DIPLOMARBEIT

„The Impact of PIP₂ on Neurotransmitter Transporter mediated Substrate Flux“

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Abstract

The serotonin transporter (SERT) as a member of the sodium-coupled neurotransmitter transporters (NSS) plays a crucial role in the termination of serotonin signalling. Alterations of intrinsic transporter function and regulation are associated with psychiatric diseases and physiological disorders. A great deal of effort has been taken to unravel SERT regulation and transport mechanism. Phosphatidylinositol-4,5-bisphosphate (PIP$_2$) is involved in several major cellular regulatory processes and was also shown to play a role in the regulation of integral membrane proteins, like ion channels (Gamper and Shapiro, 2007a). PIP$_2$ as well as cholesterol have been found to be enriched in membrane microdomains where also SERT was found to be located and regulated (Chang et al., 2012). Nevertheless, a regulatory effect of PIP$_2$ on SERT fluxes has never been investigated before. This work examined the role of PIP$_2$ on SERT transport flux by using a heterologous expression system. It could be shown that the PLC activator m-3M3FBS was able to deplete PIP$_2$ in HEK-hSERT cells resulting in reduced amphetamine-induced efflux rates but unchanged ligand influx. To preclude the possibility that observed reduction of efflux rates is caused by activation of second messengers due to cleavage of PIP$_2$ via PLC, inhibition of PIP$_2$ synthesis via the substance PAO was investigated. Indeed, the same reduction in efflux but without affecting influx rates could be demonstrated which verified that this effect was caused by PIP$_2$ depletion rather than via activation of second messengers. These results indicate that PIP$_2$ directly effects amphetamine induced transporter efflux indicating that this could be mediated via direct binding of PIP$_2$ to SERT. Computational analysis revealed two positively charged amino acids K352 (helix 6) and K460 (helix 9), located in the intracellular loops of SERT, as potential binding sites for PIP$_2$. Mutagenesis of both amino acids did not alter intrinsic transporter function but displayed the exact same effect as was observed for PIP$_2$ depletion. K352A/K460A double mutation in hSERT did affect neither substrate influx nor trafficking to the surface, but was shown to markedly reduce amphetamine induced efflux rates. Therefore it could be demonstrated that PIP$_2$ modulates SERT flux selectively, by only affecting efflux and that this modulation occurs most likely via direct binding of PIP$_2$ to the amino acids K352 K460.
1. Aim of Thesis

Neurotransmitter transporters play a crucial role in neurotransmission and maintenance of neurotransmitter homeostasis (21752877). They are the main targets of some of the most effective drugs applied in psychiatric and neurological treatment. However, still little is known about their exact function and regulation. In the human brain serotonin is one of the main neurotransmitters and the serotonergic system has been found to be dysfunctional in several diseases, like depressive disorder, anxiety, Obsessive-Compulsive Disorder (OCD), and autism (Daws and Gould, 2011). After its release, the action of serotonin at its pre- and postsynaptic receptors needs to be terminated: this constitutes the main physiological function of the serotonin transporter (SERT). It is therefore of utmost relevance to unravel the regulation of SERT and to fully understand its disease related alterations. SERT is a member of the sodium/chloride coupled transporter family and is subject to modulation by intracellular associated proteins, e.g. protein kinase C (PKC). However, recent evidence emerged that the plasma membrane itself influences transporter function (Hong and Amara, 2010; Scanlon et al., 2001). Another membrane constituent, the phospholipid PIP$_2$ (phosphatidylinositol-4,5-bisphosphate), a component of the inner leaflet of the plasma membrane is known to be involved in many regulatory events and was also shown to impact function of ion channels (Gamper and Shapiro, 2007a; Hernandez et al., 2009; Li et al., 2005). Based on preliminary results generated in the lab of Prof. Sitte, demonstrating that PIP$_2$ (i) could be depleted from HEK (human embryonic kidney)-SERT cells using the phospholipase C (PLC) activator m-3M3FBS (i) binds \textit{in vitro} to SERT via a pull-down assay by using PIP$_2$-coated beads and (iii) interacts most likely with the two amino acids K352 (helix 6) and K460 (helix 9) as was suggested by \textit{in-silico} computational modeling.

The aim of my diploma thesis was to investigate the influence of PIP$_2$ on SERT mediated substrate flux by performing uptake and efflux measurements and to further characterize the importance of two amino acids K460 and K352 for interaction of PIP$_2$ with SERT. As both amino acids are lysines (K) and therefore positively charged, they are believed to interact electrostatically with negative charged head group of PIP$_2$. Furthermore the calculated distance of 12Å would perfectly fit the size of the PIP$_2$ headgroup (~10Å). Measuring the impact of PIP$_2$ on
SERT function, uptake and release assays have been performed by treating cells with m-3M3FBS. As a PLC activator, m-3M3FBS triggers the sequestration of PIP$_2$ to diacylglycerol (DAG) and Inositol-1, 4, 5-trisphosphat (IP$_3$). This substance was used to treat cells before uptake and release (measures efflux) assays were performed. If PIP$_2$ has indeed an influence on SERT mediated currents it should have an impact on either uptake or efflux or both.

Depending on results of these experiments it was further the aim to characterize the physical SERT-PIP$_2$ interaction by creating a K352A/ K460A double mutant. Therefore two positively charged amino acids would be exchanged against neutral amino acid alanine. Further functional characterization of this double mutation would then be performed.

Taken together, two hypotheses were concluded:

1) Loss of PIP$_2$ in the plasma membrane causes a change of serotonin transporter flux in either efflux or influx (uptake) or both.

2) PIP$_2$ influences serotonin transporter flux by physically interacting with two amino acids K460 and K352 within helix 9 and helix 6.
1.1. Introduction

1.2. Structure and Function of Neurotransmitter Transporter

Neuronal cells communicate with each other by using electrical and chemical mechanisms— to be more specific by creating action potential and releasing neurotransmitter. Termination of neurotransmitter-mediated signaling can be accomplished by (1) passive diffusion away from the synaptic cleft, (2) enzymatic degradation of the neurotransmitter by enzymes within the synaptic cleft, and (3) uptake of the neurotransmitter by the presynaptic neuron. The latter is managed by neurotransmitter transporters which belong to the SLC6 (solute carrier 6) family.

The solute-carrier (SLC) family is a huge family comprising 51 groups and a total of 378 members (www.bioparadigms.org; status quo 2012). The SLCs are integral membrane proteins that catalyse the energetically unfavourable movement of solute molecules across a lipid bilayer and are involved in many different processes. By controlling uptake and efflux of crucial compounds like amino acids (e.g. SLC1, SLC7, SLC17), sugars (e.g. SLC2, SLC50, SLC37), nucleotides (e.g. SLC28, SLC35), and drugs (e.g. SLC47) they play a crucial role in maintaining important processes within and between cells. Solute carriers belong to the group of membrane transporters that selectively transport specific molecules across the membrane with the help of one or more ions. This means that their transport can be active (against the concentration gradient) but contrary to active pumps (e.g. ABC-transporter) they are not dependent on ATP (adenosine triphosphate).

With 21 members the SLC6 (Sodium-and chloride-dependent neurotransmitter transporter) group is the fourth largest (see figure 1) and contains some of the most prominent neurotransmitter transporters, like SERT (serotonin transporter), GAT (GABA transporter), NET (norepinephrine transporter) and DAT (dopamine transporter). As their group name implies nearly all transporters within this group need sodium and chloride to transport their substrate through the membrane (there are few exceptions that need only sodium). One specialty of these transporters is that they transport the substrate across the membrane by using the “naturally
occurring” sodium gradient (in<out), which in addition gave them the term Neurotransmitter:Sodium Symporter (NSS;(Busch and Saier, 2002)).

The particular clinical importance of the transporters is a result of their involvement in the treatment of neurological and psychiatric diseases. NSS are still the main targets in the treatment of certain psychiatric disorders: selective serotonin reuptake inhibitors and selective norepinephrine reuptake inhibitor (SSRI, SNRI, respectively) are successfully used in treating depressive disorder; methylphenidate is used in the treatment of ADHD (attention deficit hyperactive syndrome); DARIs (dopamine reuptake inhibitors) drew attention in the treatment of obesity. Despite their important role in disease and treatment of disease by targeting NSS, still little is known about their molecular structure and function.

Figure 1 Members of the SLC family. Bars represent the actual number of members within the different SLC groups. Therefore the SLC24 group with 46 members is the biggest group, followed by SLC22 (26 members), SLC5 (23 members), and SLC6 (21 members). Note that the SLC6 group includes also non-neurotransmitter transporters.
One reason is that there is still no crystal structure for any mammalian neurotransmitter transporter available. A first breakthrough succeeded in 2005 when Yamashita and colleagues reported the crystallization of the bacterial leucine transporter (LeuT) from *Aquifex aeolicus* in the presence of its substrate leucine (Yamashita et al., 2005). LeuT is the prokaryotic homolog to mammalian monoamine transporters of the SLC6 family. Since then the structure of LeuT has been solved at high resolutions also bound to the competitive inhibitor tryptophan (Singh et al., 2008) and several noncompetitive inhibitors, like desipramine, clomipramine and other tricyclic antidepressants (TCAs) (Singh et al., 2007; Zhou et al., 2007). As a matter of fact biochemical and genetic data from monoamine transporters matched to the obtained structural data of LeuT. Although the overall amino acid identity between LeuT and the mammalian NSS transporters is only 20-25%, the core transport machinery shows 55-67% sequence similarity (Beuming et al., 2006). Therefore we can assume structural and mechanistic details of the SLC6 transporters (Ravna et al., 2006; Wang and Lewis, 2010).

SLC6 neurotransmitter transporters (SLC6 NTTs) are mainly expressed in the CNS and play an important role in the clearance of neurotransmitter molecules from the synaptic cleft. Thus their substrates include all common neurotransmitters in the mammalian CNS. As secondary active SLC6 transporter they use the electrochemical potential of extracellular Na\(^+\) (sodium) ions as energy source for the transport of their substrate. In most of the cases extracellular chloride ions are also co-transported but the exact role of chloride in the transport process is not fully understood, however, transport by the bacterial ortholog LeuT is chloride independent (Zomot et al., 2007). The uptake of the substrate can be maintained against very large concentration gradients and the kinetics of the NTT-mediated transport follows the Michaelis-Menten model (Berg JM, 2002) with substrate K\(_M\) values in the lower micromolar range (5-50 \(\mu\)M) and maximal turnover rates ranging from 1-20 substrate molecules per second.

The fact, that many membrane proteins are organized in complex quaternary structures lead to the assumption that SLC6 NTTs are also organized in this way. Indeed there is strong evidence that Na\(^+\)/Cl\(^-\)-coupled transporters could exist in oligomeric structures (Sitte and Freissmuth, 2003). It is known that NSS possess a
GxxG-motif as well as a leucine heptad repeat. GxxG (glycine-xx-glycine) is an amino acid sequence motif that stabilizes helix–helix interactions in both membrane and soluble proteins. The GxxG sequence motif has been found in transmembrane α-helices and has been determined to stabilize the oligomerization of several membrane proteins (Kleiger and Eisenberg, 2002). The leucine heptad repeat was also found to be responsible for interactions between transmembrane segments (Gurezka et al., 1999) and for the oligomer formation of transporters, like the rat GABA transporter (Scholze et al., 2002). Furthermore these leucine heptad repeats are highly conserved throughout the evolution of transporter proteins. Besides structural evidence coming from the amino acid sequence a lot of experimental data supported this hypothesis. By co-expressing differentially tagged transporters in combination with immunoprecipitation, specific crosslinking techniques and FRET (fluorescence resonance energy transfer) it could be shown that DAT (Torres et al., 2003a), GAT and SERT (Just et al., 2004; Schmid et al., 2001) seem to form dimeric or tetrameric structures (Farhan et al., 2006). By tagging two SERT proteins with different epitope tags suggested a dimeric form of SERT with functional interactions between subunits possibly giving rise to a tetrameric structure (Kilic and Rudnick, 2000). Furthermore, studies have identified particular transmembrane domains responsible for those interactions (Beck and Shaw, 2005; Hastrup et al., 2001; Hastrup et al., 2003; Torres et al., 2003a). Amongst others, the transmembrane domains (TMDs) 11 and 12 in SERT (Just et al., 2004), Cys-306 (TMD6) as well as leucine heptad repeat in TMD2 in DAT have been identified via mutational analysis so far.

Proposed mechanisms of substrate transport by neurotransmitter transporters were generated mainly by using computational modeling approaches, electrophysiology as well as molecular biology techniques. One has to keep in mind that those mechanisms had never been observed per se but are models based on crystallographic structures of mainly LeuT and experimental data. SERT transports its substrate serotonin (5-HT) together with 1 sodium (Na⁺) and 1 chloride (Cl⁻) ion (symport) against 1 potassium (K⁺) ion (antiport). The K⁺ ion is specialty of SERT.
In doing so, energy for transport is supplied by the sodium gradient (in<out), the chloride gradient (in<out) as well as by the potassium gradient (in>out).

![Mechanism of serotonin transport](image)

**Figure 2 Proposed mechanism for the transport of serotonin by SERT.** Extracellular sodium (Na\(^+\)) binds first and facilitates substrate binding (sodium bound open-out or ‘outward-facing’ conformation). This clears the pathway to the substrate binding site (residues of TM1, 3, 6, and 8). Upon substrate and chloride (Cl\(^-\)) binding, the external gate closes to occlude the substrate from extracellular environment (occluded complex conformation). Opening of the inner gate allows substrate and ions to dissociate and exit at the intracellular face of the membrane (‘inward-facing’ conformation). Potassium (K\(^+\)) enables the reverse conformational change, leading to extracellular K\(^+\) dissociation and another cycle of transport. (Picture source :(Rudnick, 2011))

A common model for transport mechanism is the ‘alternating access mechanism’, meaning that the binding sites for substrate and ions are exposed alternately to one side of the membrane or the other (Forrest et al., 2008; Jardetzky, 1966). For detailed information, see figure 2. Earlier studies of amino acid sequence of neurotransmitter transporters (see figure 4) supposed a structure of 12 transmembrane domains (TMDs). Site-directed mutagenesis studies provided a way to access which regions were accessible from the medium and are supposed to bind
the substrate. After all it was the crystal structure of bacterial homologues that resolved the topology of the transporters. The structure of LeuT revealed that the structure of transmembrane helices 1-5 is repeated, with an inverse topological orientation, by helices 6-10 (5x5 inverted repeat motif). This topology is believed also to be true for the neurotransmitter transporters. There are several excellent reviews covering structural and functional changes during substrate transport (Forrest and Rudnick, 2009; Henry et al., 2007; Kristensen et al., 2011; Rudnick, 2011).

Many members of the NSS family exhibit substrate-independent ion currents in addition to substrate transports, so-called “leak” currents. One interesting phenomenon of sodium/chloride-coupled neurotransmitter transporter is that they can operate in two directions. Amongst other substances amphetamines are known to induce reverse transport. Reverse transport has been reported for many transporter (Roux and Supplisson, 2000; Seidel et al., 2005). Since the concentration of sodium ions in the cytoplasm is much lower than in the extracellular space, reverse transport is per se a rare event. However, if large amount of substrates are transported, as mentioned before, ion conductance becomes much larger and reverse transport is more likely to occur. There are two proposed triggers of transporter-mediated efflux (Sitte and Freissmuth, 2010):

(i) Sodium currents: accumulation of Na\(^+\) on the intracellular side favors inward-facing conformation and therefore drives efflux.

(ii) Presence of releasing substrate, like amphetamine. Amphetamine was shown to trigger inter alia Na\(^+\) influx and pushing the transporter in a channel mode.

Both oligomerization and apparently the N terminus have been shown to be of importance for the action of amphetamines. Truncation of the N terminus was found to abolish amphetamine-induced reverse transport in SERT (Sucic et al., 2010) as well as in DAT (Seidel et al., 2005). PKC- and amphetamine-stimulated phosphorylation of DAT on N-terminal serines (Cervinski et al., 2005; Foster et al., 2002) as well as of SERT (Sucic et al., 2010) have been suggested to contribute to
this mechanism by promoting a conformation of DAT favourable for reverse transport.

Furthermore it was shown that MDMA (methylenedioximethamphetamine) increases PKC translocation by two interrelated mechanisms that involve 5-HT2A/2C receptors and 5-HT transporter (Kramer et al., 1997). Based on those experimental observations, the oligomer-based counter-transport model is one possible explanation how efflux could be facilitated in the first place, since influx and efflux are not supposed to occur simultaneously (for more detailed explanation see figure 3).

**Figure 3** Oligomer-based counter-transport model. Influx and efflux pathway are separate but contained within the oligomeric quaternary structure of the monoamine transporter. Na⁺ influx promoted by the uncoupled current, which is induced by amphetamine analogues (here PCA) provides the driving force for the counter-transport; it promotes the inward-facing conformation that extrudes substrate (here 5-HT). Left: Low concentrations of PCA induces substrate (S) efflux by activating PKC mediated phosphorylation resulting in conformational change which triggers channel mode (inward-facing conformation). Right: Increase in PCA concentration causes a decline in substrate release since PCA and substrate cannot pass simultaneously. At high PCA concentration all serotonin transporters are occupied by PCA and substrate efflux is inhibited. Also, PKC activity is inhibited preventing a switch to inward-facing conformation. This model explains the experimental observation of a bell-shaped concentration-response curve of amphetamine-induced efflux (Sitte and Freissmuth, 2010).
1.3. Regulation of Neurotransmitter Transporter

It is well known that neurotransmitter transporters are regulated in a complex way. A great effort has been taken on untangling the complex networks of regulatory mechanisms that control transporter life and function. The major regulatory events are:

i. Synthesis and processing of the transporter
ii. Transport to the cell surface
iii. Activation through its substrate
iv. Phosphorylation followed by internalization
v. Recycling or degradation.

i. Synthesis and Processing of Neurotransmitter Transporters

The three human monoamine transporters SERT, DAT, and NET are localized on different chromosomes and exist (with exception of DAT) in different splice forms. The gene of hSERT (SLC6A4) has been mapped to chromosome 17q11 (24kb; 13 exons), hNET (SLC6A2) to chromosome 16q12.2 (45kb; 14 exons), and hDAT has been localized to chromosome 5p15.3 (65kb; 15 exons). Whereas several regulatory factors binding to SERT gene promoter regions have been revealed (Jayanthi and Ramamoorthy, 2005), little is known about transcriptional regulation of DAT or NET. In addition it is worth mentioning, that the promoter region of the hSERT is extensively studied in psychiatric field since it is highly polymorphic and was shown to impact its expression levels (Heils et al., 1996) and binding of regulatory proteins (Hu et al., 2006). This is one reason why there is much more information regarding SERT compared to the other monoamine transporter. Amino acid sequence analysis of DAT, SERT, and NET revealed numerous consensus sites for protein kinases and putative cytoplasmic interactions motifs for binding of second messengers (see Figure 4).
### Table

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>hDAT</td>
<td>MSSVEAPEP NAYVAPKVEIL ILVEKGNVQ L73STLTPR Q5PVRQGDRES TGKXKIFLL SYGOFAYLDA</td>
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<tr>
<td>hNET</td>
<td>MLLNHLYQQPQ IIPGDAATQGPQ PLLAIKKEL LVEEAMGQV C....LAPP IIKDAQPRQ TWQKXKIFLL SYGOFAYLDA</td>
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<tr>
<td>hSERT</td>
<td>KETTPNLNSQ QLSACEDGED QCENISVHGY VFTPGVEVSC VQISGNSAYQ PSFAGAIDTR NSIPATTTTL VAELNWQRE TWQKXKIFLL SYGOFAYLDA</td>
</tr>
<tr>
<td>hDAT</td>
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<tr>
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<tr>
<td>hNET</td>
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<tr>
<td>hNET</td>
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<tr>
<td>hNET</td>
<td>YFLSGFLQPF RELAVA C5 KEEHEL DRG L2PTQ RHN LKV G80</td>
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<tr>
<td>hSERT</td>
<td>YFLSGFLQPF RELAVA C5 KEEHEL DRG L2PTQ RHN LKV G80</td>
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Figure 4 Amino acid sequence and topology of monoamine transporters. (a) Amino acid sequence alignment of human dopamine, norepinephrine, and serotonin transporters shows a striking similarity. Sections I-XII mark the different transmembrane domains (TMDs) (see b). Conserved aspartate (D) residues, located in TMD1 are supposed to be involved in monoamine transport (yellow box). The leucine (L)-repeats in TMD2, and the glycophorin-like motif (see text) in TMD6 are supposed to play a role in oligomerization (green box). Putative residues involved in conformational changes during substrate binding are located between TMD6 and TMD 7 (blue box). The colour boxes within the parts of the intracellular carboxyl termini represent interacting sites with Hic-5 (beige), synuclein (grey), and PICK1 (purple), Rab4 (lime green box). (Picture source: adapted from (Torres et al., 2003b).
ii. Trafficking and Transport to the Cell Surface

In most neurotransmitter transporters, the C-terminal region was found to be crucial for targeting of the monoamine transporters to the plasma membrane:

Studies on GABA transporter 1 (GAT-1), a member of the SLC6 neurotransmitter transporter, identified C-terminal amino acids motif \text{VMI}^{569-571} which facilitates transport from the ER to ERGIC (ER-Golgi intermediate compartment) and finally to the cell surface (Farhan et al., 2008).

The C-terminus of SERT was shown to be critical for the delivery of SERT to the plasma membrane as deletion of 17 or more amino acid residues had a dramatically effect on uptake activity and expression on the surface. Furthermore truncation of the C-terminus resulted in decreased level of glycosylation (Larsen et al., 2006). It also contains a non-canonical PDZ domain mediating physical interactions with several proteins. Moreover, it has been demonstrated that Sec24D binds C-terminal on SERT and facilitates correct folding of the transporter (El-Kasaby et al., 2010). Sec24 is one of five proteins of the COPII (coat protein complex II) vesicle coat which mediates the selective export of membrane proteins from the endoplasmic reticulum (ER). Interestingly, in humans there are several Sec24 isoforms, which showed a selective isoform-specific transport (Wendeler et al., 2007) of a broad variety of proteins.

Similar requirements for the intracellular C-terminus have been observed for DAT and NET. However, there are differences between these transporters: amino acids at the very extreme ends of the C-terminus seem to be important for functional expression of both DAT (Foster et al., 2006; Miranda et al., 2004) and NET (Bauman and Blakely, 2002; Burton et al., 1998), whereas for SERT, deletions of 17 or more residues are necessary to affect uptake (Larsen et al., 2006).

DAT delivery to the surface has shown to be dependent on the last three C-terminal residues (LKV). It was shown in endothelial cells, that alanine substitution of Lys-590 and Asp-600 delayed the delivery of DAT to the cell membrane, due to
retention in the endoplasmic reticulum. Interestingly, mutation of Gly-585 to alanine completely blocked the exit of DAT from the ER and surface expression of the transporter (Miranda et al., 2004). Furthermore, distinct but overlapping C-terminal internalization signals in DAT are suggested to be conserved across SLC6 neurotransmitter transporters. Yet those motifs are not classical internalization motifs which suggests that SLC6 neurotransmitter transporters may have evolved unique endocytic mechanisms (Holton et al., 2005). The importance of glycosylation for efficient transport to the plasma membrane also has been found for SERT (Mercado and Kilic, 2010; Ozaslan et al., 2003) as well as for DAT (Li et al., 2004).

iii. **Activation through its Substrate**

Out of clinical studies it is known that prolonged treatment with SSRIs causes a decrease in serotonin transporter expression without affecting mRNA levels in vivo (Benmansour et al., 1999). Hence, it was suggested that inhibition of SERT must somehow lead to increased endocytosis of decreased transport to the surface. Vice versa the binding of the ligand should therefore prevent such regulatory interventions. One study showed that binding of 5-HT prevents PKC-mediated internalization of SERT. Furthermore selective serotonin reuptake inhibitors imipramine, paroxetine and citalopram could block the ability of 5-HT to limit PKC-dependent phosphorylation. Interestingly they were able to show that amphetamine reduces SERT surface expression up to 40% and that this reduction could be prevented by 5-HT. Therefore it is assumed that translocation of the substrate may result in a conformation that prevents phosphorylation via PKC and associated sequestration or alternatively may alter phosphatase or accessory protein access to PKC-dependent SERT phosphorylation sites, thereby limiting phosphorylation and sequestration (Ramamoorthy and Blakely, 1999).

iv. **Phosphorylation and Internalization**

Analysis of amino acid sequences of NSS revealed presence of several consensus sites for protein phosphorylation by cAMP-dependent protein kinase A (PKA), protein kinase C (PKC), and Ca²⁺/calmodulin-dependent protein kinase. Therefore, not surprisingly, many research groups focused on possible interaction and
regulation on transporters activity and trafficking using mentioned candidates. One major player is PKC. Although other protein kinases, like PKA (protein kinase A) and PKG (protein kinase G) are known to phosphorylate the serotonin transporter, only PKC was found to down-regulate 5-HT uptake. PKC is a single polypeptide, comprised of an N-terminal regulatory region (20-40kDa) and a C-terminal catalytic region (~45kDa). Members of the PKC family can be divided into conventional PKCs (α, βI, βII, and γ) which are regulated by Ca²⁺, novel PKCs (δ, ε, η, θ, and μ) which show no Ca²⁺ interaction, and atypical PKCs. PKC uses ATP as substrates and phosphorylates serine or threonine residues within basic amino acid sequences. Unlike its cousin PKA there seems to be no classical binding motif and PKC shows also a lower stereospecificity. PKC can be recruited to the plasma membrane via diacylglycerol (DAG) which is one of the two products of phosphatidylinositol-4, 5-bisphosphate (PIP₂) cleavage by phospholipase C (PLC). DAG causes a dramatic increase in PKCs membrane affinity and therefore serves as anchor for PKC to the plasma membrane (Nishizuka, 1992) (see figure 5). Several studies showed that the serotonin transporter is phosphorylated C-terminal by PKC, leading to internalization and reduction of substrate transport velocity (V_max) yet without affecting substrate affinity (K_M). Interestingly this regulatory mechanism seems to be a biphasic event (Jayanthi et al., 2005) for the serotonin transporter. In the early phase, activation of PKC induces phosphorylation initially on serine residues followed by a phosphorylation of threonine residues in the late phase. In addition, activation of PKC through β-PMA (β-phorbol myristate acetate) reduced uptake rates which could be restored by the addition of SERT substrates. Within 5 minutes of β-PMA treatment about two-third of inhibition of SERT activity was achieved with no detectable decrease in surface expression. After 30 minutes, when PKC activated internalization of SERT, resulting in decreased surface expression, only a modest additional decrease in transport activity was measured. So, it is likely that phosphorylation in the early phase attracts proteins that alter transport activity and SERT is internalized afterwards. Looking at those results nearly answers the question if the reduction in transport activity could be a consequence of the internalization of SERT- it is highly unlikely. Furthermore there is evidence that the direct phosphorylation itself has no influence on transporter activity. Even upon
mutation of all predicted PKC phosphorylation sites PKC-mediated reduction of 5-HT uptake was found (Sakai et al., 1997).

**Figure 5 Activation and signalling of PKC.** Phospholipase C cleaves PIP$_2$ molecule resulting in IP$_3$ (inositol-1,4,5-triphosphate) which induces Ca$^{2+}$ release from the endoplasmic reticulum, and DAG (diacylglycerol) which recruits PKC (activated by Ca$^{2+}$) to the plasma membrane. Now PKC can phosphorylate different substrates resulting in different signalling pathways. (Picture source © 2000, W. H. Freeman and Company)

Also, there are indications that the cytoskeleton may have an influence on serotonin transporter activity (Sakai et al., 2000). A recent study could show that SERT activity can be modulated depending on its membrane environment (Chang et al., 2012). Using single quantum dots (Qdot)-labeling to track single transporter molecules, they suggested that SERT can be present in two ‘compartments’. One in which SERT can diffuse freely (no-raft state) and one where SERT is immobilized by cytoskeletal proteins and constrained to membrane microdomains (tethered raft-state). Whereas in the no-raft state SERT shown reduced activity, tethering to the cytoskeleton causes SERT to display basal activity. Activation of signal molecules, like p38MAPK, is believed to relax those cytoskeletal constraints but keeping SERT
embedded within the membrane microdomain which leads to enhanced transport activity. What makes this model interesting is the linkage between cytoskeleton, membrane microdomains and SERT particularly since membrane microdomains are rich in cholesterol and PIP$_2$.

Recent studies laid focus on a very interesting protein, the MacMARCKS protein (myristoylated alanine-rich C kinase substrate (MARCKS)-related protein) which was shown to integrate Ca$^{2+}$/calmodulin and protein kinase C-dependent signals in the regulation of neurosecretion (Chang et al., 1996). MARCKS proteins consists of three major domains: the myristylation site (MS) which anchors them into the plasma membrane; the MARCKS-homology domain (MHD) with unknown function; and the effector domain (ED) which is positively charged and enables binding to calmodulin and cytoskeletal proteins (Hartwig et al., 1992). The effector domains of MARCKS and MacMARCKS are nearly identical. What makes these finding so interesting is that above mentioned effects of PKC and the cytoskeleton as well as regulatory processes involving Ca$^{2+}$ could support the role of MARCKS proteins as important interface in SERT regulation. Furthermore, MARCKS proteins are also known as “pipmodulins”. It was found, that MARCKS proteins play a critical role in the metabolism of PIP$_2$ (Kalwa and Michel, 2011) and the control of free PIP$_2$ concentrations within the cell membrane. MARCKS interacts via the effector domain with the plasma membrane. These interactions are supposed to be nonspecific electrostatically (Gambhir et al., 2004). It is suggested that MARCKS reversibly sequesters a significant fraction of the PIP$_2$ (and PIP$_3$) in the inner leaflet of the plasma membrane. Experimental data showed that overexpression of MARCKS in neuronal or epithelial cells produced an increase in the total level of PIP$_2$ in the cell which makes sense if the higher MARCKS concentration reduces the level of free PIP$_2$, causing the cell to compensate by increasing PIP$_2$ production to maintain a constant free PIP$_2$ level (Lemmon, 2003). Furthermore MARCKS was shown not to be uniformly distributed in the plasma membrane several cell type but rather restricted to certain areas in which concentrations of PIP kinases are also elevated (Doughman et al., 2003). So, MARCKS seem to accumulate PIP$_2$ around certain areas where it can be used to make local signal changes which again can be regulated through local Ca$^{2+}$ concentrations. Since the effector domain of MARCKS not only interacts with PIP2 molecules but also is the binding site of Ca$^{2+}$-bound
calmodulin (Ca/CaM), MARCKS can be hauled off the membrane via this interaction (see figure 6).

Figure 6 Regulation of PIP$_2$ availability via MARCKS protein. Low local Ca$^{2+}$ concentrations and low PKC activity allows MARCKS to associate with the plasma membrane and thereby sequestering three PIP$_2$ molecules. Increase in local calcium concentrations leads to activation and binding of Ca$^{2+}$-bound calmodulin (Ca/CAM) to the effector domain of MARCKS. This releases of MARCKS from the membrane which can set PIP$_2$ molecules free to either diffuse away or allow binding of PIP$_2$ substrates and induce local signalling.

As a consequence of Ca/CaM binding followed by MARCKS release from the membrane, PIP$_2$ molecules can laterally diffuse away from each other, abrogating local PIP$_2$ accumulation, or to clear the way for binding of PIP$_2$ substrates. Interestingly, it was shown that chronic treatment of lithium (mood stabilizing drug) and valproate (an anticonvulsant and mood-stabilizing drug) in rat hippocampus or cell culture could reduce the expression levels of PKC and MARCKS (Lenox et al., 1996). To conclude a clinically significant role for MARCKS is too early. So, taken together, PKC and MARCKS are likely to play a role in regulating serotonin transporter function, whether directly through physical interaction or indirectly by recruiting other proteins is not clear so far. The third key player and central object of this work is PIP$_2$. There is emerging evidence for PIP$_2$ to have an
important impact on the function of neurotransmitter transporter which I will discuss in the next section.

v. Recycling and Degradation

Most of the transporters rapidly internalize into the endosomes in a constitutive or stimulus-dependent manner. Internalized transporters are either recycled back to the plasma membrane or sorted to the lysosomes for degradation. Thereby posttranslational modification seems to be of great importance, especially ubiquitination (Miranda and Sorkin, 2007). SERT was found to interact with MAGE-D1, an adaptor molecule of the ubiquitin-dependent degradation pathway. Knock-out of MAGE-D1 was shown to increase expression of SERT in mice (Mouri et al., 2012). Co-immunoprecipitation and uptake experiments in platelets, which also express SERT, suggested the focal adhesion protein Hic-5 as a determinant of transporter inactivation and relocation to a compartment subserving endocytic regulation (Carneiro and Blakely, 2006). For DAT it was demonstrated, that PKC activation both increases DAT endocytic rates and decreases DAT recycling back to the plasma membrane (Loder and Melikian, 2003). Also, PKC activation leads to an increase in ubiquitinylation and therefore accelerated degradation of DAT (Miranda et al., 2005).
1.4. Regulation of Neurotransmitter Transporter by membrane components

Membranes are composed of a lipid bilayer, and organization within this structure demands that the lipids have a dual nature. Indeed lipids within the plasma membrane are amphiphilic, meaning that they consist of a hydrophobic part (tail; side chains of fatty acids) and a hydrophilic part (head; a phosphate group) which gave them the name phospholipids. The four major phospholipids within the mammalian plasma membrane are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyeline. Other phospholipids, like ionositolphospholipids are more unfrequented. But it’s them playing a major role in different cellular signaling processes as we will see. In addition there is another molecule that is also an important part of the plasma membrane-cholesterol. And cholesterol is a very interesting molecule with still unknown features. It is highly abundant in the brain and mostly resides in the nonpolar portion of the lipid bilayer. One characteristic of cholesterol is to raise the permeability barrier of the lipid membrane and influence the transition range of the membrane. The latter means that the membrane can exist in a fluid-like and solid-like state, and can be transitioned at a certain temperature range from one into another. Depletion of cholesterol was found decrease transporter activity in GAT, DAT, and SERT (Miranda et al., 2007; Scanlon et al., 2001). Interestingly in all cases not only $V_{\text{max}}$ but also $K_{M}$ was found to be reduced following cholesterol depletion. However, cholesterol is important for membrane integrity. Thereby changing membrane fluidity state by depletion of cholesterol could be responsible for reduced transporter activity in a more fundamental way. Also, the model of SERT regulation via cytoskeleton and microdomains (see previous section) could explain those observations.
Figure 7 Schematic diagram of the phospholipase C (PLC)-PIP₂ cycle. Synthesis of PIP₂ (phosphatidylinositol-4,5-bisphosphate) is facilitated in two steps by two different kinases. In the first step, phosphatidylinositol (PI) is phosphorylated on the fourth position of its inositol ring by the phosphoinositide 4-kinase (PI4-K) producing phosphatidylinositol-4-phosphate which is converted by the phosphoinositide 5-kinase (PI5-K) to phosphatidylinositol-4,5-bisphosphate (PIP₂). PI4-K activity can be inhibited by phenylarsine oxide (PAO). PLC can cleaves PIP₂ producing the second messengers inositol-(1,4,5)-trisphosphate (IP₃) and diacylglycerol (DAG). PLC activation can be facilitated by the substance m-3MFBS. DAG again activates protein kinase C (PKC) which triggers several signal transduction cascades, whereas IP₃ binds to IP₃-receptor of the endoplasmic reticulum triggering Ca²⁺ release (see figure 5). PKC activation can be prevented by the chemical substance GF109203X. DAG can be hydrolysed by DAG lipases into arachidonic
acid, a diffusible messenger, or recycled into phosphoinositide resynthesis through DAG kinase-mediated phosphorylation to phosphatidic acid (PA). IP3 on the other hand can also be recycled via several phosphorylation steps to inositol.

In vivo, seven distinct phosphoinositide species have been found and can be distinguished on the basis of the degree respectively the position of phosphorylation. Formation of distinct phosphoinositides is accomplished by phosphoinositide kinases. In mammalian, 47 genes encode 19 different phosphoinositide kinases and 28 phosphoinositide phosphatases, being both responsible for interconversions amongst phosphoinositides. The four major phosphoinositides (PI(3)P, PI(4)P, PI(3,5)P$_2$, and PI(4,5)P$_2$) are distributed in restricted to specific membrane domains. PI(3)P is enriched in early endosomes, PI(3,5)P$_2$ is present in the late endosomes and multivesicular bodies, PI(4)P is particular abundant in the Golgi complex and the endoplasmic reticulum, whereas PI(4,5)P$_2$ main distribution is found in the plasma membrane. The location of PI(4,5)P$_2$ explains their important function as being the interface between extracellular environment and the intracellular. Therefore it’s not surprising that PI(4,5)P$_2$ is involved in many important signaling processing with a high diversity of interaction partners like cytoskeletal proteins, integral membrane proteins, associated membrane proteins as well as transmembrane proteins. The enzymes that produce PIP$_2$ are the phosphatidylinositol phosphate kinases (PIP kinases), a family of lipid kinases that differs in amino acid conservation from all other lipid kinases, but contains a kinase core domain with conserved catalytic residues that bind ATP and Mg$^{2+}$. The PIP kinases are divided into three subfamilies, type I, II, and III and each subfamily produce PIP$_2$ by unique mechanisms. An activation loop within the PIP kinases defines substrate specificity (Rao et al., 1998).

So it has been shown that type I PIP kinase activity is necessary in actin rearrangements adhesion, secretion, endocytosis, and ion channel regulation as well as regulation of transporter (Huang, 2007; Kwiatkowska, 2010). There is growing evidence that PIP$_2$ influences the activity of membrane channels (Rohacs, 2009), receptors (Gamper and Shapiro, 2007b) and transporters (Huang, 2007). But the exact mechanisms still remain to be elucidated.
One possible mechanism could be direct interaction with PIP$_2$. First evidence of a direct interaction came from Huang and colleagues, demonstrating a direct binding of PIP$_2$ to inward rectifier potassium channels (Huang et al., 1998). Subsequently more and more studies provided evidence that this is also the case for many other channels (Suh and Hille, 2008). 2008, a comprehensive study identified 388 proteins/protein complexes that appeared to interact specifically with the phosphoinositide targets. Amongst others protein domains like PH, PX, PHD, MARCKS, Annexin, Snare, and HEAT have been found to directly interact with PIP$_2$ (Catimel et al., 2008). Given to the rising number of experimental data on ion channels regarding PIP$_2$ interaction, it is under consideration how the binding takes place. One hypothesis which is based on combined experimental data suggests a correlation between strong PIP$_2$ binding and high PIP$_2$ selectivity.

Phospholipids can be converted into arachidonic acid (AA) by the enzyme phospholipase A2 as well as by DAG lipase (see figure 7). Arachidonic acid belongs to the polyunsaturated fatty acids, which are highly abundant in the brain, and can act as a second messenger. Because of their structure (>1 double bond) they influence membrane fluidity by decreasing its melting temperature. Besides that, arachidonic acid was found to affect also transporter activity, like GABA (Chan et al., 1983), glutamate (Volterra et al., 1992) and DAT (L'Hirondel et al., 1995; Zhang and Reith, 1996), differently. It is supposed that arachidonic acid modulates ion channels and transporters in two ways: via direct binding to proteins and through second messenger actions of AA metabolites. Furthermore AA stimulates an inward current in oocytes expressing hDAT but did not affect the leak current. Furthermore it was found that DA potentiates the AA-induced currents in the absence of sodium and chloride which indicates that these currents arise from processes distinct from those associated with substrate transport (Ingram and Amara, 2000). Studies investigating effect of AA on SERT function are still missing, but it was found that partial knock-out of SERT in mice and treatment with SSRIs were associated with a profound reduction in arachidonic acid signalling (Fox et al., 2007; Qu et al., 2006).
Whereas most studies concerning SERT regulation have focused on regulatory proteins involved in trafficking and folding of the transporter, investigations of the influence of membrane components on SERT activity have started quite recently. Nevertheless, recent results point to an important role especially of membrane components, like PIP\(_2\), in the regulation of the transporter activity.
2. Results

SERT activity was investigated in the presence of m-3M3FBS (2, 4, 6-Trimethyl-N-[3-(trifluoromethyl) phenyl] benzenesulfonamide), an activator of PLC (phospholipase C) which leads to the hydrolysis and thereby breakdown of PIP$_2$. Therefore, it was tested if PIP$_2$ was actually depleted in HEK293 cells that stably expressed the human SERT (hSERT).

MALDI (matrix-assisted laser desorption and ionization) MS is known to be a very sensitive approach for the determination of lipid content within cell membranes (Johanson and Berry, 2009); therefore, it was used to quantify the amount of PIP$_2$ caused by treatment of cells by the PLC activator m-3M3FBS.

![Figure 8 Detection of PIP2 content by MALDI-MS.](image)

Analysis of phospholipid content in HEK-SERT cells was performed after treatment with o-3M3FBS (upper panel) and m-3M3FBS (lower panel). The amount of PIP$_2$ species was calculated relative to an internal standard (arrow). Following the treatment with m-3M3FBS, PIP$_2$ was depleted (red frames) compared to o-3M3FBS treated cells. (The signals at m/z 995.47, 1021.45 and 1047.47 correspond to the major PIP$_2$ molecular species...
detected from HEK-SERT cells). X-axis shows the mass/charge ratio (m/z); y-axis: Absolute intensity (number of ions of each species that reaches the detector). (Experiments were carried out by Dr. Gerald Stübiger and Valery Bochkov)

Indeed, m-3M3FBS treatment resulted in successful depletion of PIP$_2$ from the plasma membranes compared to its inactive analog, o-3M3FBS (2,4,6-Trimethyl-N-[2-(trifluoromethyl)phenyl]benzenesulfonamide). So, it was shown that the dramatic decrease of PIP$_2$ was caused rather by a activation of PLC than through the substance 3M3FBS itself, as was demonstrated by also using the inactive form, which is known not to be able activating PLC (Bae et al., 2003).

2.1. Analysis of PIP$_2$ depletion on serotonin transporter activity

Uptake-assays were performed to investigate whether the serotonin transporter was functionally active after PIP$_2$ depletion. Therefore, uptake velocity of serotonin was measured in the presence of m-3M3FBS or o-3M3FBS and compared to the control. No significant difference in uptake velocity ($V_{\text{max}}$) or substrate affinity ($K_m$) amongst differently treated cells (see figure 9) could be detected, showing that m-3M3FBS mediated activation of PLC, including the following activation of PKC, does not influence SERT mediated uptake.

![Figure 9 Uptake of serotonin by HEK-SERT cells treated with PLC activator m-3M3FBS and its inactive analog o-3M3FBS compared to control. No significant](image-url)
changes in transport velocity ($V_{\text{max}}$) or 5-HT affinity ($K_m$) were observed compared to control conditions. Data was analysed using GraphPad Prism® software.

<table>
<thead>
<tr>
<th>hSERT</th>
<th>Control</th>
<th>m-3M3FBS</th>
<th>o-3M3FBS</th>
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<tbody>
<tr>
<td><strong>5-HT Uptake</strong></td>
<td></td>
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<tr>
<td>$V_{\text{max}} \pm \text{S.E.}$</td>
<td>450,7 ± 30,6</td>
<td>413,5 ± 20,3</td>
<td>458,9 ± 25,3</td>
</tr>
<tr>
<td>$K_m \pm \text{S.E.}$</td>
<td>8,5 ± 1,5</td>
<td>7,0 ± 1,0</td>
<td>7,9 ± 1,2</td>
</tr>
</tbody>
</table>

**Table 1** Statistical significance was calculated using two-way ANOVA followed by Bonferroni's post-hoc test.

Next, I examined the effects of the amphetamine derivative $p$CA (para-Chloramphetamine) on efflux of radio-labelled MPP$^+$ (Methyl-4-Phenylpyridinium Acetate, N-[Methyl-3H]) in the presence of m-3M3FBS by performing a release assay. MPP$^+$ is a substance that is transported throughout the DAT, NET and SERT (Ravna and Edvardsen, 2001). By doing so, cells were loaded with radio-labelled MPP$^+$ and superfused (see chapter 6.2), and 2-min fractions were collected. At min 14, $p$CA was added to induce efflux.

![Figure 10](image-url) **Figure 10** Time course of $p$CA-induced, SERT-mediated efflux of [³H]MPP+ from HEK-SERT cells. The cells were loaded with 0.1 µM [³H]MPP+ and superfused,
and 2-min fractions were collected. The different substances were added to the superfusion buffer as indicated. At min 14, pCA (3µM) was added to induce efflux. The superfusion experiment was carried out in the presence or absence of the PKC-inhibitor GF109203X (1µM); m-3M3FBS or o-3M3FBS were present (25 µM) and DMSO was used in an equivalent volume for control purposes. The graph shows the mean data from 3 superfusion experiments performed in triplicate (n=6-9). Statistical significance was tested using Two-Way ANOVA with Bonferroni’s post-hoc test: ***: p<0.001, ns=not significant.

In these experiments, the amount of released MPP\(^+\) was significantly decreased. Contrary to uptake experiments where m-3M3FBS showed no effect on SERT mediated transport. Thus, PIP\(_2\) depletion by m-3M3FBS obviously effects efflux rather than influx rates. Next, the effect of the PKC inhibitor GF109203X on pCA triggered efflux was investigated to rule out whether the effect of m-3M3FBS is mediated by PKC. Addition of GF109203X diminished efflux rates (p<0.001) but it did not affect the efflux reduction by m-3M3FBS as it would have been expected if the observed reduction was mediated by activation of PKC via PLC. Moreover, the effect of the PKC inhibitor was shown to be more dramatic as for the activator of PLC and the effect of activating PLC on efflux rates was not influenced by PKC inhibition as was demonstrated by adding GF109203X and m-3M3FBS. Therefore, decrease in pCA mediated efflux was shown to be caused by loss of PIP\(_2\) in a PKC-independent way.

If the effect of m-3M3FBS is mediated by reduction of PIP\(_2\), an inhibitor of PIP\(_2\) synthesis should have similar effects. To investigate this I performed MPP\(^+\) release experiments and measured effects of m-3M3FBS compared to that of PI4-kinase inhibitor, phenyl-arsoneneoxide (PAO). Similar to the activation of PLC, inhibition of PAO reduced release of MPP\(^+\) although to a lesser extent (see figure 11).

These results indicate that substrate efflux (but not influx) rates are modulated by PIP\(_2\) which is most likely caused by physical interaction with SERT rather than via the second messenger PKC, as was demonstrated by using GF109203X. Also, termination of PIP\(_2\) synthesis by PAO could induce the same effect on efflux rates as sequestration of PIP\(_2\) by activation of PLC. These
results indicate that second-messenger systems do not contribute to PIP$_2$ effects on SERT efflux.

Figure 11 Changes of pCA mediated MPP$^+$ efflux in HEK-SERT cells upon treatment with PLC activator and PI4-kinase inhibitor. HEK-SERT cells were loaded with 0.1 µM [$^3$H]MPP$^+$, superfused, and 2-min fractions were collected. Different substances were added to the superfusion buffer as described in Fig 10. DMSO (control) 25µM m-3M3FBS and 30µM PAO respectively were added after 4min, after totally 14 min pCA (3µM) was added to induce efflux. Bar graphs represent efflux in percentage of total radioactivity between min 22 and 24.

Therefore, the question had to be answered where exactly PIP$_2$ physically interacts with the transporter.

Based on data generated by homology modelling (performed by Andreas Jurik and Gerhard F. Ecker) and electrostatic surface potential (by solving the Poisson-Boltzmann equation by Thomas Stockner), three amino acid residues (K460 (helix 9), K352 (helix 6), and R144 (helix2)) have been found to be putative binding sites for PIP$_2$ (see figure 12). Estimation of the distances between those three residues indicated that R144 is located in a very central position of SERT. This position excludes R144 as a putative binding site for PIP$_2$ because it simply cannot be reached from the rim of the membrane. Moreover it has been shown that the size of the PIP$_2$ head group (~10Å)
matches the calculated distance between K352 and K460 of about 12Å.

Figure 12  Putative binding sites for PIP$_2$. Left: Distance estimation within the positively charged patch (right picture) consisting of K352 and K460. The LeuT-based SERT homology model shows distances (in Angstrom) between putative interaction partners for the negatively charged PIP$_2$ headgroups (performed by Andreas Jurik and Gerhard F. Ecker). Right: Predicted electrostatic potentials. Red denotes negative potential and blue positive potential. The colored ellipses point to the positions of residues, K352 and K460 (yellow). (Performed by Thomas Stockner)

In addition preliminary results generated from pull-down assay have shown that PIP$_2$ physically interacts with SERT (data not shown). Furthermore, it was shown that single mutations K352A respectively K460A had no influence on transporter mediated flux (data not shown). Therefore it was suggested that both amino acids would be necessary to impact PIP$_2$ mediated changes on influx and efflux rates. A double mutation K352A/K460A should reveal the importance of both amino acids on modulation of SERT.

By doing so, both lysine on position 352 and lysine on position 460 have been mutated into alanine (K352A, K460A). So, two positively charged, basic amino acids have been replaced by a neutral amino acid. Based on the fact that PIP$_2$ contains a negatively charged headgroup, replacement through a neutral amino acid should disrupt PIP$_2$-binding to the transporter. Thereby we would expect similar behavior of SERT like it was shown for PIP2 depletion by m-3M3FBS, meaning reduced efflux rates with eventually unchanged influx rates.
2.2. Examination K352A/K460A SERT double mutant

A double mutation K352A/K460A has been introduced to YFP tagged version of human SERT (hSERT) via Quickchange Lighntning Kit (Agilent technologies). The mutations were proofed by sequencing, and this construct was used to transfect HEK293-cells. After treatment with G418, cells stably expressing the mutant version were selected. To ensure that transport of SERT to the plasma membrane was not impaired by the introduced mutations, stably transfected HEK-hSERT-K352A/K460A cells were examined via confocal laser microscopy. In addition a biotinylation assay was performed to confirm intact surface expression.

![Figure 13 Visualization of surface expression via confocal laser microscopy.](image)

Left: YFP-hSERT Right: K352A/K460A double mutant YFP-hSERT. No difference in surface expression could be detected between WT and double mutant SERT. YFP was visualized using a 514nm argon laser and a 505-530nm bandpass filter (30 to 45% input power).

For biotinylation assay, cells have been incubated with EZ-Link Sulfo-NHS-SS-Biotin (Thermo Scientific) and cell lysates have been pulled down via streptavidin (a strong ligand for biotin)-coated beads. Purified cell surface proteins have been separated by SDS-page and SERT was quantified via western blot (see figure 13).
Figure 14 Validation of surface expression by biotinylation assay. a) Bar graphs represent biotinylated in percentage compared to the wild type. There was no significant difference in cell surface expression between the K352A/K460A double mutant and wild type SERT (p=0.8863). Statistical analysis was performed using GraphPad®Prism5.0 software package. B) ELISA: Quantification of hSERT was facilitated using rabbit Anti-GFP (1:10,000) antibodies and mouse anti-Tubulin antibodies (1:20,000) for tubulin. Tubulin was used as a loading control. Experiments were performed 4 times.

The double mutation had no influence on cell surface expression. Therefore, by introducing a double mutation had no impact on trafficking to the cell surface.
To investigate the functional properties of K352A/K460A hSERT I performed inhibition experiments. By using a constant concentration of radio-labeled 5-HT ligand and simultaneously increase of inhibitor pCA concentrations, pCA competes increasingly with 5-HT for its uptake. Therefore, this can be used to investigate substrate affinity as well as transport ability. Since PIP₂ was found to affect only substrate efflux rates and not influx rates, meaning uptake, deletion of putative PIP₂ binding sites should not significantly influence pCA mediated inhibition of SERT uptake.

Inhibition of radio-labeled 5-HT ligand uptake was measured in the presence of rising inhibitor, pCA, concentrations (0, 1-100µM). Mutational loss of positively charged amino acids providing potential binding sites for PIP2 did not alter SERT inhibition by competitors (see figure 15).

**Figure 15 Inhibition of 5-HT uptake at increasing pCA concentrations.** Percentage of 5-HT[H³] (0,1µM) uptake relative to control (0µM pCA) at increasing pCA concentrations (0, 01-100µM). Background was measured at 10µM paroxetine. EC50 values of hSERT (1,029) and double mutant K352A/K460A (0, 5030) were calculated using Graph Pad Prism software.
Taken together, my results imply PIP$_2$ alters amphetamine induced SERT efflux while substrate uptake (influx) remains unchanged. It was also shown that modulation of SERT efflux was not caused via second messenger signalling of PIP$_2$ as could be demonstrating by using PKC-inhibitor GFX. These data point to a modulation of SERT by PIP$_2$ by a direct mechanism. Furthermore, deletion of amino acids which are supposed to be potential binding sites for PIP$_2$ did show the same effects. It could be excluded, that mutation of the amino acids K352 and K460 causes altered transporter efflux by either influencing transporter function itself or altered trafficking of the transporter as it was shown in inhibition experiments and surface expression assays. However, a direct interaction of PIP$_2$ could not be determined yet.
3. Discussion

The serotonin transporter (SERT) is one of the major neurotransmitter transporters and plays an important function in the termination of serotonin signalling. Deregulation of SERT is observed in several psychiatric and also physiological diseases, since the serotonin transporter is also expressed peripheral. Most of the efforts taken by so far rather deled with membrane localization, identifying domains important for correct folding and interaction with proteins necessary to target transporters to their site of action (El-Kasaby et al., 2010; Sucic et al., 2011; Sucic et al., 2013). Nevertheless, a large amount of data could be generated by using mutagenesis, heterologous expression systems, pharmacokinetic experiments and recently computational as well as crystallographic approaches. Several interacting protein-candidates have been found to regulate SERT mediated currents, SERT expression and SERT trafficking. With arrival of heterologous expression systems and transporter specific antibodies in the mid 1990 evidence emerged for involvement of several proteins in regulation of SERT surface expression and transporter function. Amongst those regulatory proteins cellular kinases have been found to regulate neurotransmitter transport by affecting intrinsic transport activity or by controlling transporter cell surface expression via phosphorylation. One of the most common observations was that application of protein kinase C (PKC) activators led to a reduction in amine transport capacity, revealed by changes in $V_{\text{max}}$ with little or no change in $K_m$ (Blakely et al., 1998). Furthermore, immunoprecipitation of phosphorylated SERT linked phosphorylation also to other kinases, like PKA or PKG (Ramamoorthy et al., 1998). However, PKA and PKG failed to influence intrinsic transporter capacity. Up to now the exact mechanisms that underlie these regulatory properties were not fully understood. Activation of PKC and its recruitment to the plasma membrane is facilitated via PIP$_2$ break-down by phospholipase C (PLC) into the second messengers IP3 and DAG. Furthermore, PIP$_2$ not only serves as a substrate for PLC, but was also shown to directly interact with intracellular proteins and is involved in a vast amount of various and fundamental processes (Berridge, 2009; Koch and Holt, 2012; Moss, 2012). Recent observations showed an involvement of PIP$_2$ in the regulation of membrane
channels as well as ion transporters (Hilgemann et al., 2001; Logothetis et al., 2010; Wang et al., 2011). In addition, PIP$_2$ was shown to accumulate at sphingolipid/cholesterol-based rafts following activation of distinct membrane receptors or be sequestered in a reversible manner due to electrostatic constrains provided by proteins like MARCKS (Kwiatkowska, 2010). Investigating the interaction between PIP$_2$ and potassium channels it was suggested that a direct binding of PIP$_2$ prevents its inactivation (Hilgemann, 1997). However, regulation of PIP$_2$ on the SERT has not been reported before.

This work investigated the effect of PIP$_2$ on SERT mediated flux as well as the interaction of PIP$_2$ with SERT. Following successful PIP$_2$ depletion using the PLC activator m-3M3FSB, it could be demonstrated that only substrate efflux of SERT induced by the amphetamine analogue pCA, but not substrate uptake was affected by a loss of PIP$_2$ (see figure 9 and 10) thereby excluding general regulatory mechanisms such as changes in surface expression. Depletion of PIP$_2$ through activation of PLC caused a significant decrease of the SERT efflux rates pointing to the fact, that PIP$_2$ supports amphetamine induced efflux by probably keeping the transporter in a favourable inward facing conformation via direct or indirect interactions. Stimulating PLC to deplete PIP$_2$ from the cell membrane causes a proportional increase in second messenger molecules IP$_3$ and DAG which again increase PKC activity. So, one could argue that the observed decrease in efflux rates could be probably the result of enhanced activation of PKC. Therefore, in addition pCA induced substrate efflux was measured in the presence of the PKC inhibitor GFX was measured. The results show that PKC could not be responsible for the change in efflux rates, since inhibition of PKC led to comparable efflux rates as the control. Still, production of second messengers through activation of PLC could have an effect on efflux rates. Therefore I altered the phosphoinositide pool in a different way, reducing PIP$_2$ without activating at the same time IP3 and DAG. This was achieved using the PI4-kinase inhibitor PAO. PI4-kinase phosphorylates inositol phosphate (IP) creating PI4P, the precursor of the PIP$_2$ synthesis. Accordingly, application of PAO also reduced SERT efflux rates although not so dramatic as m-3M3FBS, indicating that the phosphoinositide pool obviously plays an important role.
These results indicate that PIP2 modulates SERT efflux without the contribution of second-messenger systems.

The next question was based on the assumption that the interaction between PIP$_2$ and SERT is highly likely direct. Thus a computational approach by Andreas Jurik, Gerhard F. Ecker and Thomas Stockner revealed two possible amino acids which due to their charge, location and distance are potential candidates for a direct PIP$_2$ binding. Therefore the two positively charged amino acids K460 and K352 within the intracellular loops of the helix 9 respectively helix 6 have been used for further approaches. Exchange of positively charged lysines against neutral alanines was expected to eliminate PIP$_2$ binding to the serotonin transporter thereby causing similar effects on substrate efflux as depletion of PIP$_2$. But previous to substrate efflux measurements functional characterization of the K352A/K460A hSERT double mutation was necessary to show that the exchange of those two amino acids does not alter intrinsic SERT function. Heterologous expression of YFP tagged K352A/K460A hSERT A in HEK revealed normal transport to the cell surface using confocal laser microscopy (see figure 13). This observation could be confirmed by a biotinylation approach combined with ELISA (see figure 14). No impaired trafficking due to introduction of the double mutation could be observed. In a next step a pharmacological approach was used to investigate substrate flux in K352A/K460A hSERT. According to observed effects of PIP2 depletion on SERT substrate flux, the double mutation was expected to show altered efflux but normal influx behaviour. Substrate influx in the presence of rising concentrations of competitive pCA (see figure 15) as well as uptake experiments (data not shown) indicated unchanged influx behaviour of the double mutant compared to wild type SERT. As expected the introduction of the double mutation did not alter substrate influx. Parallel experiments performed by Florian Buchmayer investigated also substrate efflux of the K352A/K460A hSERT. Amphetamine induced substrate efflux was drastically reduced (see figure 16) in the presence of PLC activator m-3M3FBS as well as in the presence of its inactive analogue o-3M3FBS. This result shows that the introduction of the double mutation had the same if not stronger effect on substrate efflux and that the addition of PLC activator had no effect on that.
Figure 16 pCA-induced, SERT-mediated efflux of [³H]MPP+ from HEK293 cells expressing hSERT-K352A-K460A. The cells were loaded with 0.1µCi [³H]MPP+ and superfused, and 2 min fractions were collected. 25µM m-3M3FBS or o-3M3FBS were added to the superfusion buffer at min 4. At min 14, pCA (3µM) was added to induce efflux (Experiments performed by Florian Buchmayer).

Taken together, it could be proven that the phosphoinositide PIP₂, which was already reported to influence other membrane proteins, impacts SERT efflux but not influx behaviour and that the two amino acids K352 and K460 are essential for those changes. Still, the proof for a direct interaction of PIP₂ with those amino acids is still missing, and the question how exactly PIP₂ manage to change SERT flux behaviour still remains. Somehow PIP₂ seems to stabilize the inward conformation of SERT and thereby facilitating substrate efflux in the first way. Although results strongly indicate that PIP₂ binds directly to those amino acids it cannot be excluded that a unknown protein could act as scaffold protein by binding PIP₂ and triggering conformational change by simultaneous binding of SERT. One possible candidate could be the MARCKS (myristoylated alanine-rich C-kinase substrate) protein that was shown to sequester PIP2 and also induce conformational changes in NMDA and EGF receptors (McLaughlin and Murray, 2005). Thus, further experiments have to be conducted to unravel the exact binding mechanism of PIP₂.
4. Materials and Methods

4.1. Mutagenesis and Cell Transfection

Transfection of HEK 293 fibroblasts

HEK293 cells were transfected following the Ca-PO₄ precipitation method (Chen and Okayama, 1988). HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/liter) and l-glutamine (584 mg/liter) and µg/ml pen/strep. To select for cells stably integrated the transfected constructs for SERT variants, cells were selected for 10 days in the presence of 250µg/ml G418. Colonies found after this time were picked and tested for functional expression of SERT. Cells stably expressing the constructs were afterwards propagated in medium supplemented with 50µg/ml G418.

Mutagenesis

Mutation of YFP-SERT constructs were performed using Quickchange lightning KIT (Agilent). Following primers were used:

K352A: TGGCTTTTGCTAGCTACAACCGCGTTCAACAACAACACTGCTACC
K460A: GTTCCCACACGTCTGCGCAGCGCGCCGGGAGCGGTT

PCR conditions:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
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</tr>
<tr>
<td>95°C</td>
<td>30sec</td>
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<tr>
<td>56°C</td>
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</tr>
<tr>
<td>68°C</td>
<td>4min</td>
</tr>
<tr>
<td>72°C</td>
<td>5min</td>
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</tbody>
</table>
4.2. Pharmacological Assays

Uptake and release measure the substrate influx respectively efflux per minutes per cell. Since substrate binding and release follows the rate law of Michaelis-Menten enzyme kinetics:

Reaction mechanism: \[ E + S \rightleftharpoons [ES] \rightarrow P + E \]

Velocity of product formation:

\[ v = \frac{d[P]}{dt} = \frac{V_{\text{max}}[S]}{K_M + [S]} \]

Figure 17 Michaelis-Menten kinetics in theory and praxis. Left: schematic plot of an enzymatic reaction. Right: experimental data of an uptake assay. Experimental data are plotted as a function of reaction velocity (unit: pmol/mio/min) against concentration of substrate (or ligand). The Michaelis constant (\(K_M\)) is the substrate concentration at half-maximum enzyme velocity (\(V_{\text{max}}/2\)).

To measure release of neurotransmitter or other ligands, a superfusion experiment has to be performed (see figure 16). Therefore the ligand is added at a desired concentration to a microporous filter layered with cells in bulk. Because released molecules would hit receptors and transporters, triggering chain reactions and therefore a change in release of that molecule, any released molecule is immediately removed by the superfusion medium. The
superfusion medium is applied in a constant up-down flow which prevents reuptake and minimizes indirect effects. Concentrations of released ligands are measured by high performance liquid chromatography (HPLC).

Figure 18 Schematic setup of a superfusion experiment. Release of neurotransmitter would cause a chain reaction by stimulating transporters and receptors and therefore changing the release of that neurotransmitter. The application of a constant up-down superfusion any releases neurotransmitter is immediately removed. (Picture source: Nature Reviews Neuroscience 13, 22-37 (January 2012))

HEK cells stably expressing YFP tagged WT-hSERT and mutated hSERT (K352A/K460A) respectively were used.

**Uptake Assay:**

Cells were plated on 24-well plates at a density of 1*10^5 cells per PDL-coated well. Cells were preincubated with o-3M3FBS (25µM) and m-3M3FBS (25µM) respectively for 10 min. Medium was removed by aspiration and cells were covered with 750µl KHP buffer (5mM D-glucose) at room-temperature.

**Determination of non-specific uptake:** cells were pre-incubated with 10 µM paroxetine for 5 min following incubation with 60µM [³H] 5HT for 1 min at RT.

**Determination of specific uptake:** To determine K_m and V_max values, nonlabeled substrate was used at increasing concentrations to dilute the specific activity of [³H] 5HT. Therefore, 0,1-60µM [³H] 5HT (containing 30-50nM [³H]) were added and incubated for 1min at RT.

After incubation for 1min at RT, cells were washed with ice cold uptake buffer and immediately lysed with 1ml 1%SDS. Finally, cell suspension was
transferred to scintillation vials and 2 ml scintillation cocktail was added and counted in a beta counter.

Analysis of cpm (counts per minute) data was performed using following formula:

\[ x = \frac{(cpm - BK) \times df}{t \times TA \times CpW} \]

BK= cpm at 10 µM paroxetine  
df= dilution factor (concentration of 5HT/concentration \([^3H]\))  
t= uptake time (1minute)  
CpW= cells per well  
x= pmol/Mio/min  
TA= total activity (cpm/concentration of \([^3H]\))

Data evaluation was done using GraphPadPrism®5.0 software packages. Thereby pmol/Mio/min values were plotted against concentration of 5-HT and analyzed using non-linear curve fitting to calculate \(V_{max}\) and \(K_m\).

**Substrate efflux assay:**

Culture medium was removed from stably or transiently transfected HEK cells (4\(\times\)10\(^5\) cells per well grown on coverslips in 96-well plates), and the cells were pre-incubated with 0.4 µM \([^3H]\)5HT or with 0.1 µM \([^3H]\)MPP+ for 20 min at 37°C in a final volume of 0.1 ml uptake buffer per well. The coverslips were transferred into chambers and excess radioactivity was subsequently washed out with buffer at 25°C, for 45 min at a perfusion rate of 0.7 mL/min. Once stable efflux of radioactivity was achieved, following the initial wash, 2 min fractions were collected and samples were counted in a beta counter. Data evaluation was performed using Microsoft Excel and GraphPadPrism®5.0 Software packages.
**Inhibition assay:**

Cells were plated on 24-well plates at a density of $1 \times 10^5$ cells per PDL-coated well. After washing cells with 750µl uptake buffer, cells were incubated with 0.01-100µM pCA respectively 10µM paroxetine for 5min at room temperature. After incubation cells were washed with ice-old uptake buffer and incubated with same concentrations pCA in addition to 0,1µM $[^3]$H 5HT ligand for 1min. After incubation for 1 at RT, cells were washed with ice cold uptake buffer and immediately lysed with 1ml 10%SDS. Finally, cell suspension was transferred to scintillation vials and 2 ml scintillation cocktail was added and counted in a beta counter.

Analysis of cpm (counts per minute) data was performed using following formula:

$$ x = \frac{cpm - BK}{cpm 100\%} $$

$x=\%$ uptake relative to control (0µM pCA)

$BK= cpm$ blank (10µM paroxetine)

$cpm 100\% = cpm$ values at 100% inhibition (at 10µM paroxetine).

Data evaluation was done using GraphPadPrism® Software. Thereby “% uptake relative to control” values were plotted against concentration of logarithmic concentrations of PCA and analyzed using “One site competition” to calculate EC50 and $K_i$ values.
4.3. Biotinylation Assay

HEK cells stably transfected with hSERT and hSERT (K352A/K460A) respectively were used. Cells were grown overnight on 6-well plate (PDL-coated) at ~80% confluency. Serum starvation was performed by changing DMEM (+FCS) medium against serum-free DMEM medium followed incubation of cells for 2.5h. After starvation, cells were immediately put on ice and washed three times with 2ml ice-cold PBS²⁺ buffer. Cells were incubated with 750µl sulfo-NHS-SS-biotin (Thermo Scientific) (1mg/1ml PBS²⁺) for 15 min at 4°C shaking vigorously. After a washing step with ice-cold PBS²⁺, incubation with biotin was repeated. Free biotin was removed by incubating cells twice with quench solution for 15min at 4°C on a shaker. After removing quench solution by washing cells three times with 2ml ice-cold PBS²⁺ buffer, cells were lysed by adding 250µl RIPA buffer and scraping. Lysates were cleared by centrifugation (13,000 rpm x 10', 4°C) and protein concentration was determined by BCA protein assay BSA standards in RIPA using TECAN plate reader (562nm absorbance; 24°C). To separate biotinylated and non-biotinylated proteins a pull down of 100µg cell lysate was performed o/n at 4°C using Streptavidin-agarose suspension (Thermo Scientific). On the next day lysate-beads suspension was separated via centrifugation and remaining beads have been treated with strong reducing 2X SDS-PAGE sample buffer/100mM DTT to release bead-bound proteins.

Purified lysate was separated on a 10% SDS-PAGE gel and plotted on nitrocellulose membrane. ELISA was performed by using rabbit Anti-GFP (1:10.000) for SERT-GFP and mouse anti-Tubulin antibodies (1:20.000). Immunoreactive bands were detected using the enhanced chemoluminiscence (Biorad) method and band densities were quantified by ImageJ.
Figure 19 BSA standard curve. BSA standards at concentration 0.01-1 mg/ml were measured at absorbance wave length of 562nm at 24°C.

Lysates were diluted in 96-well plate with three BSA standards at each measurement as internal control. Absorbance of 562nm was measured in triplicates by using TECAN infinite 200Pro plate reader. Temperature of measurement was kept at 24°C each time. Protein concentrations were calculated using the linear equation of BSA-Standard (see figure 16).

To calculate percentage of SERT surface expression, band density was measured and following formula was used:

\[
\% (\text{total on surface}) = \frac{\text{density of biotinylated band}}{\text{density of total lysate band}} \times 100
\]

Total lysate: The sample of 25µg lysate before loading on beads occurred (before pull-down)
Biotinylated: Sample of << 100µg purified lysate (after pull-down).

Data evaluation was done using GraphPadPrism® Software.
Confocal laser scanning microscopy

HEK293 cells stably expressing either YFP-SERT or mutant versions were grown on 15 mm coverslips in DMEM plus 10% FCS. Live cell imaging was performed using a Zeiss LSM 510 confocal microscope, equipped with an Argon laser (30 mW) and a Helium-Neon laser (1 mW), and a 63X oil immersion Zeiss Plan-Neofluar 1.4 objective. JHC1-64 was visualised using a 543nm HeNe laser line and a 585nm long pass filter. YFP was visualized using a 514nm argon lazer and a 505-530nm bandpass filter at 30 to 45% input power. The thickness of the optical sections was between 0.8 µm and 1.5 µm (frame-scan) at 30 to 45% input power.
4.4. Substances and Solutions

Compounds:

**GFX**: GF109203X is a potent and selective competitive inhibitor of protein kinase C (PKC) and of glycogen synthase kinase-3 (GSK-3) (Lee and Stern, 2000). This substance was supported from Sigma-Aldrich (Milwaukee, WI, USA).

**MPP⁺**: N-methyl-4-phenylpyridinium is a positively charged active neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-terahydropyridine was found to be specifically transported by SERT (Scholze et al., 2000; Sitte et al., 2001; Torres-Altordo et al., 2008). This substance was supported from Sigma-Aldrich (Milwaukee, WI, USA).

**m-3M3FBS**: 2,4,6-Trimethyl-N-[3-(trifluoromethyl)phenyl]benzenesulfonamide directly activates phospholipase C (PLC) (Bae et al., 2003). This substance was supported from Sigma-Aldrich (Milwaukee, WI, USA). Its inactive analogue (used as negative control) **o-3M3FBS** (2,4,6-Trimethyl-N-[2-(trifluoromethyl)phenyl] benzenesulfonamide) was supported from Tocris Bioscience (Bristol, UK).

**PAO**: phenylarsine oxide (arsorosobenzene) specifically inhibits phosphatidylinositol 4-kinase (Wiedemann et al., 1998). This substance was supported from Sigma-Aldrich (Milwaukee, WI, USA).

**pCA**: para-Chloroamphetamine [1-(4-chlorophenyl)propan-2-amine] is a chlorinated analog of amphetamine. It is a substrate for the serotonin transporter which also induce an outward movement or exchange of 5-HT from the cytoplasm through SERT (Rudnick and Wall, 1992). This substance was supported from Sigma-Aldrich (Milwaukee, WI, USA).
Buffers

PBS$^{2+}$
PBS 1,0 mM MgCl$_2$; 0,1 mM CaCl

Quench Solution
PBS$^{2+}$ 100mM Glycine

RIPA Buffer
10mM Tris (pH 7.4); 150mM NaCl; 1 mM EDTA; 1% Triton-X-100; 0.1% SDS; 1% Na deoxycholate

Sulfo-NHS-SS-Biotin
Stock solutions: 200 mg/ml in DMSO.

Uptake Buffer
25mM HEPES; 120mM NaCl; 5mM KCl; 1,2mM CaCl$_2$; 1,2mM MgSO$_4$; 5mM D-glucose (pH 7,4).

Cell Culture Medium
Dulbecco's Modified Eagle's Medium (Sigma Aldrich); 10% FCS; 1%Pen Strep (Penicillin Streptomycin).
Maintenance of stable cell lines: 50 μg/ml G418 was added to DMEM.
5. References


monoamine transporters is a lever required for the action of amphetamines. The Journal of biological chemistry 285, 10924-10938.


6. Appendix

ABSTRACT GERMAN

ABSTRACT ENGLISH
The serotonin transporter (SERT) as a member of the sodium-coupled neurotransmitter transporters (NSS) plays a crucial role in the termination of serotonin signalling. Alterations of intrinsic transporter function and regulation are associated with psychiatric diseases and physiological disorders. A great deal of effort has been taken to unravel SERT regulation and transport mechanism. Phosphatidylinositol-4,5-bisphosphate (PIP$_2$) is involved in several major cellular regulatory processes and was also shown to play a role in the regulation of integral membrane proteins, like ion channels (Gamper and Shapiro, 2007a). PIP$_2$ as well as cholesterol have been found to be enriched in membrane microdomains where also SERT was found to be located and regulated (Chang et al., 2012). Nevertheless, a regulatory effect of PIP$_2$ on SERT fluxes has never been investigated before. This work examined the role of PIP$_2$ on SERT transport flux by using a heterologous expression system. It could be shown that the PLC activator m-3M3FBS was able to deplete PIP$_2$ in HEK-hSERT cells resulting in reduced amphetamine-induced efflux rates but unchanged ligand influx. To preclude the possibility that observed reduction of efflux rates is caused by activation of second messengers due to cleavage of PIP$_2$ via PLC, inhibition of PIP$_2$ synthesis via the substance PAO was investigated. Indeed, the same reduction in efflux but without affecting influx rates could be demonstrated which verified that this effect was caused by PIP$_2$ depletion rather than via activation of second messengers. These results indicate that PIP$_2$ directly effects amphetamine induced transporter efflux indicating that this could be mediated via direct binding of PIP$_2$ to SERT. Computational analysis revealed two positively charged amino acids K352 (helix 6) and K460 (helix 9), located in the intracellular loops of SERT, as potential binding sites for PIP$_2$. Mutagenesis of both amino acids did not alter intrinsic transporter function but displayed the exact same effect as was observed for PIP$_2$ depletion. K352A/K460A double mutation in hSERT did affect neither substrate influx nor trafficking to the surface, but was shown to markedly reduce amphetamine induced efflux rates. Therefore it could be demonstrated that PIP$_2$ modulates SERT flux selectively, by only affecting efflux and that this modulation occurs most likely via direct binding of PIP$_2$ to the amino acids K352 K460.
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01.08 - 01.09.08
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01.05 - 01.06.07
„C.elegans- mapping, complementation analysis, epistasis, RNAi silencing and cytogenesis“. (Dr. Jantsch-Plunger Verena; IMBA)

01.06 - 01.07.06
„Examining genes relevant for pigment patterning in Danio rerio“. (Dr. Hofinger Bernhard; MFPL)

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