) with CaMKII or empty vector (pcDNA3.1) was transfected along with the donor and acceptor DNA. The calculated BRET signals were plotted as a function of the DNA ratio (GFP²-tagged construct/Rluc-tagged construct), and data was analyzed by non-linear regression curve fitting using Prism 4.0 software (GraphPad Inc., San Diego, CA, USA).

7.12 Gel electrophoresis, staining and Western blot analysis

Protein samples were subjected to SDS-PAGE on a 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA). Coomassie-staining was performed using Colloidal Blue Staining Kit (Invitrogen, Carlsbad, CA, USA) following the protocol supplied by the manufacturer. Silver-staining was performed as previously described. For Western blots, proteins were transferred to a PVDF membrane (Invitrogen, Carlsbad, CA, USA) and blotting was performed using a SNAP i.d. Protein Detection System (Millipore, Molsheim, France). PBS-T (0.1% Tween 20 in PBS) was used as washing buffer and 0.2% non-fat dried milk in PBS-T as blocking reagent. For detection of SERT, blots were incubated 10 min with primary mouse anti-hSERT antibody (ST51, Mab Technologies, Stone Mountain, GA, USA) diluted 1:1,000 (for detection of hSERT) or goat anti-hSERT antibody (sc-1458, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200 (for detection of rSERT), followed by 10 min incubation with secondary HRP-linked
anti-mouse antibody (W4021, Promega, Madison, WI, USA) diluted 1:10,000 or anti-goat antibody (SC2020, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:4,000. For detection of GluA1-pS381, blots were incubated 10 min with primary rabbit anti-GluA1-pS831 antibody (AB5847, Millipore, Temecula, CA, USA) diluted 1:500 followed by 10 min incubation with secondary HRP-linked anti-rabbit antibody (W4011, Promega, Madison, WI, USA) diluted 1:10,000. All blots were developed using enhanced chemoluminescence detection reagents (GE Healthcare, Buckinghamshire, UK) and visualized using a DNR MicroChemi System (DNR Bio-Imaging Systems Ltd, Jerusalem, Israel).

7.13 Purification of His-tagged hSERT

COS7 cells transiently expressing His-tagged hSERT growing in 150 mm tissue culture Petri plates were washed with PBS supplemented with 1 mM EDTA to detach cells from the plate. In a typical experiment, cells from five Petri plates were used. Cell suspension was centrifuged at low speed (700 × g) at room temperature for 5 min. The cell pellet was re-suspended in cold H2O containing protease inhibitors (complete mini, Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (5 mM NaF, 5 mM β-glycerophosphate, 1 mM Na3VO4) and frozen at -20 °C for 1 h. The suspension was thawed on ice and subjected to 10-15 passages through a 21-gauge needle to disrupt cells. The homogenate was transferred to cold 2 ml microcentrifuge tubes and centrifuged at 18,000 × g, 4 °C for 30 min. The supernatant was aspirated, and the pellet was re-suspended in binding buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 1% triton X-100 and 5 mM imidazole) supplemented with protease inhibitors and phosphatase inhibitors at a protein concentration of 2-6 mg/ml. The protein solution was filtered and loaded onto a pre-equilibrated (5 ml binding buffer) 1 ml HisTrap HP column connected to an ÄKTAprime system (both GE Healthcare, Freiburg, Germany). A flow rate of 0.5-1.0 ml/min was used. The column was washed with 15-20 column volumes of binding buffer and bound protein was eluted using a linear gradient of 5-500 mM imidazole in binding buffer over a period of 20 min, with collection of 1 ml fractions. Eluted fractions were analyzed by dot-blot (samples were spotted directly onto a PVDF membrane followed by Western blotting) and fractions with hSERT activity was further analyzed by SDS-PAGE and Western blotting. hSERT-containing fractions were combined, added 4 volumes of ice-cold acetone and allowed to precipitate ON at -20 °C, followed by centrifugation at 25,000 x g, 4 °C for 20 min. The resulting pellet was dissolved in SDS-PAGE loading buffer and proteins separated by SDS-PAGE. The band containing SERT was excised from the gel for further processing and LC-MS/MS analysis.

7.14 Synthesis of aminomethyl (S)-citalopram

(S)-Citalopram (3 mmol) was dissolved in 40 ml dry tetrahydrofuran and 15 ml 1 M LiAlH4 (3 eq) was added drop wise under vigorous stirring. The mixture was refluxed overnight at 70 °C under nitrogen atmosphere. The reaction was quenched with 20 ml cold H2O and poured into ice. The mixture was then
allowed to thaw at room temperature. The aqueous phase was filtered, extracted 4 times with 25 ml ethyl acetate, and the combined organic phases were dried with anhydrous sodium sulphate. The organic phase was filtered and then evaporated to dryness, giving the crude product as a clear oil. Purification was carried out in 7 potions by preparative HPLC. The crude product was dissolved in 5 ml HPLC solvent A and eluted with a linear gradient of 0-60% HPLC solvent B over 60 min. Fractions were analyzed by LC-MS, and the pure fractions combined and lyophilized, yielding 0.360g (36.45%) of aminomethyl (S)-citalopram as a yellow oil. LC-MS, [M+H]⁺ = 329.2; purity (ELSD), 95%; microanalysis, w-% C = 52.43, w-% H = 4.72, w-% N = 5.46; ¹H NMR (300 MHz, CD3OD), δ [ppm] = 7.57 – 7.35 (m, 5 H), 7.06 – 7.00 (m, 2 H), 5.19 (q, J = 17.5 Hz, J = 21 Hz, 2 H), 3.13 (t, J = 15 Hz, 2 H), 2.78 (s, 6 H), 2.33 – 2.26 (m, 2 H), 1.70 – 1.61 (m, 2 H).

7.15 Generation of an (S)-citalopram-derived affinity resin

Aminomethyl (S)-citalopram was coupled to N-hydroxysuccinimide (NHS) -activated sepharose 4 fast flow (GE Healthcare, Uppsala, Sweden) as follows: 6 ml NHS-activated sepharose (~120 μmol NHS) was washed with 90 ml 1 mM HCl, drained and added 360 μmol aminomethyl (S)-citalopram dissolved in 6 ml 50% DMSO in 0.25 M NaCl/0.2 M NaHCO₃, pH 8.3. pH of the sepharose/ligand mixture was adjusted to 8 and allowed to react under rotation for 3 h at RT followed by ON at 4 °C. The resin was drained and non-reacted groups blocked by incubation with 0.5 M ethanolaime/0.25 M NaCl, pH 8.3 for 2 h at RT. The resin was washed with 18 ml 0.1 M Tris-Cl, pH 8.5 followed by 18 ml 0.1 M CH₃COOK/0.4 M NaCl, pH 4.4. The washing procedure was repeated 6 times. The resin was stored at 4 °C in 50 mM Na₂HPO₄ containing 0.1% NaN₃, pH 7 until use.

7.16 (S)-Citalopram-based affinity purification of SERT

Membrane preparation, heterologous expression: Membranes from HEK293 cells transiently expressing hSERT growing in 150 mm tissue culture Petri plates were prepared as described above for COS7 cells expressing His-tagged hSERT. After the final centrifugation, membranes were resuspended in 5 mM Tris-HCl, pH 7.4 containing protease inhibitors and phosphatase inhibitors.

Preparation of rat brain synaptosomes: Synaptosome preparation was essentially carried out as described. Briefly, rats (Sprague Dawley) were killed by guillotine, their brains were rapidly dissected and the cortex was placed in 8 ml ice-cold homogenization buffer (5 mM Tris-HCl, pH 7.4, 0.32 M sucrose, 1 mM EDTA containing protease inhibitors and phosphatase inhibitors (Phosphatase Inhibitor Cocktail Set II, Merck Chemicals, Nottingham, UK). The tissue was cut into small pieces and homogenized using a Teflon/glass homogenizer with 6 up and down strokes at 700 rpm. The homogenate was centrifuged at 4 °C for 10 min at 3,200 rpm using a Beckman J2-MC centrifuge equipped with a JA-20 rotor. The supernatant was brought to 17 ml with cold homogenization buffer and dithiothreitol (DTT) was added to a final concentration of 0.25 mM. Percoll gradients (8 per rat brain) were prepared in 12 ml centrifuge
tubes by layering of 23%, 10% and 3% percoll in 5 mm Tris-HCl, pH 7.4, 0.32 M sucrose, 1 mM EDTA, 0.25 mM DTT (2.5 ml of each percoll reagent) and kept at 4 °C ON. 2 ml of rat brain supernatant was layered onto each percoll gradient and centrifuged at 4 °C, 3,200 rpm for 5 min at speed. The synaptosomal fraction was collected from the interface between the 10% and the 23% percoll layer, brought to 80 ml with cold homogenization buffer and centrifuged at 4 °C for 15 min at 16,000 rpm. The supernatant was discarded and the last step repeated. The resulting pellet was suspended in PBS containing protease inhibitors and phosphatase inhibitors.

**Purification of SERT using (S)-citalopram-based affinity chromatography:** HEK293 membranes or synaptosome preparations were solubilized in cold solubilization buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 1% digitonin containing protease inhibitors and phosphatase inhibitors (Phosphatase Inhibitor Cocktail Set II, Merck Chemicals, Nottingham, UK)) at a protein concentration of 0.5-8 mg/ml. Solubilization was allowed to proceed at 4 °C for 1 h under rotation followed by centrifugation at 18,000 x g, 4 °C for 30 min. The supernatant was loaded onto an equal volume of affinity resin pre-equilibrated with solubilization buffer and binding was allowed to proceed for 2 h at 4 °C under rotation. The affinity resin was washed with at least 25 volumes of washing buffer 1 (25 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.1% digitonin containing phosphatase inhibitors) followed by at least 10 volumes of washing buffer 2 (25 mM Tris-HCl, pH 7.4, 150 mM KCl, 0.1% digitonin containing phosphatase inhibitors). Bound protein was eluted in washing buffer 2 containing 5 mM (S)-citalopram and protease inhibitors. The eluted fractions were analyzed by SDS-PAGE and Western blotting. Fractions containing SERT were combined and concentrated using Amicon ultra 4 centrifugal filter devices (30 kDa) (Millipore, County Cork, Ireland). The resulting material was mixed with SDS-PAGE loading buffer and proteins separated by SDS-PAGE. The band containing SERT was excised from the gel for further processing and LC-MS/MS analysis.

### 7.17 In-gel digestion, enrichment of phosphopeptides and LC-MS/MS analysis of purified SERT

Gel pieces were destained by washing thoroughly with 50% acetonitrile in 25 mM NH₄HCO₃ followed by dehydration in acetonitrile. Disulfide bonds were reduced by re-swelling the gel pieces in 50 μl 10 mM DTT/25 mM NH₄HCO₃ for 45 min and free Cys residues were alkylated by 30 min incubation in 50 μl 55 mM chloroacetamide/25 mM NH₄HCO₃. After dehydration in acetonitrile, proteins were digested ON with 50 μl 12.5 ng/μl trypsin (modified sequencing grade, Promega, Madison, WI, USA) in 25 mM NH₄HCO₃. 10% aqueous TFA was added to a final TFA concentration of 3% followed by centrifugation for 2 min at 0.5 x g. The supernatant was collected and the gel pieces washed with a) 30% acetonitrile/3%

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The present description covers the analysis of purified His-hSERT from COS7 cells, which was performed by post doc Alicia Lundby, Centre for Protein Research, University of Copenhagen. The purified hSERT from HEK293 cells was analyzed by Proteome Factory (Berlin, Germany) by a procedure similar to the one described here.
TFA in H₂O; b) 80% acetonitrile/0.5% acetic acid in H₂O and c) acetonitrile, 50 µl of each, with centrifugation (2 min at 0.5 x g) between each wash. The combined supernatants were dried using a vacuum centrifuge and redissolved in 0.5% aqueous acetic acid. The peptide mixtures were either directly used for LC-MS/MS analyses or further processed to enrich the phosphopeptides, which was performed essentially as described previously. Briefly, a slurry of TiO₂ beads precoated with 2,5-dihydrobenzoic acid was prepared by mixing 10 µg of titansphere TiO₂ beads (GL Sciences, Torrance, CA, USA) with 20 µl of 30 mg/ml 2,5-dihydrobenzoic acid in 80% acetonitrile in H₂O. 5 µl of this 2,5-dihydrobenzoic acid/TiO₂ slurry was added to the acidified peptide mixtures extracted from in-gel digests. The peptide mixtures were shaken for 30 min at 4 °C, and then spun down in a microcentrifuge. The pelleted TiO₂ beads were washed twice with 30% acetonitrile/3% TFA in H₂O, and peptides were eluted with 15% NH₄OH/40% acetonitrile in H₂O (pH >10.5). Finally, the eluates were dried in a vacuum concentrator and reconstituted in 0.5% aqueous acetic acid. Extracted peptides were subsequently analyzed by nanoflow-LC-MS/MS on a Thermo Scientific LTQ-Orbitrap Velos instrument. Raw MS files were processed with MaxQuant software and sequences were identified by searching against primate sequences in the SwissProt database (version 51.6), using the Mascot search engine (Matrix Sciences, Boston, MA). The search included variable modifications for oxidation of Met, protein N-terminal acetylation, and phosphorylation of Ser, Thr, and Tyr.
References


Appendix 1: Supplementary figures and tables

Supplementary figures:

Figure 1. hSERT phosphorylation site prediction by NetPhosK

Figure 2. Co-expression of hSERT and kinases using the pCI-IRES vector

Figure 3. Activity of CaMKII and PKC expressed in COS7 cells

Figure 4. 5-HT transport activity of GFP$^2$ and Rluc fusion proteins of hSERT

Figure 5. hSERT oligomerization studied by the BRET$^2$ saturation assay

Figure 6. 5-HT transport activity of His-tagged hSERT

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**NetPhosK 1.0 Server - prediction results**

Technical University of Denmark

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**Supplementary Figure 1. hSERT phosphorylation site prediction by NetPhosK**

[http://www.cbs.dtu.dk/services/NetPhosK/](http://www.cbs.dtu.dk/services/NetPhosK/) The prediction was carried out for full length hSERT (UniProt-SwissProt accession number P31645), with use of the ESS filter (considering evolutionary conservation of the sequence motif) and a threshold of 0.5. The criteria for selection of kinases were either a) the presence of a score >0.6 or b) the presence of minimum three scores >0.5 for residues within the intracellular hSERT regions. Two kinases; cdk5 and GSK3, fulfilled one of these criteria and were included in the assay. (Only kinases not already included in the assay based on previous reports about their involvement in SERT phosphorylation were considered.)
Supplementary Figure 2. Co-expression of hSERT and kinases using the pCI-IRES vector
cDNAs encoding hSERT and constitutively active mutants of PKC, CaMKII and p38MAPK were inserted pair
wise in a bicistronic mammalian expression vector (pCI-IRES). Mammalian cells can indeed translate bicistronic
mRNAs (i.e. RNAs with two independent open reading frames) provided that the second open reading frame is
preceded by an internal ribosomal entry site (IRES)\textsuperscript{1,2}. Use of the pCI-IRES constructs ensured expression of
both hSERT and kinase in all transfected cells. Shown is the hSERT-IRES-PKC vector construct. Depicted
protein structures: left, LeuT (PDB 2A65); right, PKC\textsubscript{α} (PDB 3IW4).


2. Trouet D, Nilius B, Voets T, Droogmans G and Eggermont J. Use of a bicistronic GFP-expression vector
to characterise ion channels after transfection in mammalian cells. Pflugers Arch \textbf{1997}, \textit{434}:632-638.
**Supplementary Figure 3. Activity of CaMKII and PKC expressed in COS7 cells**

To ensure that expression of the CaMKII and PKC mutants indeed induces constitutive activity in COS7 cells, these kinases were co-expressed with the glutamate receptor GluA1, which is known to contain a CaMKII/PKC phosphorylation site at Ser831\(^1,2\). 48 h post transfection, cells were lysed and proteins were separated by SDS-PAGE. Western blotting with anti-GluA1-pS831 phospho-antibody (Millipore, Temecula, CA, USA) showed increased WT GluA1 phosphorylation upon co-expression with PKC or CaMKII to the same extent as when endogenous PKC was pharmacologically induced with \(\beta\)-PMA. No bands were seen for cells expressing GluA1 S831A or for non-transfected cells.


Supplementary Figure 4. 5-HT transport activity of GFP² and Rluc fusion proteins of hSERT
Representative concentration-response curves from at least three independent experiments of saturation [³H]5-HT uptake in COS7 cells transfected with cDNA encoding hSERT WT or the respective hSERT fusion proteins. Data points are normalized to Vₘₐₓ for hSERT WT and represent mean ± S.E.M. from triplicate determinations.
Supplementary Figure 5. hSERT oligomerization studied by the BRET^2 saturation assay

BRET^2 saturation experiments with the four possible hSERT donor/acceptor pairs. Increasing amounts of GFP^2-tagged construct and decreasing amounts of Rluc-tagged construct were co-transfected in COS7 cells. BRET values in mBRET are plotted as a function of the DNA ratio of GFP^2 to Rluc. Data points represent mean ± S.E.M from 16 wells.
Supplementary Figure 6. 5-HT transport activity of His-tagged hSERT

Representative concentration-response curves from at least three independent experiments of saturation $[^3H]5$-HT uptake in COS7 cells transfected with cDNA encoding hSERT WT or hSERT encompassing a 6-histidine tag at either the N-terminus (His-hSERT) or C-terminus (hSERT-His). Data points are normalized to $V_{\text{max}}$ for hSERT WT and represent mean ± S.E.M. from triplicate determinations.
### Supplementary Table 1. Buffer composition for *in vitro* phosphorylation assays

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Supplementary Table 2. $K_d$ values for RTI-55 at hSERT phospho-mimicking and -blocking mutants

The $K_d$ values for RTI-55 were determined at hSERT WT and point mutants in a [125I]RTI-55 whole cell saturation binding assay. Values are presented as mean ± SEM from at least three independent experiments each performed in triplicate. * $p<0.05$ $K_d$ value significantly different from hSERT WT (Student’s $t$-test).

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Supplementary Table 3. hSERT-specific peptides identified in the LC-MS/MS analysis of His-hSERT purified from COS7 cells using nickel affinity chromatography

The MS/MS data were processed using the MaxQuant software and searched against primate sequences in the SwissProt protein database (version 51.6) using the Mascot database search software (Matrix Sciences, Boston, MA). Shown is the list of identified peptides derived from hSERT.

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Supplementary Table 4. hSERT-specific peptides identified in the LC-MS/MS analysis of hSERT purified from HEK293 cells using (S)-citalopram-based affinity chromatography

The MS/MS data were searched against human sequences in the SwissProt protein database (version 51.6) using the Mascot database search software (Matrix Sciences, Boston, MA). Shown is the list of identified peptides derived from hSERT.

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