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Phosphorylation–mediated regulation of the Serotonin Transporter

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Contents

Abbreviations

5-HT	5-hydroxytryptamine, serotonin
[^{3}H]5-HT	tritiated 5-hydroxytryptamine
5-HTTLPR	Serotonin-transporter-linked polymorphic region
A_3AR	A3 adenosine receptor
ACN	Acetonnitrile
ADP	Adenosine -– diphosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAD	Cath.-a-differentiated
cDNA	complementary DNA
CNS	Central nervous system
COP	Coat protein complex
DAT	Dopamine transporter
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphate
DPBS	Dulbecco's phosphate-buffered saline
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmatic reticulum
ERGIC	ER-Golgi intermediate compartment
ETDA	Ethylenediaminetetraacetic acid
GABA	γ-Aminobutyric acid
GAT	GABA transporter
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GlcNAc	N-acetylglucosamine
GTP	Guanosine triphosphate
H_20_2	Hydrogen peroxide
HCL	Hydrochloric acid
HEK	Human embryonic kidney
hSERT	Human serotonin transporter

kDa	Kilo-Dalton
KHP	Potassium hydrogen phthalate
LC-MS	Liquid chromatography-mass spectrometry
LeuT	Leucine transporter
L-TRP	L- Tryptophan
MAPK	Mitogen-activated protein kinase
NET	Norepinephrine transporter
OCD	Obsessive-compulsive disorder
p38 MAPK	P38 mitogen-activated protein kinase
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDL	Poly-D-lysine
PIC	Protease inhibitor cocktail
PKC	Protein kinase C
PKG	Protein kinase G
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
RSB	Reduced sample buffer
RT	Room temperature
S.E.M.	Standard error of mean
SDS	Sodium dodecylsulfate
SSRI	Serotonin selective reuptake inhibitor
SERT	Serotonin transporter
SLC	Solute carrier
SNARE	Soluble N-ethylmaleimide-sensitive-factor attachment receptor
β-PMA	ß-Phorbol 12 – myristate 13-acetate
Syn1A	Syntaxin1A
TEMED	Tetramethylethylenediamine
TGN	Trans-*Golgi* network
TM	Trans membrane
tsa	Transformed *HEK-293* cell
UV	Ultraviolet radiation
YFP	Yellow fluorescent protein

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Abstract

Posttranslational modifications of proteins play an important role in several molecular processes. Especially, phosphorylation seems to play an important role in the coordination of the cellular network due to the presence of many different kinases and phosphatases in eukaryotes. Among other proteins, this rule also applies to the serotonin transporter (SERT). In particular, the regulation of SERT trafficking, activity and interaction with other proteins is regulated by phosphorylation, although it still contains many unexplored molecular processes. SERT contains consensus sites for kinases, such as protein kinase C (PKC) and p38 MAPK, which are already known to influence SERT activity and surface expression. However, specific phosphorylation sites involved in the regulation of transporter activity and trafficking have not been well identified.

Therefore, it was aimed to determine the cellular compartment distribution of SERT by kinase activation and identify specific phosphorylation sites implicated in the SERT function and surface expression/ER retention.

Immunoblot analysis showed that the ER retained form of SERT was decreased in HEK293 cells stably expressing YFP-SERT due to treatment of a p38 MAPK activator anisomcyin. By contrast, treatment with β-PMA, a PKC activator, caused an increase of the ER-resident SERT expression. Anisomycin-mediated effect on ER retention form was detected in SERT but not in DAT expressing cells, leading to the assumption of specificity of anisomycin-mediated redistribution for SERT.

In addition, in vitro phosphorylation assay followed by mass spectrometry only identified only phosphorylation sites in the C-terminal of SERT specific for p38 MAPK and PKC. Even though further in vivo phosphorylation experiments determined S52 and S62 as phosphorylation sites induced by anisomycin or okadaic acid, a phosphatase 1/2 A inhibitor, generated phosphomimetic and dephosphomimetic mutants of both sites still showed the same redistribution of SERT by anisomycin treatment as observed in the wild type SERT. However, dephosphomimetic mutant at identified p38 MAPK-specific Thr616 phosphorylation site showed a significant reduction of the anisomycin effect on ER-resident SERT. Taken together, phosphorylation at the C-terminus of SERT but not at the N-terminus regulates the ER retention of SERT.

1 Introduction

1.1 Phosphorylation of proteins

Posttranslational modification (PTM) of proteins such as glycosylation or phosphorylation plays an important role in the function and regulation of cellular processes (Hunter et al., 2000). Especially, phosphorylation has a great significance in the coordination of cellular network due to the presence of many different phosphatases and kinases in eukaryotes (Rubin et al., 2000). During the process of phosphorylation, a phosphate residue is transferred to the protein with the help of partly specific kinases, which use ATP as co substrate. Hydrolysis of phosphoric acid ester is catalyzed by phosphatases. These modifications mainly take place at side chains of serin, threonine or tyrosine residues within the protein (Cozzone et al., 1993).

The reversible modification represents a mechanism allowing the cell to react directly to signals and act as a molecular switch (Hubbard and Cohen, 1993). Phosphorylation or dephosphorylation of specific amino acids induces protein conformational change and further affects protein activity without a change in gene expression or protein synthesis.

Besides the activation or deactivation of proteins, the PTM also affects protein stability, interaction with DNA or proteins and trafficking between cell compartments. The kinases not only lead to phosphorylation of specific proteins but also act as substrates for other kinases, resulting in phosphorylation cascades that can influence many different cellular processes (Nestler and Hyman, 2002).

Due to its important role in different cell processes (cell cycle) and cellular signaling cascades, incorrect phosphorylation has been connected with many diseases such as cancer, diabetes and Alzheimer´s disease (Cohen, 2001).

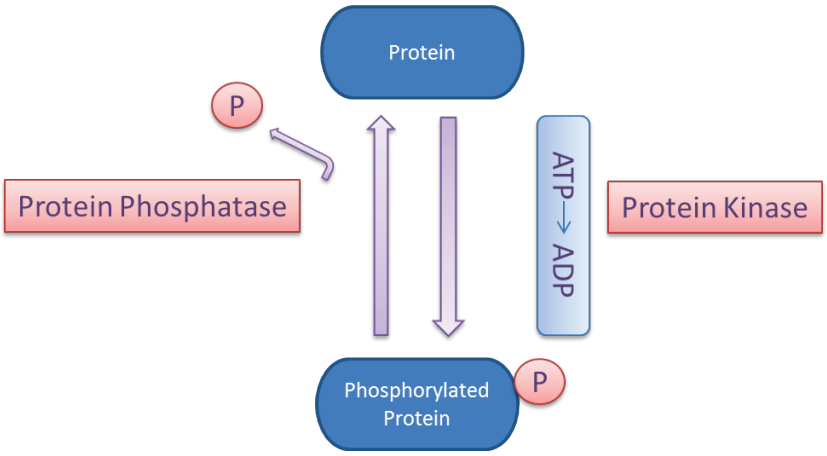

Fig. 1 Reversible protein phosphorylation

Protein kinase catalyzes the attachment of a phosphate to a cellular protein by means of ATP. Here, serine, threonine or tyrosine side chains of the protein are modified. The protein is deactivated by phosphatases due to hydrolysis of the phosphate group.

(modified from http://www.scq.ubc.ca/protein-phosphorylation-a-global-regulator-of-cellular-activity, Illustrator: Jane Wang)

1.2 Monamine transporters

Two different classes of neurotransmitter transporter exist: intracellular vesicular transporters and plasma membrane transporters (Hahn and Blakely, 2002). The former are responsible for transporting neurotransmitters from cytoplasma into synaptic vesicles and can be divided into three subclasses: SLC (Solute Carrier) 18, SLC32 and SLC17 family (Gether et al., 2006). Plasma membrane transporters that sequester released neurotransmitters from the synaptic space can be divided into two major subclasses: SLC1, the high-affinity glutamate transporter family; and SLC6, the high-affinity sodium (Na^+) and chloride (Cl^-) dependent transporter family (Kristensen et al., 2011).

The SL6 family is the largest class with 20 genes encoding for different yet more-or-less similar transporters. This family comprises four subfamilies, including neurotransmitter transporters for monoamines, GABA, as well as the amino acid glycine (Gether et al., 2011). Monoamine transporters are important for cellular signaling by regulating the concentration of extracellular monoamine neurotransmitter. The family includes the serotonin (SERT), dopamine (DAT) and norepinephrine (NET) transporter, which are expressed in the central nervous system (Pramod et al., 2013; Kristensen et al., 2011). They mediate recycling, release and reuptake of the specific neurotransmitter across a synapse (Hahn and Blakely, 2002; Holton et al., 2005).

To retrieve further information about the transporter function and structure, a bacterial homologue $LeuT_{Aa}$ was crystalized and resolved at high resolution (Yamashita et al., 2005). LeuT is isolated from the thermophilic bacterium *Aquifex aeolicus* and its crystal structure is highly homologous with that of monoamine transporters (Steiner et al., 2008): the similarity is about 40-45 %, whereby the sequence identity of 20-25 % is highly conserved in the active binding site of the transporters (Yamashita et al., 2005). The structure is considered a suitable template for homology models of these transporters (Loland, 2014). This enables the use of homology-based modeling approaches to provide a better investigation of domains that are involved in substrate recognition and the substrate transport (Steiner et al., 2008; Gether et al., 2006).

The membrane transporters function as Na^+-dependent co-transporters using the Na^+ concentration gradient (provided by the Na^+/K^+ pump) to transfer neurotransmitter against their concentration gradient from the extracellular space into the cell. By contrast to SLC1 transporter, SLC6 members additionally transport Cl^- (Masson et al., 1999).

The proteins comprise approximately 500-600 amino acids, have 12 transmembrane alpha –helices and intracellular amino and carboxyl termini (Gether et al., 2011; Ramamoorthy et al., 1993). In addition, a large N-glycosylated loop is located between transmembrane domains 3 and 4. This extracellular loop 2, resulting from disulfide bridge building by cysteine residues, plays an important role in trafficking and stability with its consensus sites for posttranslational modification like glycosylation (Melikian et al., 1996).

Due to their functional importance at the synapse, monoamine transporters are related to neurological disorders such as epilepsy and mood abnormalities like depression and schizophrenia (Pramod et al., 2013, Kristensen et al., 2011). Mutations in transporter genes are known to result in altered function and expression of the affected protein, whereby disruptions of transmitter signaling occurs (Kilic et al., 2003; Hahn et al., 2003).

A few missense mutations in transporters like DAT are already known to be associated with neuropsychatric disorders. Coding variants of the DAT gene SLC6A3 caused the ER retention of DAT and greatly reduced its surface expression (Kurian et al., 2009) These variants cause ADHD and dopamine transporter deficiency syndrome (Kurian et al., 2009). Other missense mutations in SLC6A3 induce structural faults, which reduce dopamine uptake activity without a change in surface expression or protein folding. These mutations are connected to adult parkinsonism and ADHD (Hansen et al. 2014).

In terms of their role in neuropsychiatric disorders, monoamine transporters serve as a target for drugs that allow treating these diseases that display phenotypically abnormalities in neurotransmitter homeostasis. The substances bind specifically to the transporters and block the reuptake of the neurotransmitter into the presynaptic cells. For this reason,

the signaling between neurons is enhanced due to a higher neurotransmitter concentration in the synaptic cleft (Jayanthi and Ramamoorthy, 2005). If sustained over a period of days, the increased extracellular neurotransmitter concentration leads to symptom relief (Brown et al., 1993).

Changes in genes often lead to misfolded or unfolded proteins, which are incapable of exiting the ER to their final destination and thus alter synaptic transmission (Torres et al., 2003, Hahn and Blakely, 2002). In addition, variations of the protein structure could lead to alteration in their affinity for binding partners responsible for trafficking or function (Farhan et al., 2010).

In addition to being target for therapeutic drugs, they act as a target for drugs of abuse, which inhibit neurotransmitter transport in the same way as antidepressants (Rothman and Baumann, 2003). Cocaine and amphetamine inhibit DAT, SERT and NET, and antidepressants such as SSRI (selective serotonin reuptake inhibitor) and tricyclic agents affect SERT (Jayanthi and Ramamoorthy, 2005).

The trafficking of transporters from the ER to their final destination includes different possibilities for regulation by PTMs (Ramamoorthy et al., 2011). During the transport to their destination, transporters are modified by glycosylation and reach their destination in their mature form.

Furthermore, signal transduction pathways modulate the neurotransmitter transporters in their function and distribution via the presence of several consensus sites for PTMs and protein-protein interaction motifs (Ramamoorthy et al., 2011; Vaughan et al., 2004). Despite the high identity of the amino sequences among the different transporters, the function of transporters may be specified by variation of motifs for PTM and interacting proteins in amino- and carboxyl termini (Kristensen et al., 2011).

1.2.1 Trafficking of monoamine transporters

Monoamine transporters are synthesized like other proteins on ribosomes associated with the ER membrane, followed by trafficking to different cell compartments such as Golgi and plasma membrane. Plasma membrane proteins follow the secretory pathway during their transport from the ER to the cell surface (Reiterer et al., 2008). Protein localization is controlled and achieved by several pathways. Different kinases like MAPKs seem to play a particularly important role in the ER-export or the internalization of the transporter (Wang and Lucocq, 2007, Farhan et al., 2010). Besides direct phosphorylation of the transporter, vesicle formation, which is essential for the export of the proteins, involves several phos-

phorylation-dependent components regulated by theses kinases (Palmer et al., 2005, Ramamoorthy et al., 2011).

Different types of coated vesicular carriers play a role in the trafficking between the compartments: clathrin-coated, COPI-coated and COPII-coated vesicles (Rothman and Wieland, 1996; Kirchhausen, 2000).

COPI and COPII represent coat proteins that support the trafficking between ER and Golgi (Cosson and Letourneur, 1997); however, COPII transfers proteins from the ER to the Golgi and COPI is responsible for the retrograde transport.

Along the trafficking pathway, each protein is processed to its mature form by PTMs such as glycosylation. The process of carbohydrate chain modifications takes place stepwise and is connected to specific compartments, whereby the compartment localization of proteins can be observed by analyzing the presence of sugar chains (Lodish et al., 2000).

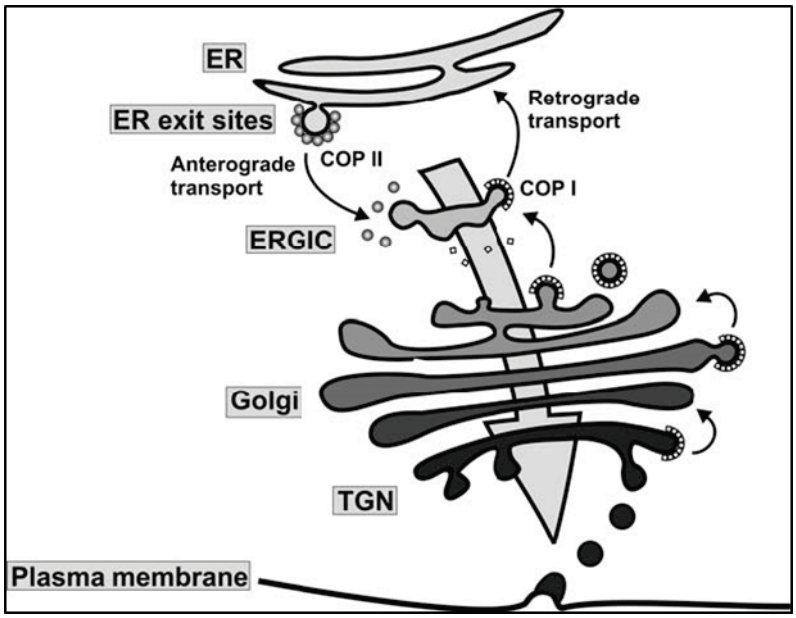

Fig. 2 Secretory pathway

Synthesized secretory proteins are transported in COPII vesicles from the ER to ERGIC. After migration and modification through golgi cisterns the proteins are packed in vesicles and fuse with the membrane for releasing.

(from http://www.gerritbouw.com/thesis)

Newly synthesized membrane and secretory proteins reach, via their N-terminal signal peptide, the endoplasmic reticulum, where folding of the native form takes place. Specific covalent bindings of carbohydrates occur during migration from the ER membranes to the trans-site.

At the ER exit site, the export of the proteins is mediated by COPII-coated vesicles. COPII is a multiprotein coat, which components are essential for cell viability (Klumperman, 2000). It comprises SEC23 and SEC24, which form the inner coat, as well as SEC13 and SEC31 forming the outer coat. In addition, COPII includes the small GTPase SAR1, which controls the conformation of COPII (Delisle et al., 2009). Its activation by GTP for GDP exchange by guanine nucleotide exchange factor (SEC12) enables binding to the ER membrane and recruitment of the SEC23/SEC24 compound, followed by SEC13/31 complex, leading to vesicle forming (Budnik and Stephens, 2009). Recruitment of SEC13/31 enhances SAR1-GTP activity and its GTP hydrolysis, which initiates uncoating of the vesicle (Bi et al., 2007).
A few proteins planned for ER-export contain a specific signal, whereby they can be recognized as cargo proteins and connected to SEC24 for COPII transport. Due to the different isoforms of SEC24, many different export signals can be recognized and specific cargo selection occurs (Wendeler et al., 2007). The C-terminus of SLC6 family transporter appears responsible for the ER-export.
It has been shown that GAT1 contains an export motif (566RL567), which is necessary for SEC24D binding and oligomerization (Farhan et al., 2006, 2007). Defects in this motif abolished correct binding to SEC24 and resulted in a failure of ER-export of GAT1. The motif is conserved among the other transporters supporting the presumption of its relevance in COPII formation and the ensuing trafficking. In SERT and GAT4, this conserved motif requires SEC24C for transporter export in contrast to SEC24D-depedent GAT1 and DAT export (Sucic et al, 2011).
It has been also shown that the oligomerization of transporters is a prerequisite for trafficking (Schmid et al., 2001; Scholze et al., 2002).
Mutation at dimerization motifs in specific transmembrane (TM) domains of DAT (TM6) and GAT1 (TM2) resulted in intracellular retention of transporters, supporting the role of transporter oligomerization in the ER-export and subsequent trafficking of transporters to the plasma membrane (Sitte and Freissmuth, 2004).

COPII formation is regulated by different factors affecting the aforementioned COPII components, including the p38 MAPK kinase (Wang and Lucocq, 2007). Several kinases and phosphatases regulate the organization or trafficking of the ER-Golgi system. The MAPK pathway is involved in trafficking by activating the Raf–MEK–ERK cascade, which influences COPII budding and the transport to the Golgi (Farhan et al., 2010).

In respect to these findings and studies demonstrating the phosphorylation of SEC31 (Salama et al., 1997) as well as SEC16, which is a target of ERK2 (Farhan et al., 2010), phosphorylation of COPII components seems to play a basic role in trafficking.

After COPII assembling, the budded vesicles use the dynein motor and migrate along the microtubules to the ERGIC. They are directed to their specific target and mediated fusion by SNARE interaction. SNAREs are ubiquitous proteins located on vesicles (v-SNAREs) and target membranes (t-SNAREs) and mediate vesicular trafficking and exocytosis (Chen et al., 2001). The interaction between specific pairs of v- and t-SNARES, a process that needs Rab GTP-binding proteins, enables a direct transport of protein vesicles to their point of destination and the fusion of both membranes (Miller et al., 2002).

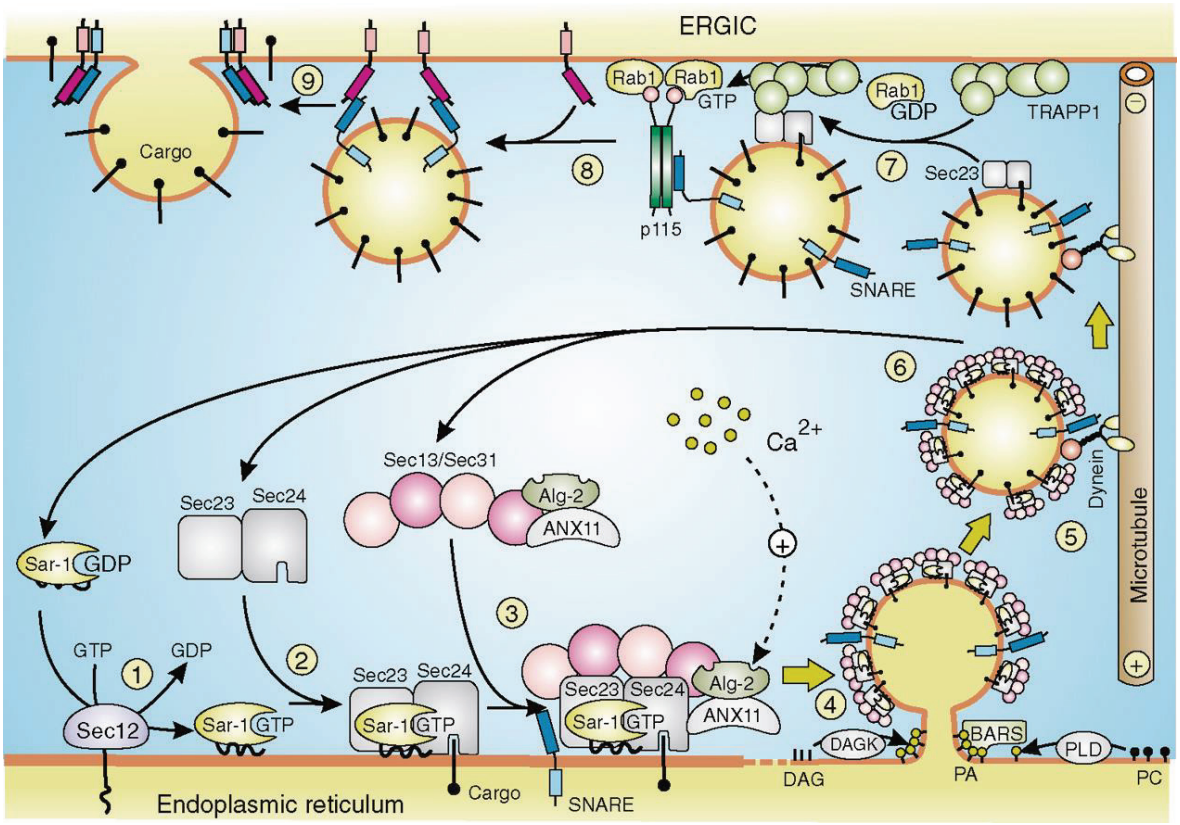

Fig. 3 COPII assembling and transport to the Golgi

The assembling of transport vesicle COPII components is mediated by activation of SAR1. First, SEC23/SEC24 complex binds to SAR1 followed by cargo binding of the Sec24 subunit. Subsequently, recruitment of SEC13/Sec31 terminates the vesicle forming and the coats are transported along microtubules by means of the dynein motor to ERGIC. There specific SNARE pairs ensure fusion of the vesicle with the early Golgi membrane.

(from Berridge, M.J., 2012)

Within the Golgi complex, the transported proteins are modified and sorted to be retained in the ER/Golgi complex or transported to the surface membrane.

During the migration from the cis-site to the trans-site cisternae, the proteins are modified by addition or elimination of oligosaccharides, before subsequently being released again (Lodish et al., 2000). Once the final location of action is reached, the membrane proteins are not constrained in a static way; rather, they are internalized, degraded or recycled and return to the cell surface (Melikian, 2004), in processes mediated by de-/phosphorylation of the proteins, among other things (Ramamoorthy et al., 2011).

Many molecular processes, and especially MAPK signaling and interactions, are part of trafficking as a process in itself. Therefore, trafficking represents a crucial part in monoamine transporter regulation and activity: specifically, this can be achieved in terms of their surface expression or endocytosis rate.

1.3 Serotonergic system

1.3.1 Serotonin

Serotonin is a neurotransmitter that is synthesized in two steps from the amino acid L-Tryptophan (L-Trp). The cytosolic tryptophanhydroxylase transforms L-TRP into 5-hydroxytryptophan, which is converted to serotonin (5-HT) by means of 5-hydroxytryptamindecarboxylase (Maximino, 2012).

Synthesis of 5-HT mainly takes place in the CNS and enterochromaffin cells of the gastrointestinal tract. The largest components of serotonergic neurons are located in the midbrain area of raphe nucleus, from where they branch out into several brain areas (Lesch, 2001).

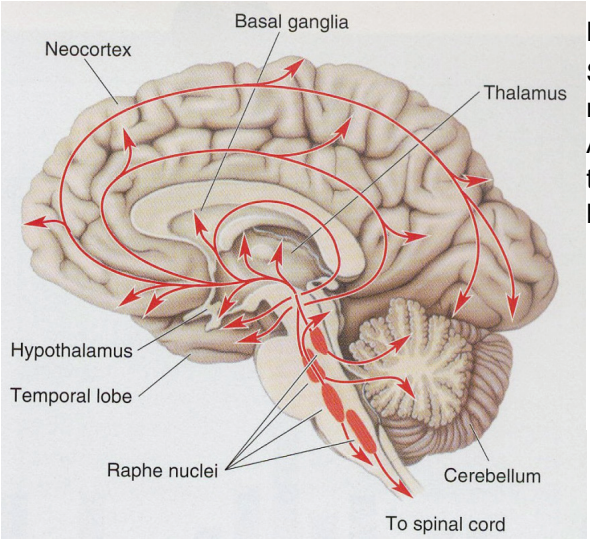

Fig. 4 Serotonin system
Serotonergic neurons are mainly situated in the raphe nuclei along the midline of the brainstem. Axons of the neurons form a good branching transmitter system so that nearly every part of the brain is included.

(from www.nxdomain.nl/~anja/brains/hersenen.html)

Distribution of the serotonergic neurons in different brain areas suggests that the serotonergic system is an essential part of neuronal processes (Diksic and Young, 2001).The neurotransmitter is associated inter alia with cell proliferation during prenatal development of the brain, as well as pain perception, sleep disorder, aggression and anxiety (Oberlander, 2010; Seo et al., 2008). In neurons, the synthesized transmitters are transported to the end of axons of the presynapse and saved in vesicles until they are released into the synaptic cleft after a depolarizing stimulus. Subsequently, the binding to specific postsynaptic serotonin receptors promotes the signal transmisson between the two cells. In addition, serotonin also binds to a plasma membrane transporter located at the presynaptic cell and is subsequently taken up to end neurotransmission (Lovinger et al., 1997; aan het Rot et al., 2009).

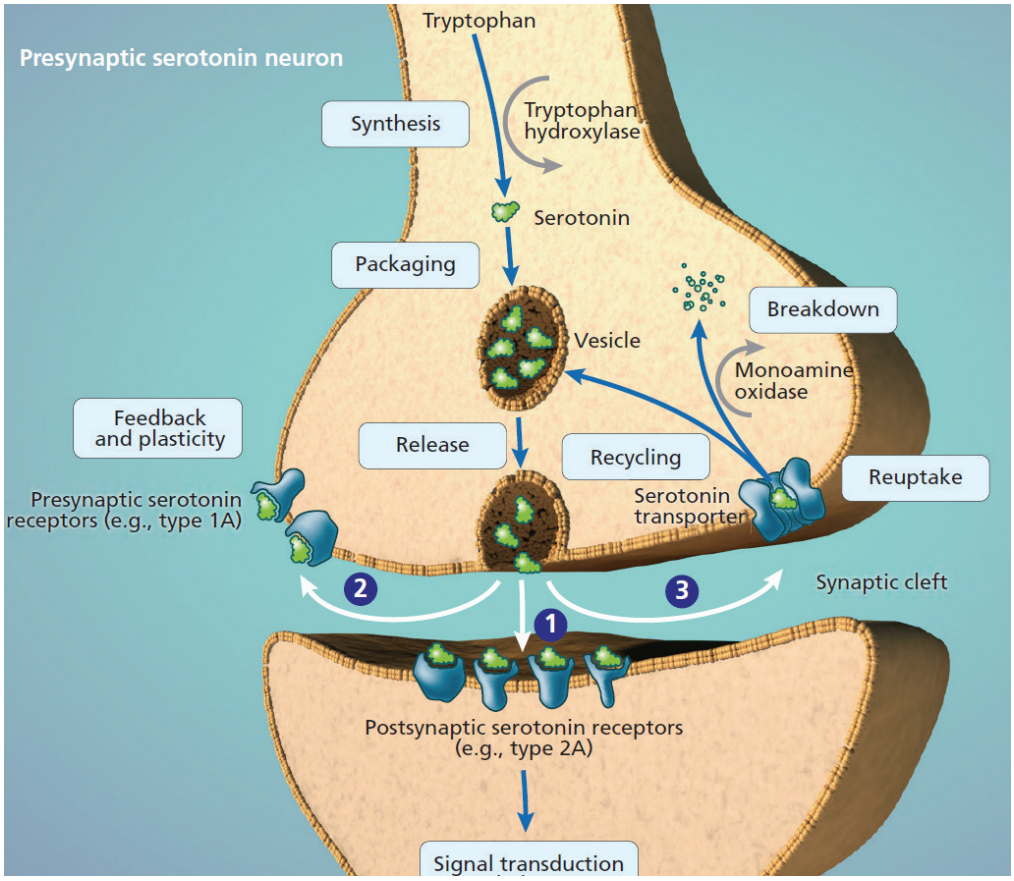

Fig. 5 Serotonin synthesis and signaling

After synthesis of serotonin from tryptophan by means of tryptophan hydroxlase the transmitter is packed into vesicles. Stimulation of the cell induces the release of serotonin into the synaptic cleft. Extracellular 5-HT is able to bind to postsynaptic resceptors of other neurons following by signal transmission or to presynaptic receptors of its origin neuron, whereby the neuron receives feedback and can regulate plasticity.Reuptake of 5-HT by the serotonin transporter interrupts signal transmission and reduces the extracellular transmitter concentration. Afterwards 5-HT is recycled and stored in vesicle for another release or degraded by monoamine oxidase.

(from aan het Rot et al., 2009)

1.3.2 Serotonin Transporter (SERT)

The serotonin transporter (SERT) belongs to the SLC6 transporter family and is thus related to dopamine and norepinephrine transporter (Blakely et al., 1991). It terminates the serotonergic neurotransmission by the process of reuptake of its specific neurotransmitter 5-HT (Ramamoorthy et al., 1998).

The polytopic, integral membrane SERT protein is encoded by the SLC6A4 gene. The gene is located on chromosome 17 and organized into 14 exons. It compromises 630 amino acids with 12 transmembrane spanning domains and cytoplasmic N- and C-termini (Lau and Schloss, 2012).

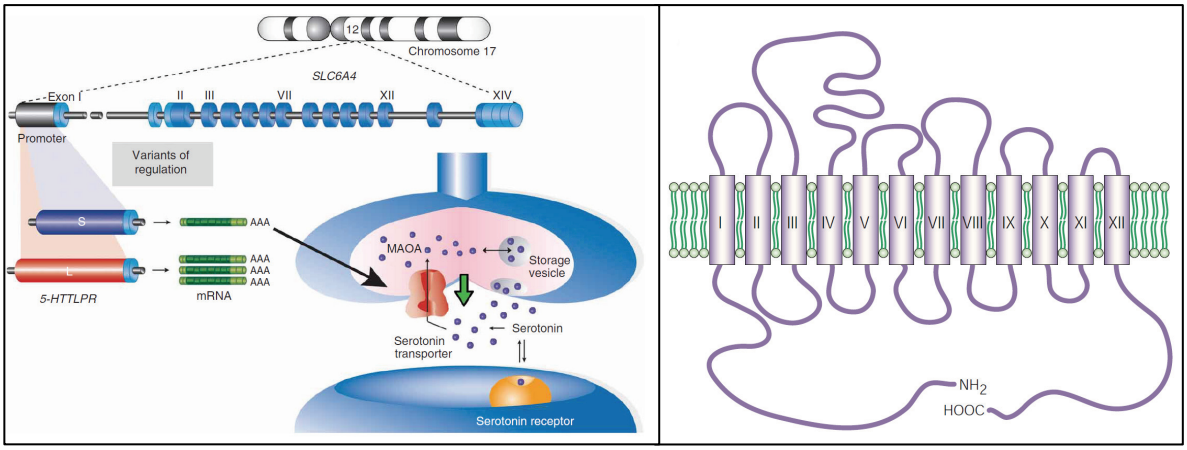

Fig. 6 Serotonin transporter

SERT is member of the SLC6 transporter family. It is located at chromosome 17 and consists of 630 amino acids and 12 trans-membrane-spanning domains (right). Due to variants within the 5-HTTLPR region of the transporter, two different lengths result, which produce different amounts of transporter mRNA. The short variant is associated with different diseases.

(from Canli and Lesch, 2007 (left);Torres et al., 2003 (right))

It is concentrated in the raphe nuclei, the prefrontal cortex and in thalamocortial afferents. (Maximino, 2010). It is not only expressed in neurons but also in specialized non-neuronal cells such as intestinal crypt epithelial cells (Wade et al., 1996), platelets (Carneiro et al., 2008), blood lymphocytes (Gordon et al., 2003) or adrenal chromaffin cells (Schroeter et al., 1997).

SERT activity is dependent on extracellular Na^+ and extracellular Cl^-. SERT exists as a homooligomer (Sitte et al., 2003, 2004) and uses the gradients of Na and Cl for the reuptake of 5-HT. The maintenance of the Na^+ concentration is regulated by Na^+/K^+ pumps, which transport three Na^+ ions out of the cell and two K^+ ions into the cell. For reuptake into the presynaptic cell, SERT binds Na^+ first, followed by 5-HT (Chen et al.,

2004). Binding of Cl^- to the transporter terminates formation of the complex and leads to a conformational change of the transporter, whereby the neurotransmitter and ions can be released into the neuron. By means of K^+ binding, the transporter is able to reform to its starting point for renewed reuptake (Hahn and Blakely, 2002; Kristensen et al., 2011).

The serotonin transporter has been studied to gain a better knowledge of its connection to diseases, including the extent to which regulation of the transporter might play a role. Due to inter alia a common polymorphism in the promoter of SERT (Homberg and Lesch, 2011), a disruption of serotonergic neurotransmission results in brain disorders and related diseases. Different promoter constructs arise from the polymorphism in the promoter region 5-HTTLPR. It is related to a short allele containing 14 repeats and a 44bp longer allele with 16 repeats (Heils et al., 1996). The promoter variants lead to different SERT expression levels. The 'l' variant of SERT is expressed significantly higher than 's' variants in different cell lines (Heils et al., 1996, 1997). The 's' variant is known to be associated with anxiety-related traits and depression (Lesch et al., 1996; Nardi et al., 2013).
Altered SERT expression in different types of psychopathology may emphasize the important role of functional SERT in brain function. Consequently, several studies have shown that SERT is connected to autism (Huang and Santangelo, 2008), depression (Clarke et al., 2010; Caspi et al., 2003), anxiety (Mazzanti et al., 1998) and obsessive–compulsive disorders (OCD) (Lin, 2007), diseases caused by dysfunction of signal transmission due to a lack of neurotransmitter in the synaptic cleft and thus reduced activity of signaling.
Six variants of SERT were identified in connection to OCD and autism, leading to an increase activity of SERT (Prasad et al., 2005, 2009). Other studies displayed a decreased SERT density in the brain of patients troubled with depression compared to non-affected people (Leake et al., 1991; Arango et al., 1995).

Consequently, SERT represents a target for clinically relevant drugs like antidepressants (Carrasco and Sandner, 2005). The drug treatment results in higher extracellular 5-HT concentrations and the associated stronger postsynaptic signaling of serotonin due to the inhibition of 5-HT reuptake. A subtype of these drugs are the SSRI with prototypical members such as paroxetine, fluoxetine and citalopram (Hiemke and Härtter, 2010), which bind specific to SERT, in contrast to other antidepressants binding several transporters and/or receptors (Sarker et al., 2010).

Besides its role in brain disorders and the therapeutic site of action, SERT is also connected to drugs of abuse (Jayanthi and Ramamoorthy, 2005; Chen et al., 2004; Schloss et al., 1998, Sitte and Freissmuth, 2010). SERT serves as a target for drugs such as am-

phetamine and cocaine, leading to increased extraneuronal 5-HT levels and enhanced serotonergic signaling.

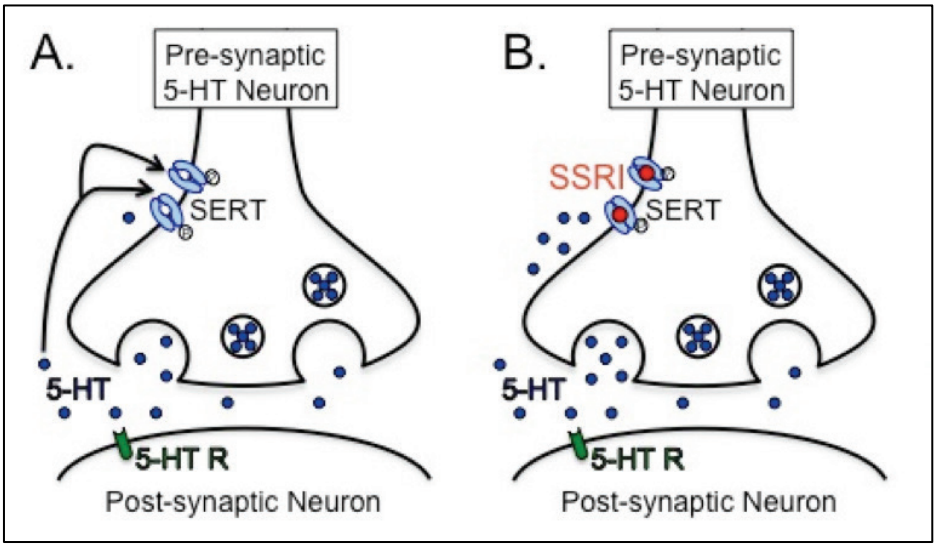

Fig. 7 Effect of SSRI on serotonergic signaling
The reuptake of 5-HT into the neuron by SERT is blocked due to the presence of SSRI which directly binds the monoamine transporter. The concentration of extracelluar 5-HT increases resulting in an enhanced serotonergic signaling.

(modified from http://blogs.scientificamerican.com/scicurious-brain/files/2012/11/ssri-action.png)

1.3.3 Regulation of SERT

Due to the fact that altered SERT expression is associated with brain diseases and the transporter serves as a target for drugs, its regulation by cellular and molecular mechanism seems to be a critical part in the maintenance of 5-HT homoeostasis.

The activity of SERT is dependent upon 5-HT concentration, its interaction with other proteins and its distribution in the cell (Haase et al, 2001). It has been presented by SDS-PAGE separation that two major SERT populations exist in the cells: the post-Golgi (cell surface)- and the ER-form (Steiner et al., 2008; Zhang and Rudnick, 2010).
A range of cellular and molecular mechanisms can regulate the monoamine transporter by affecting the activity, expression or trafficking of SERT. Several studies have reported that SERT belongs to the group of phosphoproteins (Ramamoorthy et al., 1998; Qian et al., 1997; Samuvel et al., 2005). Consequently, changes of the protein phosphorylation state seem to have a crucial function in SERT regulation.
Several proteins have already been identified to bind the N- and C-terminus of SERT, which could be mediators or components of SERT trafficking between ER and plasma membrane and thus also responsible for its regulation. One of these interacting proteins is

protein phosphatase 2 (PP2A), an ubiquitous enzyme that is known to play a role in regulating many cellular processes such as signal transduction or regulation of membrane protein trafficking (Molloy et al., 1998). The subunit PP2Ac was identified to bind to SERT (Bauman et al., 2000) and this interaction may be involved in the regulation of the surface expression of transporters. Because it is recognized that p38 MAPK activates PP2A (Westermarck et al., 2001; Liu et al., 2003) and associates with surface SERT, PP2A inhibitors such as okadaic acid or calyculin A binding to catalytic subunit result in loss of its activity and is connected to increased SERT phosphorylation and PKC-mediated internalization (Bauman et al., 2000).

Additional interacting proteins of SERT are syntaxin 1A (Quick, 2003) SEC24C (see above). Syntaxin 1A is integrated into a neurotransmitter release process by supporting the fusion of synaptic vesicles and presynaptic membrane.

In addition, studies have showed an interaction of syntaxin 1A with plasma membrane transporters including SERT (Ciccone et al., 2008; Haase et al., 2001; Quick, 2003), regulating its transport stoichiometry and affecting surface expression.

Beside the motifs for interaction with other proteins, both termini of SERT contain consensus sites for kinases and serve as a connection between interaction and regulation processes (Ramamoorthy et al., 2002; Vaughan et al., 2004). It has already been displayed that p38 MAPK and PKC are involved in the regulation of SERT and partially other monoamine transporters such as DAT and NET (Ramamoorthy et al., 1998; Qian et al., 1997; Samuvel et al., 2005). Both kinases act by phosphorylation of hydroxyl groups of serine and threonine residues of proteins. Alterations of the phosphorylation state in the transporter and associated conformational change may be responsible for the transporter distribution within the cell (Vaughan, 2004).

p38 MAPK belongs to the MAPK family, which includes several serine-threonine kinases connecting to regulation of protein expression and biological activities (Roux and Blenis, 2004). Stimuli such as cytokines and stress regulate protein kinases followed by their influence on homeostasis and expression of 5-HT.

Investigations of SERT regulation with synaptosomes have displayed a decreased reuptake of extracellular 5-HT followed by p38 MAPK inhibitor (PD169316 and SB203580) treatment in a time- and dose-dependent manner and in correlation with a reduced surface expression (Samuvel et al., 2005). By contrast, the p38 MAPK activator anisomycin induced an elevation of 5-HT uptake associated with no increase of cell surface expression in synaptosomes or heterologous cells (Samuvel et al., 2005; Zhu et al., 2005). Other stimulants of p38 MAPK such as H_2O_2, UV and interleukin-1ß also increased the 5-HT uptake or affinity of SERT for 5-HT, but seem to have no influence on transporter trafficking (Zhu et al., 2005, 2010).

Activation of G-protein coupled A3 adenosine receptor (A_3AR) affects 5-HT signaling via p38 MAPK-dependent or PKG-dependent enhancement of SERT activity (Zhu et al., 2004, 2007). Because stimulation of PKG also leads to activation of p38 MAPK, resulting in an increase of SERT activity, it seems that the phosphorylation cascades with multiple kinases are involved in the regulation of SERT.

In contrast to p38 MAPK, PKC is associated with the internalization of SERT and other monoamine transporters and downregulation of their activity at the surface (Ramamoorthy et al., 1998; Melikian, 2004). The PKC activation resulted in a reduction of 5-HT uptake and substrates of SERT (e.g. 5-HT) attenuated the PKC-mediated SERT downregulation (Ramamoorthy and Blakely, 1999). By using PKC activator ß-phorbol 12–myristate 13-acetate (β-PMA), a loss of surface form of SERT was observed together with reduced 5-HT uptake (Jayanthi et al., 2005).

It seems that the loss of surface transporter molecules takes place due to a rapid internalization and redistribution from the plasma membrane to an intracellular compartment mediated by PKC activation (Samuvel et al., 2005; Ramamoorthy et al., 2011), as already described for DAT. DAT internalization is the result of an increase endocytosis resulting in degradation of the transporter due to lysosomal pathway (Daniels and Amara, 1999).

Furthermore, a biphasic effect of activated PKC on SERT has been reported in platelets (Jayanthi et al., 2005). Kinetic studies have shown a significant decrease of SERT transport activity after 5 minutes β-PMA incubation, while significant reduction of surface SERT expression was observed after 30 minutes incubation of β-PMA. Therefore, it is assumed that β-PMA treatment in early periods affects the transporter activity and in the later periods increases the redistribution of SERT into intracellular space. Both regulatory steps are linked to an increase of phosphorylation of specific amino acids: initially, serine residues become phosphorylated, whereas threonine residues are modified later on (Jayanthi et al., 2005).

PKC activation is also attributed to a decrease in association between SERT and PP2A (Ramamoorthy et al., 1998), resulting in the reduced activity of the transporter. Substrates of SERT like 5-HT are able to attenuate the PKC-mediated effects on SERT (Ramamoorthy and Blakely, 1999).

Although PKC is a possible upstream activator of the MAPK pathway (Roberson et al., 1999), PKC activation or p38 MAPK inhibition may mediate a down regulation of SERT and thus the processes seem to be independent of each other, yet play important parts in orchestrating the regulation of SERT.

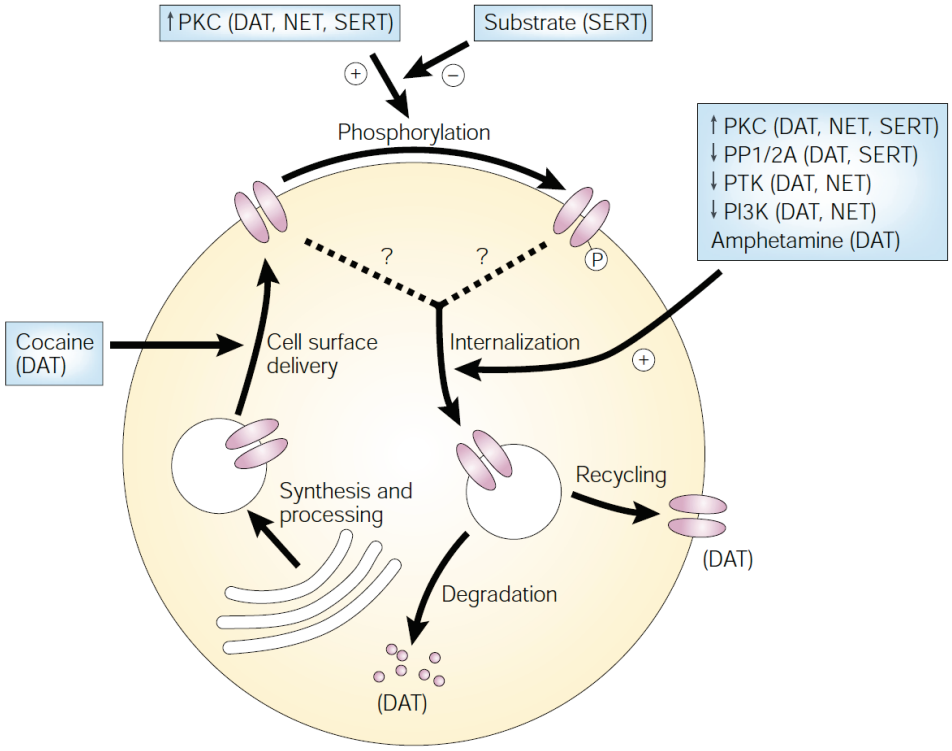

Fig. 8 Regulation of monoamine transporter

After synthesis the monoamine transporter are affected by kinases, phosphatases and substrates to regulate transporter activity, trafficking and endocytosis.

(from Torres. et al., 2003)

1.4 Aim of this study

The regulation of monoamine transporter trafficking, activity and interaction with other proteins is a complex topic compromising many still unknown molecular processes.

Kinases and phosphates are involved in this regulation and seem to operate among each other by signal cascades to affect different regulation parts.

However, specific phosphorylation sites of SERT required for regulation processes remain enigmatic.

Accordingly, the main focus of this study was to investigate phosphorylation-mediated regulation of the serotonin transporter. Especially, the process of trafficking from endoplasmic reticulum to plasma membrane based upon ER retention of SERT was examined.

Accordingly it was intended to:

- determine the impact of PKC and p38 MAPK activation on SERT surface/ER expression'
- contrast different transporter to show the requirements of different stimulation for trafficking;
- identify kinase specific phosphorylation sites by in vitro phosphorylation and mass spectrometry; and
- characterize the implication of the identified phosphorylation in SERT function and redistribution.

2 Materials and Methods

2.1 Materials

Cell culture

Cell line	Media (+ Additive)
CAD	DMEM/F12 (PAA) + 10 % FCS, + 1 % Penicillin/Streptomycin
HEK293	DMEM high glucose (4.5 g/l) (PAA) + 10 % FCS, + 1 % Penicillin/Streptomycin
SS52/62AA	DMEM high glucose (4.5 g/l) (PAA) + 10 % FCS, + 1 % Penicillin/Streptomycin, + G418 (Gibco)
tsa	DMEM high glucose (4.5 g/l) (PAA) + 10 % FCS, + 1 % Penicillin/Streptomycin
YFP-hSERT, YFP-hDAT	DMEM high glucose (4.5 g/l) (PAA) + 10 % FCS , + 1 % Penicillin/Streptomycin, + G418 (Gibco)
Solution	**Composition**
10x PBS	80 g NaCL, 2 g KCL, 14,4 g Na_2HPO_4 , 2,4 g KH2PO2 H_2O up to 1 l
Chemical	**Company**
Trypsin/EDTA	Cellgro
Tools	
Cell culture incubator	Forma Scientific Water-Jacketed Incubator 3250
Cell culture Lamina	clanLAF
Material	
6, 24, 48, 96 - *Well* plate	SPL Life Science
Cell culture dishs (35 mm, 10 cm)	Sarstedt
Cell culture flasks (25 cm², 75 cm²)	Cellstar

Transfection

Solution/ Reagent	Company
Turbofect reagent	Fermentas
Serumfree DMEM_high glucose (4,5 g/l)	PAA

Drug treatment

Drug	Company
β-PMA (1 mM Stock)	Sigma
Anisomycin (1 mM Stock)	Sigma
Okacaid acid (1 mM Stock)	Sigma
H_2O_2 (1 mM Stock)	Sigma

Lysis

Solution/Reagent	Company/Composition
Lysis buffer	1 M Tris-HCl (pH 8.0), 3 M NaCl, 20 % Triton X-100, 0,2 M EDTA,0.2 M NaF, 0.1 M $Na_4O_7P_2$, 0,2 M Na_3VO_4
Protease inhibitor (PIC)	Roche (1 tablet in 1 ml MQ; 20 µl per 1 ml lysis buffer)

BSA quantification

Solution/Reagent	Company/Composition
BSA	Pierce
BSA reagent A,B	Pierce
Lysis buffer	1 M Tris-HCl (pH 8.0), 3 M NaCl, 20 % Triton X-100, 0,2 M EDTA, 0.2 M NaF,0.1 M $Na_4O_7P_2$, 0,2 M Na_3VO_4
Tool	
Multi-label Microplate Reader	Victor

Western blot

Solution	Composition
10x Running buffer	125 mM Tris-base, 2 M Glycine, 1 % SDS H_2O up to 1 l
Washing solution	100 ml 10x TBS, 3 ml Tween 20 H_2O up to 1 l
5x Transfer buffer	15,15 g Tris-base, 72 g Glycine H_2O up to 1 l (1x Transfer buffer with 20 % Methanol)
2x RSB	125 mM Tris (pH 6,8), 4 % SDS, 20 % Glycerol, 10 % ß-Mercaptoetanol, 0,02 % bromophenol blue in H_20
10x TBS	24,22 g Tris-base, 87,66 g NaCL H_2O up to 1 l ; ph 7,5
Blocking solution	1,25 g milk powder, 25 ml 1x TBS, 0,05 % Tween 20
1,0 M Tris-HCL	121 g Tris-Base H_2O up to 1 l, pH 6,8
Reagent	**Company/ Composition**
PageRuler Plus (10-250 kDa)	Thermo Scientific
ECL reagent	Pierce
Chemicals	
SDS	Sigma
Acrylamide mix	BioRad
Ammonium persulfate	Sigma
TEMED	Merck
Antibody	
Goat anti-SERT	Polyclonal, Santa Cruz Biotechnology
Rabbit Anti-GFP	Life technologies
Tools	
Glass plate (1 mm ,1,5 mm thickness)	BioRAD
Gel caster	BioRAD
Gel comb	BioRAD
Semi-Dry BLOT	BioRAD
Gel chamber	BioRAD
Whatman paper	Protran
Nitrocellulose Membrane	BioRad

Surface biotinylation

Reagents	Company
Sulfo-NHS-SS biotin	Thermo scientific
Biotin –LC-hydrazide	Thermo scientific
Streptavidin Agarose Resins	Thermo scientific
Solutions	**Composition**
PBS^{2+}	PBS, 1,0 mM $MgCl_2$, 0,1 mM $CaCl_2$
Quench solution	PBS^{2+} , 100 mM Glycine
Lysis buffer (w PIC)	1 M Tris-HCl (pH 8.0), 3 M NaCl, 20 % Triton X-100, 0,2 M EDTA, 0.2 M NaF,0.1 M $Na_4O_7P_2$, 0,2 M Na_3VO_4
2x RSB	125 mM Tris (pH 6,8), 4 % SDS, 20 % Glycerol, 10 % ß-Mercaptoetanol, 0,02 % bromophenol blue in H_20
10mM sodium periodate/ 100mM sodium acetate	2,14 mg/ml in 100 mM sodium acetate
100mM sodium acectate	8,2 g in 1 l H_2O
DPBS	8 g, NaCl, 0,2 g KCl, 1,15 g Na_2HPO_4 , 0,2 g KH_2PO_4 H_2O up to 1 l (pH 7,3)

Quick-Change mutagenesis (PCR)

Reagents/ Components	Company
10x reaction buffer	Fermentas
Primer fw, rw (10 µM)	VbC biotech
dNTP (10 µM)	Fermentas
Quick solution reagent	Agilent Technologies
QCL enzyme (polymerase)	Agilent Technologies

Plasmid purification

Kit	Company
NucleoSpin Plasmid QuickPure kit	Machery -Nagel
NucleoBond® Xtra Midi kit	Machery -Nagel

Transformation

Solution	Company
Sterile LB-Medium	10 g tryptone, 5 g yeast extract, 10 g NaCl H_20 up to 1 l
Antibiotics	
Kanamycin	Sigma
Material	
Agarplates (kanamycin-resistant)	----------

Deglycosylation

Reagents	Company
10x buffer	New England Biolabs
10x G7 buffer	New England Biolabs
10x G5 buffer	New England Biolabs
NP-40	New England Biolabs
Enzymes	
PNGase F	New England Biolabs
Endo H	New England Biolabs

Uptake

Solutions	Company / Composition
1x KHP	10 mM HEPES, 120 mM NaCL, 3 mM KCL, 2 mM $CaCl_{2,,}$ 2 mM $MgCl_2$ H_2O up to 1 l
Reagents	
SDS	Sigma
1 mM 5-HT Stock	Perkin Elmer
[H^3] 5-HT	Perkin Elmer
Tool	
Counter	TRI-CARD 2300 TR

In vitro phosphorylation

Solutions	Company / Composition
50 mM NH_4HCO_3	39,5 mg in 10 ml H_2O
50 % ACN/50 mM NH_4HCO_3	5 ml ACN + 5 ml 50 mM NH_4HCO_3
DTT	2 mg/ml in 50 mM NH_4HCO_3
Iodoacetamide	10 mg/ml in 50 mM NH_4HCO_3
Trypsin / chymotrypsin	10 ng/µl in 25 mM NH_4HCO_3
Extraction buffer	50 % ACN/ 5 % formic acid
Comassie blue staining	Invitrogen
Reagents	
10 mM $MgCl_2$	(Roth) 2,0 mg in 1 ml H_20
1,67 mM $CaCl_2$	(Roth) 1,1 mg in 10 ml H_20
ATP	Thermo Scientific
Kinases	**Company**
PKC alpha	Life technologies
P38 MAPK	Life technologies
Tool	
SpeedVac, Concentrator plus	Eppendort

Confocal microscope

Solutions	Composition
10x PDL	5 mg PDL in 100 ml of H_2O
Material	**Company**
6-well plate	SPL Life science
Tool	
Confocal microscope, LSM 780	Zeiss

General

Tools	Company
Centrifuge : Mini Spin; 5418R	Eppendorf
Shaker	Stuart
Fluorescence microscope Axiovert 40 CFL	Zeiss
Thermomixer R	Eppendorf
LSE Vortex Mixer	Corning

2.2 Methods

2.2.1 Cell culture

The different cell lines were cultivated in 10 cm dishes, 35 mm dishes, 6 well– or 48 well-plates at 37 °C.
After a growth period of 2-7 days (depending on different cell lines and seeding), the cells had a confluence of 80-90 % and were passaged to allow further cell division.
First, cells were detached from the 10 cm dishes by removing the medium, washing twice with 8 ml PBS and adding 2 ml prewarmed Trypsin/ETDA to cleave proteins bonding of the cultured cells to the dish during a 2-minute incubation at 37 °C. Depending on the necessary dilution, a specific amount (40–350 µl) of this cell suspension was transferred to a new dish containing 10 ml warm fresh medium.

2.2.2 Transfection

To introduce DNA into cells, the cationic polymer Turbofect reagent of Fermentas was used. The reagent shows a high efficiency of transfection and a simple performance in comparison to other transfection variants. The technique is based upon the formation of positively charged and stable DNA-polymer complexes, which are endocytosed easily by cells.
Cells growing in 35 mm dishes were used for transfection at 80 % confluency.

Transfection approach was prepared in a reaction tube. 100 µl serumfee DMEM, 1 µg cDNA and 5 µl of transfection reagent were combined and incubated for 15 minutes in the hood followed by adding 100 µl of the mix drop-by-drop to the cells. After 24 or 48 hours, the cells were used for further experiments.

2.2.3 Drug treatment

For the investigations of the influence of specific drugs on protein trafficking, cells (80-100 % confluence) were treated with a definite concentration and time at 37 °C following by lysis.

2.2.4 Lysis

Cells with a confluency of 100 % were washed twice with 1 ml cold PBS, detached by addition of cold 250 µl lysis buffer(with PIC) and transferred to a reaction tube. Ensuing, the cells were incubated for 30 minutes at 4 °C on a rotator and cell debris pelleted by

subsequent centrifugation for 10 minutes at 12.000 x g. The protein containing supernatant was collected in a new reaction tube and stored at -20 °C until use.

2.2.5 BSA quantification

Determinations of protein amount in the lysates were performed by using BSA Protein Assay. Bovine serum albumin is a reference protein for total protein quantification and was present in a 2 mg/ml stock.
To obtain a standard curve for the following protein quantification of samples, 7 standards A-G were prepared in the combination shown in table 1.

Table 1: BSA standard approches

Standards in µl	A	B	C	D	E	F	G
BSA	0	1	2,5	5	10	12,5	17,5
Lysis buffer	2	2	2	2	2	2	2
H_20	18	17	15,5	13	8	5,5	0,5

For each standard and sample (2 µl lysate + 18 µl H_20), 200 µl BSA reagent A and 1/50 of amount A of solution B are necessary. Both components were combined and added (200 µl) to the tubes followed by transferring the mixes into wells of a flat 96 well plate, which was incubated at 37 °C for 30 minutes.
Based upon the development of different colors during the incubation time, the Multi-label Microplate Reader Victor analyzed the protein amounts in each sample.
The values were entered to Excel and the exact protein concentration was calculated based on the standard curve of BSA.

2.2.6 Western Blot

Western blot is a method to detect specific proteins in cell lysates by using SDS-PAGE, transfer and detection by specific antibodies.
To separate proteins by SDS-PAGE, an 8 % stacking and 8 % resolving gel with a thickness of 1 mm were prepared in gel casters comprising glass plates. After polymerization, the gels were transferred into the gel chamber, which was filled up with 1x running buffer. A specific amount of 2x RSB buffer was added to each sample as well as to the molecular

weight marker, centrifuged and loaded into the gel wells. The run was performed at 20 mA per gel for approximately 1 hour.

The transfer of separated proteins onto a nitrocellulose membrane was carried out by a semi-dry transfer chamber. 4 Whatman papers and a nitrocellulose membrane per gel were equilibrated in 1x transfer buffer for a few minutes, followed by preparing a sandwich of all components with likewise equilibrated gel located on the membrane in the middle of the sandwich. At max. 25 V and constant 230 mA, the transfer was completed after 90 minutes. Membrane was cut to size and blocked with 5 % milk solution on a shaker for 60 minutes.

After membrane blocking, the specific primary antibody was added to 8 ml 5 % milk, pipetted onto membrane and incubated overnight at 4 °C.

Removing primary antibody was carried out by washing three times with a washing buffer, followed by a corresponding secondary antibody. The incubation took 90 minutes at room temperature. In the final step the membrane was washed again and prepared for detection. The two components of ECL reagent (Pierce) were mixed together with the identical volume (1 ml each) to obtain the detection solution with which the membrane was incubated for 1, 5 minutes, ensuing transfer to the cartridge.

Depending on protein expression intensity, the films were exposured with different periods and developed in the dark room.

Table 2: Stacking and resolving gel recipes

8 % stacking gel	2 ml total per gel	**8 % resolving gel**	5 ml total per gel
H_20	1.4	H_20	2.3
1,0 M Tris-HCL (pH 6,8)	0.25	1,5 M Tris-HCL (pH 6,8)	1.3
10 % SDS	0.02	10 % SDS	0.05
30 % Acrylamide mix	0.33	30 % Acrylamide mix	1.3
10 % ammonium persulfate	0.02	10 % ammonium persulfate	0.05
TEMED	0.002	TEMED	0.003

2.2.7 Surface biotinylation

Using this method, proteins of the cell surface could be labeled with biotin on lysine residues via impermeant disulfide-coupled biotinylation reagent and a comparison between labeled and unlabeled proteins could take place.

Cells were cultivated in 35 mm dishes until 90-100 % confluence and washed three times with 1 ml cold PBS^{2+} afterwards. To each sample, 750 µl of 4 mM sulfo-NHS-SS biotin/PBS^{2+} was added and incubated for 15 minutes at 4 °C on a shaker (gently). 2 ml ice cold quench solution was used for two quenching steps under the same conditions as before. Removing quench solution was performed by washing three times with cold 2 ml PBS^{2+}. The lysis of the cells was carried out with 500 µl lysis buffer containing protease inhibitor, as described in 2.2.4. The supernatant was aspirated and transferred into a new tube representing “Input”.

For the separation of biotinylated and non-biotinylated proteins, 40-70 µl 50 % streptavidin agarose beads were added to the lysate and incubated overnight at 4 °C on a rotator. In the following day the beads were spun down (5 minutes, 4 °C, 12.000 x g) and the supernatant, containing the unbound proteins, carefully aspirated by a nozzle and transferred into a new tube. The beads were washed three times with 1 ml of lysis buffer (w/PIC), whereby after lysis addition the sample was centrifuged for 2 minutes and the supernatant was carefully aspirated and discarded. Biotinylated proteins were extracted with 200 µl 2x RSB at 95 °C for 3 minutes, transferred into a new tube and used for western blot analysis.

In addition to sulfo-NHS-SS-biotin, we tested biotin –LC-hydrazide for labeling of the surface proteins.
There are only some differences compared to the procedure with sulfo-NHS-SS-biotin.
For the washing steps, DPBS was used and the quench solution comprises 10 mM sodium periodate/100 mM sodiumacetate. After the washing steps in the beginning, 2 ml of the quench solution was added to the cells. The incubation took 30 minutes at 4 °C in the dark. The solution was removed by several washing steps and 2 mM biotin–LC-hydrazide/100 mM sodium acectate added for 30 minutes at 4 °C in the dark. Following steps were performed in accordance with the protocol previously described. Directly before using the samples for western blot, it is necessary to boil them for 3 minutes at 95 °C.

2.2.8 CO-IP

Confluent cells in 35 mm dishes were washed twice with cold PBS and solubilized in a reaction tube with 1 ml lysis buffer for 30 minutes at 4 °C on a rotator. The resulting supernatant after 10 minutes of centrifugation at 14.000 x g was used for immunoprecipitation. 50 µl of the supernatant was kept for “Input” and 500 µl lysate was used for overnight incubation at 4 °C on a rotator with specific primary antibody. Equilibrated 50 % protein G sepharose beads were added (35 µl) to the sample and incubated again on the rotator for 4 hours. The beads were pelleted by centrifugation at 10,000 x g for 30 seconds and the supernatant collected in a new tube (“Flowthrough”). Using a needle and syringe, the beads were washed 4-6 times with 1 ml lysis buffer, including 30 sec centrifugation at 10.000 x g after each washing step. Protein extraction was carried out by adding 100 µl of fresh prepared 2x RSB and heating at 95 °C for 3 minutes followed by centrifugation for 1 minute. Extracts were collected in a new reaction tube.

Finally, all samples (Input, Flowthrough and Extract) were loaded with specific amounts onto an 8 % gel and a usual western blot was performed.

2.2.9 Quick-Change Site-directed mutagenesis

To introduce point mutations in a DNA sequence, the Quick-Change lightning site-directed mutagenesis kit from Agilent Technologies was used. For this method, two primers containing the desired mutations and a double stranded vector with the sequence for mutation are necessary. Designed primers are complementary to opposite strands of the vector and are extended during the PCR by PfuUltra high fidelity DNA polymerase. Due to the extension of the primers, the directed introduction of mutations into the plasmid DNA takes place. Ensuing treatment of the PCR with Dpn I leads to digestion of the non-mutated parental DNA owing to its methylation status. The enzyme digests specific methylated and hemimethylated DNA. The remaining mutated plasmids were transformed into competent *E. coli* cells.

Plasmid	Mutation
pcSERT-YFP	T603A,D

kanamycin-resistant

PCR

Table 3: PCR approach

Components	Volume/Mass
10x reaction buffer	7,5 µl
DNA template (plasmid)	50 ng
Primer 1 fw (10 µM)	1,8 µl
Primer 1 rw (10 µM)	1,8 µl
dNTP (10 µM)	1,5 µl
ddH_20	7,5 µl
Quick solution reagent	2,25 µl
QCL enzyme (polymerase)	1,5 µl
total	25 µl

Table 4: PCR program

Temperature (°C)	Time	Cycles
95	2 min	1
95	30 sec	20x
59	20 sec	
68	3,5 min	7Kb
72	5 min	1
4	Endless	1

Total time: 2,5-3h

After PCR, 1 µl DpnI was added to 10 µl each PCR product to digest the parental DNA.

Transformation

Incubation took 1 hour at 37 °C followed by transformation, in which 2,5 µl of the sample was combined with 50 µl of competent *E. coli* in a reaction tube and placed on ice for 20-25 minutes. A heat shock at 42 °C for 45 seconds and 2 minutes of ice placement followed the first step. 2 ml of sterile LB-medium was added to each sample, which had to incubate at 37 °C for 1 hour after the addition.
The tube was centrifuged for 5 minutes at 10.000 x g and the supernatant was removed, apart from 50-100 µl in which the pellet was resuspended and plated out on selective agar plates (including kanamycin). The plates were incubated overnight at 37 °C.

Only *E.colis* containing the mutation were able to grow on the selective agar.
Positive single transformed colonies were picked and transferred to a falcon with 3 ml LB-medium containing 3 µl specific antibiotic and incubated overnight at 37 °C in a shaker.

Plasmid purification

The next day, a mini prep was performed with the NucleoSpin Plasmid QuickPure kit for plasmid purification, according to manufacturer's protocol.
After sequencing by Microsynth AG positive samples were used for Midi prep to increase the DNA concentration. 2-3 ml of the mini culture was transferred into an autoclaved Erlenmeyer flask with 200-250ml LB-media and the specific antibiotics followed by overnight incubation at 37 °C on a shaker.
The following day, the suspension was transferred to sterile GSA cups and centrifuged for 20 minutes at 4500 rpm. The pellet was used for midi DNA purification with NucleoBond® Xtra Midi kit.

2.2.10 Deglycosylation

During protein biosynthesis, most of the polypeptides undergo posttranslational modifications to obtain their final function and structure and as a result they are able to transport to their final destination in the cell. This manifests itself in adding of functional groups such as carbohydrates, lipids or phosphates. N-linked glycans are attached to plasma membrane proteins in the ER, which are further remodeled and become complex during their trafficking to the cell surface, especially in the Golgi.
The technique of deglycosylation is used to control protein trafficking and characterize molecular weights. It uses the property of the specificity of enzymes to digest proteins in defined stages during the transport.

Endo H and PNGase F were used to confirm the ER-form and surface form of the serotonin transporter. Proteins that are located in the ER are sensitive for Endo H digestion, whereby asparagine-linked mannose rich oligosaccharides are cleaved. Highly processed complex oligosaccharides that already entered the Golgi are non-sensitive for Endo H, aside from PNGase F (cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins).

For these experiments, the reagents shown in table 5 were combined in a reaction tube and incubated for 2-3 hours at 37 °C.

Table 5: Deglycosylation with PNGase F and Endo H

Components	PNGase F approach (µl)	Endo H approach (µl)
Lysate	9	9
10x buffer	1	1
10x G7 buffer	2	
10x G5		2
NP40	2	
PNGase F/ Endo H	1	1
H_20	5	7
Total	20	20

Finally 20 µl of 2x RSB were added and 8 µl of the mix was used for western blot.

2.2.11 Confocal microscope

CAD cells were seeded into 6-well plates containing cover slips, which were coated with 1x PDL for a short time at 37 °C. After two days of cultivation, transfection with YFP-SERT was performed. 48 hours later, the cover slips were monitored under a confocal microscope at the CEMM building.

2.2.12 In vitro phosphorylation

In vitro phosphorylation assays provide the possibility to identify kinase specific phosphorylation sites in proteins, including whether the protein is a substrate for the kinases at all.
YFP-hSERT stable cell line was used for the assay to detect possible phosphorylation sites specific for p38 MAPK and PKC in the N- or C-term of SERT.
The cells were cultivated and harvested as previously described in 2.2.1.
Subsequently, GST-protein purification of N- and C-term of SERT was performed by Thomas Steinkellner.
3 µg of each purified sample was incubated in 50 µl of a kinase reaction mix (table 6) for 20 minutes at 30 °C.

Table 6: In vitro phosphorylation

Components	PKC alpha approach (µl)	Components	P38 MAPK approach (µl)
20 mM Tris-HCL	10	20 mM Tris-HCL	10
10 mM $MgCl_2$	5	10 mM $MgCl_2$	5
1,67 mM $CaCl_2$	0,835	1 mM DTT	0,5
1 mM DTT	0,5	1 mM EGTA	0,5
500 µM ATP	1	200 µM ATP	1
150 ng PKC alpha	0,4	120 ng P38 MAPK	0,25
H_20	Up to 50	H_20	Up to 50

Adding a 50 µl 2x RSB buffer stopped the phosphorylation reaction. A 10 % acrylamide gel with five slots was prepared and filled with 100 µl of each sample after polymerization. 7 µl of marker and 85 µl 2x RSB buffer was combined for molecular weight identification. The gel ran for approximately 70 minutes at 20 mA.
For visualization of the resolved proteins, a coomassie blue staining (Invitrogen) was carried out overnight on a shaker at room temperature. The gel was transferred into a plastic box containing 100 ml coomassie blue mix in accordance with the manufacturer's information.

The next day, the specific bands were cut out on a glass plate with a scalpel, divided into two pieces for the use of two different enzymes in gel digestions, reduced to small pieces

and subsequently placed into Eppendorf tubes. After each band, the glass plate was cleaned to prevent contamination.
For coomassie staining and SDS removal, 200 µl of 50 % ACN/ 50 mM NH_4HCO_3 was added to each tube and incubated overnight on a rotator at 4 °C. The solution was removed by pipette and 200 µl of pure ACN was added, which was removed immediately afterwards. To dry the gel pieces, the tubes were placed into a SpeedVac for 10 minutes.

Alkylation and reduction of the samples was achieved by the following steps. 1 ml of DTT solution was added for 2 hours at 56 °C for re-hydration of the gel pieces, before it was replaced by 1 ml 54 mM iodoacetamide solution, which had to incubate for 1 hour at room temperature in the dark. This was followed by three washing steps with 100-200 µl (pieced should be covered) 50 % ACN/50 mM NH_4HCO_3. For each run, the samples were placed for 10 minutes on a mixer and shortly spun at 1300 x g (20 °C).
After the supernatant was removed, the tubes were placed again into the SpeeVac for 50 minutes to completely dry the pieces.

In gel digestion started with the addition of trypsin or chymotrypsin solution (10 ng/µl in 25 mM NH_4HCO_3) to the sample tubes to cover all gel pieces. The volumes varied between 40 µl and 160 µl due to the different sizes of the gel pieces. The samples were re-hydrated at 4 °C for 10 minutes and incubated overnight at 37 °C. The following day, the enzyme solutions were transferred into new tubes after centrifugation. Extraction of the peptides was carried out by adding extraction buffer to the gel pieces. The volumes were oriented towards the volumes of the enzyme solutions and differ between 150-350 µl. After two hours on a mixer at 37 °C, the supernatant was aspirated and united with the previous collected solution. For the second extraction, a 250 µl buffer was used and the process was performed again. The combined solutions were reduced with SpeedVac to a volume of approximately 40 µl and prepared for Maldi-mass spectrometry.

2.2.13 Uptake

To identify the functional activity of the YFP-hSERT cells, an uptake assay was performed, with a confluent 10 cm dish used to split the cells into a 48 well plate. The cells were detached with 500 µl trypsin (2 minutes incubation at 37 °C) after washing twice with 10 ml cold PBS. The solution was added into 30 ml fresh medium and the cell number was determined by counting chamber. The average value of different counting processes were used to calculate a dilution factor ((average value/100) /0,4 = dilution factor) regarding a correct cell number for uptake after two days of cultivation. Due to the fact that 500 µl per well was necessary, a total volume of 13 ml was prepared, whereby the total volume divided by the dilution factor resulted in the volume of cell suspension, which was

refilled with fresh media up to 13 ml. 500 µl of this solution was added into each well of a 48 well plate, which was prepared with 1 ml 1x PDL incubation (overnight at 37 °C) and washing with 500 µl MQ. After two days incubation at 37 °C, the cells were almost confluent and thus were used for uptake.

Before uptake, different solutions of 5HT concentrations with 0,15 µM [^{3}H]5-HT were prepared in KHP, in accordance with the data shown in table 7. For blank 10 µM, paroxetine was used.

Table 7: Uptake pipetting schema for 5-HT

		finalconc 3H [µM]	endvolume: µL				finalconc [µM]	
		0,15	650				10	
eppis #	finalconc [µM]	3H-conc **(10x)**	5HT [10µM]	5HT [100µM]	5HT [1mM]	KHP	bk/PAR [µM]	
		1,5	10	100	1000		100	Endvolume
1	1	65	55,25	xxx	xxx	530	xxx	650
2	5	65	315	xxx	xxx	270	xxx	650
3	10	65	xxx	64	xxx	521	xxx	650
4	30	65	xxx	195	xxx	390	xxx	650
5	100	65	xxx	xxx	65	520	xxx	650
bk	100	65	xxx	xxx	65	455	65	650
Prä	xxx	xxx	xxx	xxx	xxx	585	65	650
			370,50	259	130			
vol. 3H-solution [µL]		405						
3H-5HT [µL]		16,9						

The uptake procedure started with aspiration of the supernatant followed by directly adding 750 µl cold KHP. Subsequently, the cells were incubated with increasing concentrations of 5-HT and a final concentration of 0,15 µM of hot radioactive 5-HT for 1 minute at RT. The uptake was terminated by aspiration of the solutions, the cells were instantly washed with 750 µl KHP, lysed in 500 µl SDS and transferred in counter tubes containing 4 ml szintillation cocktail. The assay was conducted in triplicates.

Analysis of the samples was carried out by counter and cell number was counted by counting chamber.

3 Results

3.1 Characterization of the serotonin transporter in Hek293 cells stably expressing YFP-hSERT

Before investigating the effects of kinase activation on SERT distribution in different sub-cellular compartments, characterizations of the wildtype SERT stably expressed in HEK293 cell line were first performed by immunoblotting, immunocytochemistry and 5-HT uptake assay.

Expression of YFP-tagged SERT in the cells was determined by western blot analysis. The cell lysate was loaded on an 8 % polyacrylamide gel and resolved by SDS-page for 1 hour, followed by protein electrotransfer by a semi-dry system. With a goat polyclonal anti-SERT antibody, the blot revealed two major immunoreactive bands at approximately 100 kDa and 70 kDa, which probably represent the surface (upper band) and the ER retention form of SERT (Fig.9).

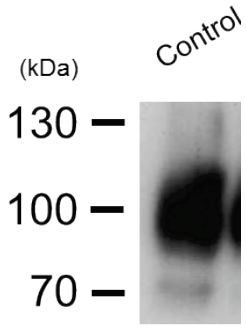

Fig. 9 Western blot of SERT

Cell lysates from YFP-hSERT stable cell line were used for serotonin transporter detection with Western blot technique (goat anti-SERT antibody). The blot shows the glycosylated surface (~100 kDa) as well the unglycosylated ER retention form (~ 70 kDa) of SERT.

A deglycosylation assay was performed to confirm that the bands represented the different cellular localizations of SERT. Lysates of the stable cell line were incubated in the presence or absence of Endo H and PNGase F for 3 hours, resolved by SDS-PAGE and immunoblotted with GFP- antibody.

Endo H only showed a sensitivity to the ER retention form of SERT (lower band). This treatment led to a reduction of about 2 kDa due to the removal of high mannose and hy-

brid types of N-linked carbohydrates, which is not preceded by Golgi mannosidase II and thus Endo H sensitive lower band represents an ER retention form of SERT. Deglycosylation by PNGase F affected both bands by additional cleavage of complex oligosaccharides that are attached during protein trafficking.

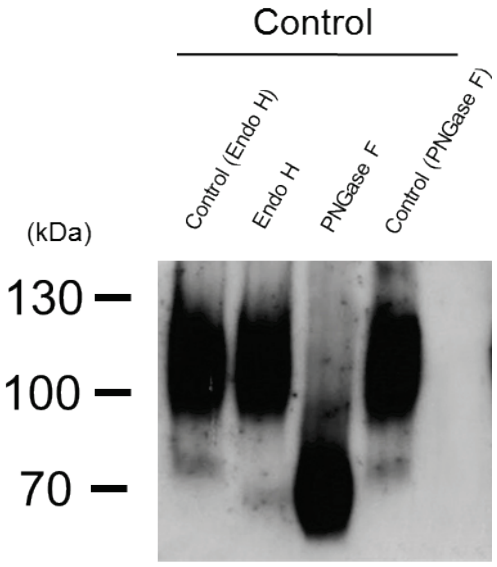

Fig. 10 Deglycosylation of SERT examined by western blot.
Cell lysates from YFP-hSERT stable cell line were added with Endo H, PNGase F or with the specific enzyme buffers as control. For western blot GFP- antibody was used.

Besides western blot detection of SERT, attempts were made to visualize and quantify the cellular distribution of SERT within the cells by confocal microscopy. In addition, the aim was to investigate whether the distribution is in accord with the immunoblot results.
Cultivated CAD cells were seeded on coated coverslips and transfected with wildtype SERT at a confluence of approximately 90 % for 48 hours. The fluorescence signal of SERT was primarily observed at the surface, as well as in the intracellular parts of the transfected cells, which represented the immature proteins (Fig.11).

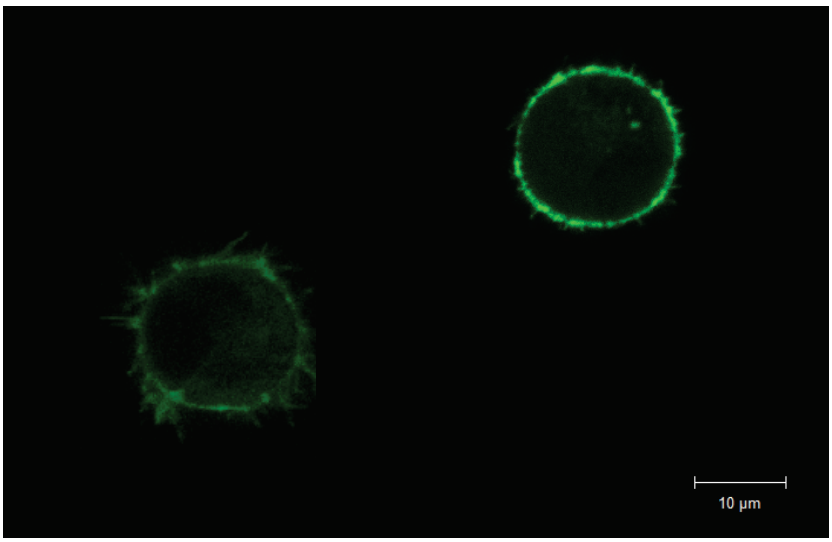

Fig. 11 Confocal microscopy of transiently transfected CAD-YFP-SERT cells

CAD cells were cultivated on coverslips in a 6 well-plate and transfected with 1,5 µg YFP-wildtype SERT cDNA for 48 hours followed by testing fluorescence and localization of SERT by confocal microscopy.

The transporter activity of SERT in the stable cell line (YFP-hSERT) was investigated by an uptake experiment and measured in triplicates. Cells were cultivated in a 48-well plate until a confluence of almost 100 % and were used for the functional study as described in 2.2.12. The affinity of SERT for 5-HT was determined and showed a K_m value of 4,2 µM, which is a normal value for wildtype SERT cells (Fig.12).

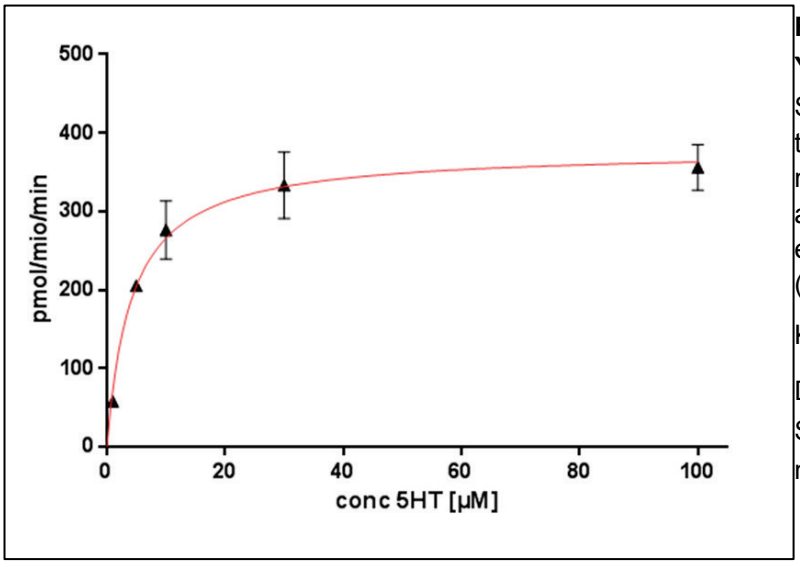

Fig. 12 Uptake activity in YFP-hSERT cells

Stable cell line expressing YFP tagged –hSERT were used to measure their [^{3}H] 5-HT uptake activity in the absence or presence of 10 µM paroxetine (blank).

K_m =4,2 µM

Data points represent mean ± S.E.M. from triplicate determinations.

3.2 SERT redistribution due to anisomycin or β-PMA treatment

Both kinase activators anisomycin (p38 MAPK) and ß-PMA (PKC), which are already known to be important in regulation of monoamine transporters (Ramamoorthy et al., 1998; Qian et al., 1997; Samuvel et al., 2005), were used for cell treatment to identify their impact on the cellular distribution of SERT.

Cells treated with 1 µM of anisomycin and those treated with 1 µM of β-PMA for 1 hour were analyzed via western blot pertaining to SERT expression and distribution. Through the treatment of anisomycin, the ER retention form was reduced, which differs from the β-PMA effect of an increase retention form in comparison to the control (Fig.13).

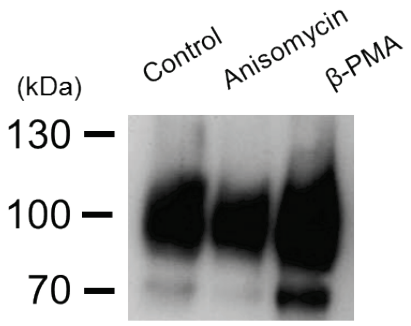

Fig. 13 Impact of anisomycin and β-PMA on SERT
YFP-SERT cells, treated with 1 µM β-PMA or anisomycin for 1 hour, showed different effects on the two SERT forms and therefore distribution.

Again, a deglycosylation assay was performed to confirm the different cellular localization of SERT after drug treatment. In Fig.14, it can be seen that the drug treatment influences the specific two SERT bands like in the control.

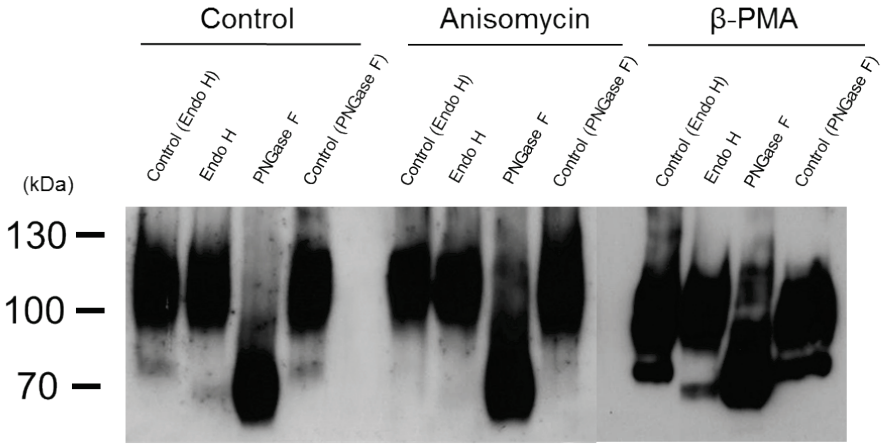

Fig. 14 Deglycosylation assay after drug treatment of YFP-SERT cells
Cell lysates from YFP-hSERT stable cell line treated with anisomycin or β-PMA (1 hour, 1 µM) were added with Endo H, PNGase F or with the specific enzyme buffers as control. For western blot GFP- antibody was used.

3.3 Time- and dose-dependence of SERT distribution by anisomycin or β-PMA treatment

It is known that the p38 MAPK activator anisomycin influences the activity of SERT in different cells (Zhu et al., 2005). The stimulation of the kinase is associated with a higher 5-HT uptake. Previous results have shown a different cellular localization of SERT by anisomycin treatment (1 µM) in comparison to untreated cells (Fig.13 and 14). In line with this observation, the aim was to establish a possible connection between drug concentration or treatment time and SERT distribution within the cell.

YFP-hSERT cells were treated with different concentrations (0,1–20 µM) of anisomycin for 1 hour during the cultivation. After lysis and immunoblotting with a goat anti-SERT antibody, concentration dependence in connection with the decreased ER retention form of SERT was observed. A concentration of 0,5 µM of anisomycin already leads to the highly reduced unglycosylated form of SERT due to enhanced trafficking of the SERT to the surface.

A similar effect of treatment dependence could be observed after incubation of 1 µM anisomycin for different time periods (3–120 minutes). With a longer duration, a reduction of the ER retention signal was monitored, which achieved a very clear effect after 60 minutes compared to the control.

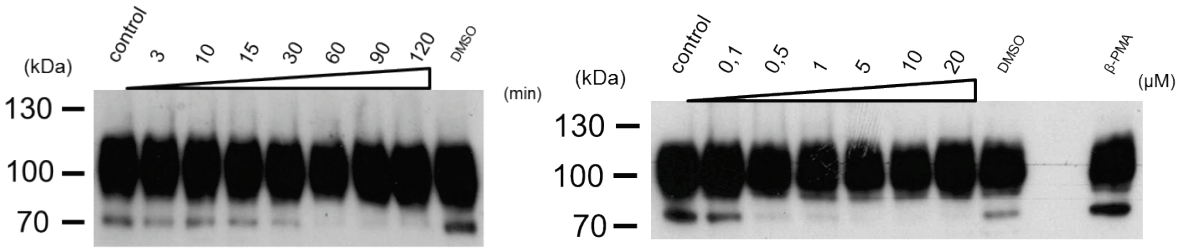

Fig. 15 ER retention form of SERT is decreased in a concentration- and time-dependent manner by anisomycin treatment

YFP-hSERT cells were treated with 1 µM for different time periods or for 1 hour with different concentrations of anisomycin and compared with untreated (control) and DMSO treated lysates. The proteins (1,5 µg) were separated by SDS-PAGE, transferred on to membrane and immunoblotted with anti-SERT antibody.

Ten independent immunoblots were performed using these optimal conditions for anisomycin treatment (1 hour, 1 µM) to determine the treatment effect on the ER retained form in comparison to the ER retained form of the control. The blots were developed, scanned and the signals were finally quantified by *Image Studio software (Li-Cor)*.

Analysis showed (Fig.16) a significant effect on the unglycosylated form after anisomycin treatment. Anisomycin treated cells only displayed 19,2 % of the control ER retention form, representing a clear loss of unglycosylated SERT.

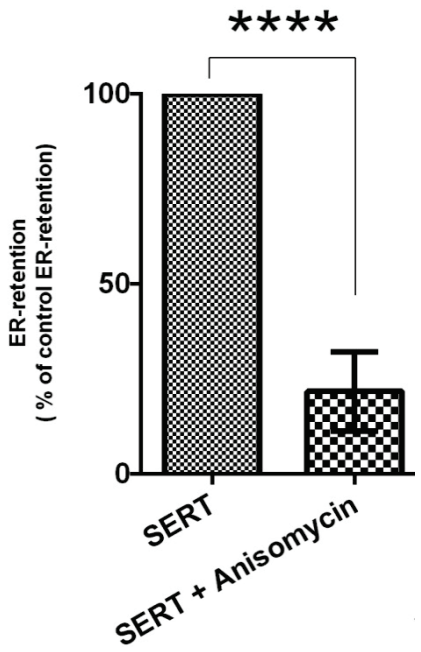

Fig. 16 Decreased expression of ER resident of SERT by anisomycin

Analysis of western blots of untreated or anisomycin treated (1 µM, 1 hour) YFP-hSERT stable cell line lysates showed a significant reduction of ER retention expression after anisomycin incubation compared to SERT control. Data are presented as mean +/- SEM, ****, $p < 0,0001$ (t-test), n = 10

The opposite effect on SERT distribution could be observed after incubation with β-PMA: this compound is known to induce internalization of SERT via the activation of PKC and thereby a reduction of transporter activity (Ramamoorthy et al., 1998).

Again, I aimed to determine the connection between drug dose or treatment time and the subsequent subcellular localization of SERT.

Therefore, I treated cultivated cells first with different concentrations (0,01-0,1 µM) of the PKC-activating phorbol ester for 1 hour, followed by lysis, SDS-PAGE and transfer of the proteins from the gel onto a membrane. Immunological detection of SERT was performed with an anti-GFP antibody.

The expression pattern showed a steady rise of the ER retention form expression with an increasing concentration of β-PMA. A concentration of 0,01 µM already effected a weak increase of the unglycosylated form expression compared to the control. The highest intensity of the ER retention form expression was observed at a concentration of 0,075 µM rather than 0,1 µM, the highest concentration in this experiment. The reason for this is a higher protein amount in the sample, despite the previous normalization of all samples. In comparison to all other expression patterns, it could be observed that the glycosylated form is also enhanced at this concentration. Taking this into account, it can be pointed out that SERT distribution is dependent on the β-PMA concentration.

Despite the results of concentration dependence, 1 µM of β-PMA was also used to check the stable cell line on time dependence. In addition, the concentration of 1 µM was used in different paper for experiments and already indicated good results in other cell lines.

Similar to the experiments with anisomycin, I used the same duration of 1 hour for the incubation with PMA. Interestingly, an increase of the ER retention form was observed over time: after 60 minutes of treatment, the enhancing effect was clear. As a consequence of this observation and knowledge of the toxicity of β-PMA, the following experiments were carried out with 1 µM for 1 hour.

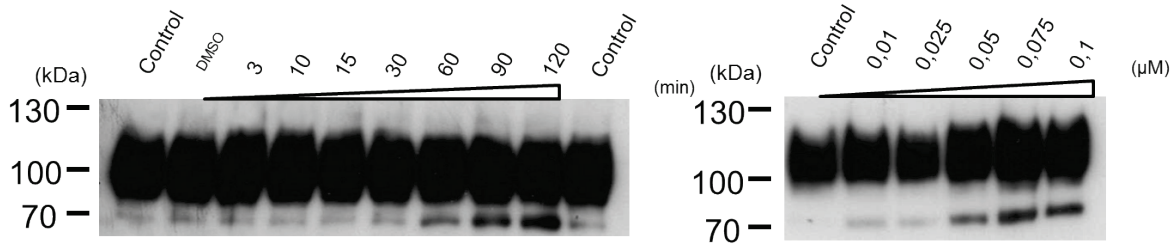

Fig. 17 In response to β-PMA, the ER retention form of SERT is increased in a concentration- and time-dependent manner

Cell lysates from YFP-hSERT stable cell line were incubated with β-PMA for 1 hour with different concentrations or with 1 µM for 0-120 minutes. As control untreated and DMSO treated lysates were used. The samples were normalized on 1,5 µg.

3.4 Anisomycin-mediated decrease of ER retention form specific for SERT

The previous experiments showed that anisomycin treatment has a clear impact on SERT expression by reducing the ER retention. Next, I wanted to ensure that the observed effect was specific for SERT and tested another stable cell line expressing the SLC6 family member DAT in the same experimental setting. DAT is relatively similar to SERT due to its structure and thus represents a suitable candidate to check the specificity of the anisomycin-SERT interaction.

Both cell lines were cultivated under the same conditions and treated with anisomycin (1 µM for 1 hour) at a confluency of approximately 90-100 %. By means of a BSA assay, the protein concentration was determined in each cell lysate, whereby only 1 µg of each sample was used for SDS-PAGE. The transporter expression was visualized by western blot with an anti-GFP antibody.
Figure 18 shows the different expression pattern of the transporters. Based upon the blots, all samples of DAT expressing cells had almost the same expression intensity of surface and ER retention form. Therefore, I concluded that anisomycin treatment had no influence.
Quantification analysis of several independent anisomycin treatment experiments with SERT or DAT expressing stable cell lines showed a significant result of ER retention form reduction compared to untreated cells only for SERT.

In comparison to SERT, the other anisomycin treated cell line only showed a minor impact on ER retention form reduction in relation to total ER retention expression in their specific control. SERT showed a clear reduction of control ER retention form (80,8 %) in contrast to DAT, which only showed a reduction of 5,6 % of the control ER retention form by anisomycin treatment.

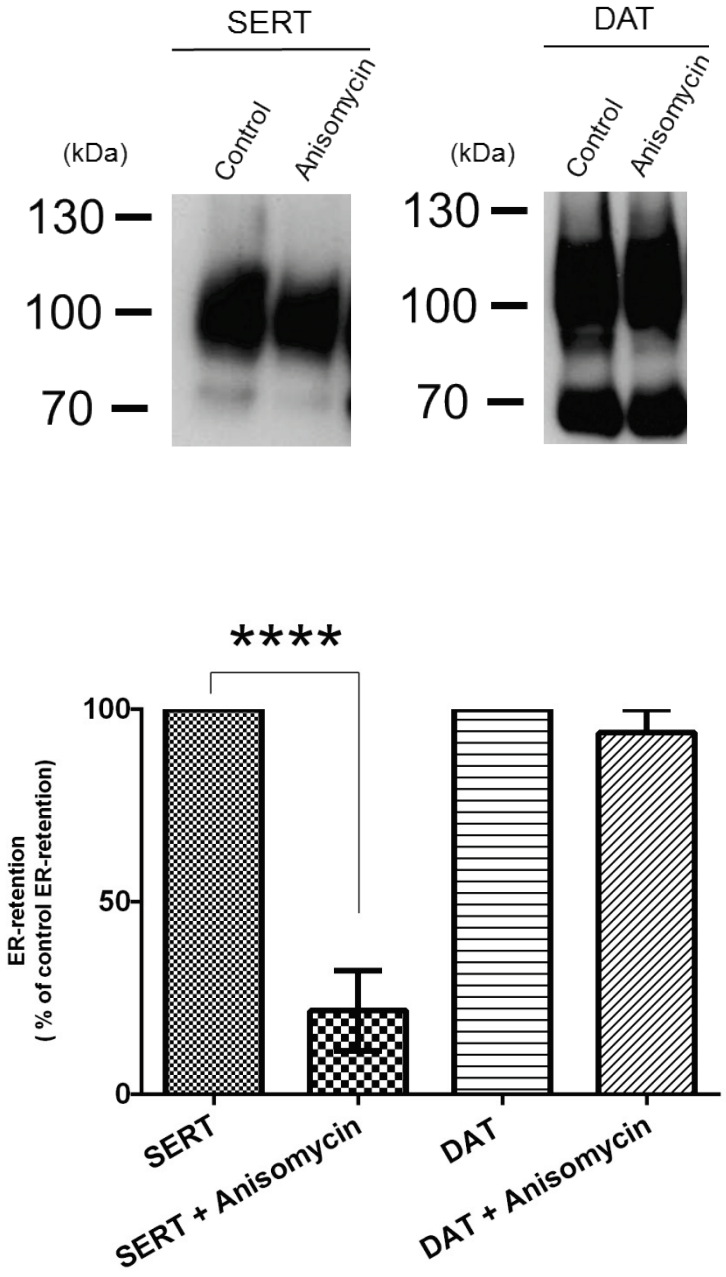

Fig. 18 Effect of anisomycin is specific for the serotonin transporter

Stable cell lines of wildtype SERT and DAT (all YFP-tagged) were treated with or without 1 µM anisomycin for 1 hour. DAT samples in the western blot showed no clearly decrease of the ER-retention form in contrast to SERT by anisomycin treatment.

Quantitative analysis of different western blots (n= 5 for DAT, n= 10 for SERT) with *Image Studio* acknowledged the specificity of anisomycin effect for SERT. The bars display percentage of total control ER-form expression after anisomycin treatment. Data are presented as mean +/- SEM. ****, $p < 0{,}0001$ (t-test).

3.5 In vitro phosphorylation

In vitro phosphorylation is a method to uncover kinase specific phosphorylation sites in proteins. Taking my previous results into account, I based the focus on p38 MAPK and PKC to find possible phosphorylation sites that could be involved in this trafficking.

GST fusion proteins with each C- and N-terminus of SERT were blended with the two specific and purified kinase PKC and p38 MAPKs and incubated for 20 minutes at 30°C. The following steps were carried out corresponding to 2.2.12 and analyzed via mass spectrometry.

The analysis of the samples showed a detection of two phosphorylation sites in the C-terminus of SERT. In vitro phosphorylation displayed a phosphorylation of T616 in the C-terminus of SERT induced by active p38 MAPK and T603 induced by active PKC. In this case, the N-terminus remained unphosphorylated despite the detection of p38 MAPK associated phosphorylation sites S52 and S62 via previous in vivo phosphorylation. In addition, T616 was also detected in this context (Jae-Won Yang, data not published).

Both phosphorylation sites are located close to the endocytic or ER-export signal of SERT.

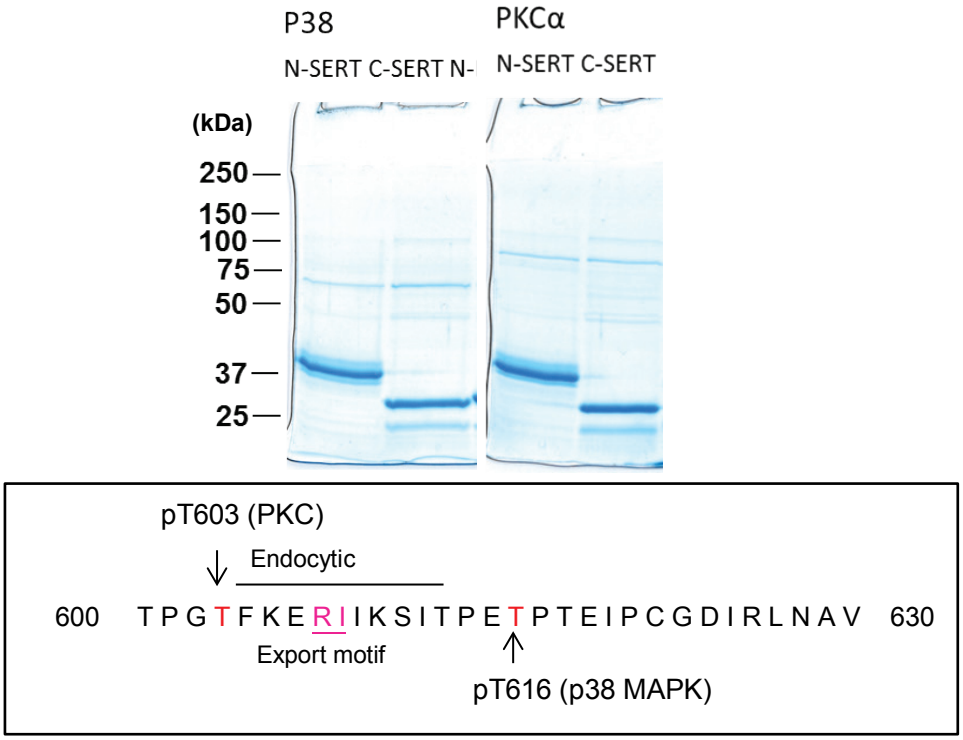

Fig. 19 Kinase specific in vitro phosphorylation sites of in SERT identificated by mass spectrometry

Coomassie blue stained bands of specific phosphorylated GST tagged N- and C-term of SERT were excised from the gel and prepared for MS via in gel digestion. Both detected phosphorylation sites are located in C-term of SERT close to endocytic and ER export signal.

3.6 N-terminal phosphorylation does not control ER retention form of SERT

The N-terminal phosphorylation sites S52 and S62, previously detected by in vivo phosphorylation assay with anisomycin treatment (Jae-Won Yang, unpublished data), were investigated for a possible correlation to an anisomycin-mediated decrease of the ER retention form of SERT.

CAD cells were transfected with double mutant S52/S62AA, phospho-mimicking or phospho-blocking mutant of this sites and incubated with typical conditions for anisomycin. Based upon phosphorylation avoidance, the mutants whose phosphorylation site replaced to alanine (A) should display no effect of anisomycin in SERT expression if an anisomycin-induced decrease of ER-resident SERT occurs via their phosphorylation.

Detection of the SERT signal with anti-GFP antibody displayed that all mutants except S52D still showed a reducing effect of the ER retention form after anisomycin treatment.

Consequently, it can be excluded that these phosphorylation sites play a role in trafficking of SERT due to activation of p38 MAPK by anisomycin. Indeed, this is even true despite the fact that identification by mass spectrometry was achieved.

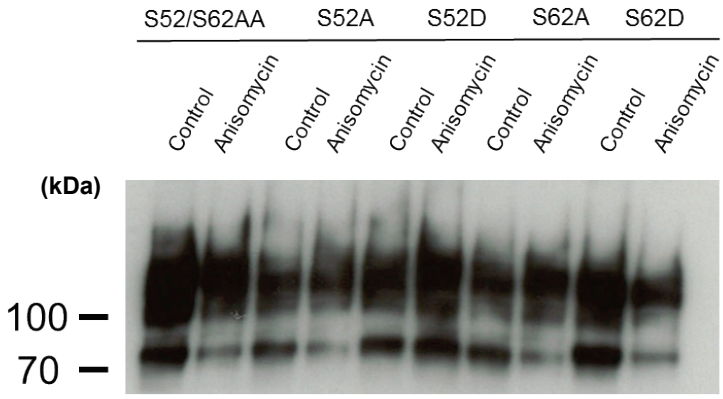

Fig. 20 Anisomycin-mediated effects on SERT localization occurs in mutants S52 and S62

CAD cells were transiently transfected with SERT cDNAs mutated at S52, S62 or S52/S62AA for 48 hours followed by incubation for 1 hour with or without 1 µM ansiomycin. Detection of SERT was performed by western blot with anti-GFP antibody.

Phosphorylation was also detected at S52 and S62 by okadaic acid, which inhibits the PP1/PP2A (Jae-Won Yang, unpublished data). Therefore, I set out to reveal whether the

increase of phosphorylation status at S52 and S62 through the inhibition of PP1/PP2A affects SERT redistribution in wildtype SERT cells.
Wildtype SERT cells were treated with okadaic acid with different concentrations for 30 or 60 minutes and with 30 µM H_2O_2 for 15 minutes. Treatment with H_2O_2 was performed due to its attribute of activating p38 MAPK.

In figure 21, it can be seen that there is no clear change in SERT expression due to the treatment in comparison to the control sample. Thus, it can be excluded that there is an impact on ER retention of SERT via phosphorylation at S52 and/or S62 through the use of PP1/PP2 inhibitor or H_2O_2.

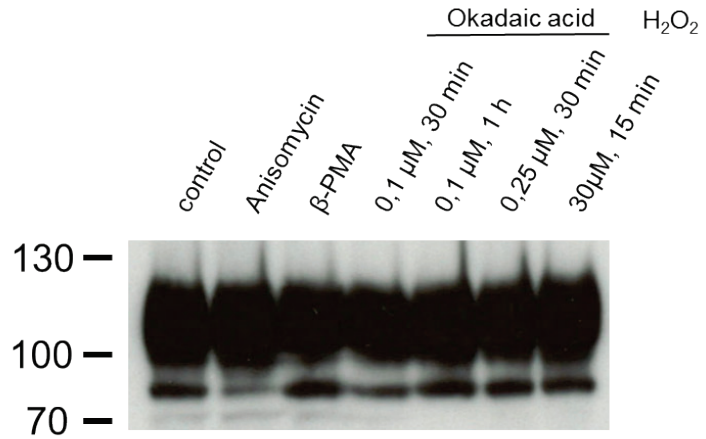

Fig. 21 No impact on SERT ER-retention by PP1/PP2 inhibition

YFP-hSERT cells were incubated with 0,1 µM PP1/PP2 inhibitor okadaic acid for 30 minutes and 60 minutes or 0,25 µM for 30 min as well with 30 µM H_20_2 for 15 minutes to investigate their effect on SERT distribution. For expression comparison untreated, ansiomycin and β-PMA (each 1 µM, 1 hour) treated cells were used for western blot.

3.7 p38 MAPK phosphorylates SERT at T616 and induce its surface trafficking

P38 MAPK activity is known to phosphorylate and regulate SERT (Samuvel et al., 2005). Accordingly, the next step was to examine the role of the identified C-terminal phosphorylation site T616 in anisomycin-mediated p38 MAPK activity and redistribution of SERT. Phosphomimetic (T616D) and dephosphomimetic (T616A) mutants were generated by Quick-Change mutagenesis and transfected into CAD cells.

The analysis was performed by western blotting, albeit with an anti-SERT antibody in this case.

In contrast to the phosphomimicking mutant T616D and wildtype SERT, the dephosphomimetic mutant showed a less reduction effect on the unglycosylated form of SERT (Fig. 22). In the investigations of this treatment, a significant result of a reduced influence of anisomycin on the T616A mutant could be shown. After analysis of all blots with *Image Studio*, the average of a mutant displayed an ER-form expression of 72 % regarding control ER-form expression. However, wildtype SERT and T616D have almost the same value (~ 41 %) due to anisomycin treatment.

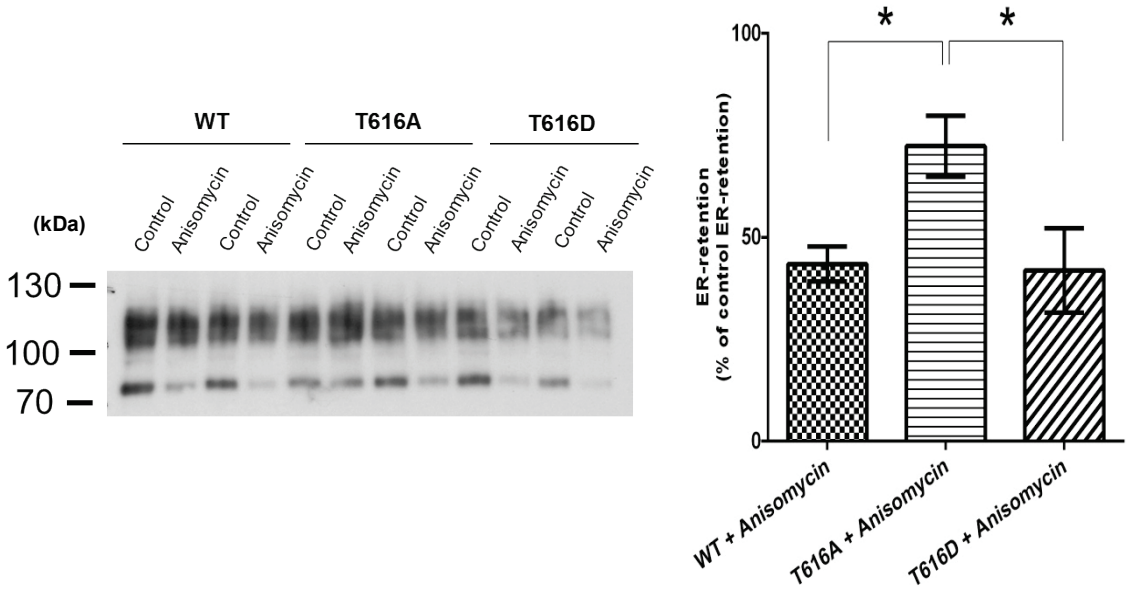

Fig. 22 Effect of Anisomycin on resident ER-form of wildtype SERT and T616 mutants

CAD cells transfected with wildtype SERT or mutants T616A or T616D were incubated with 1 µM Anisomycin for 1 hour, lysed and used for western blot (A). (B) The bars show the percentage of total control ER- form expression of the treated cells. Anisomycin caused a significant reduction of ER resident SERT in all cells. But in comparison T616A mutant showed a much less decrease (~72 % of control ER-form expression). Band densities of SERT ER-form (% of control) were quantitated with Image Studio and are presented as mean +/- SEM. One-way ANOVA. *, p <0,05 . (n= 4)

4 Discussion

The monoamine transporter SERT is a member of the SLC6 family and mediates specific reuptake of extracellular 5-HT following the release of the transmitter (Chen et al., 2004). Due to this function, SERT plays an important role in serotonergic signaling by controlling the 5-HT concentration within the synaptic cleft. This process is affected by the posttranscriptional modification of SERT (Lesch et al., 1993; Ramamoorthy et al., 1993) and the regulation of its protein biosynthesis (Qian et al., 1997; Ramamoorthy et al., 2002).

Here, the focus wasplaced upon the trafficking dependent regulation of SERT, which is known to play a prominent role in neurotransmitter signaling. For the experiments, YFP-SERT cells were used, which showed a K_m value of 4,2 µM in an uptake experiment, representing a normal [^{3}H]-5HT binding specificity in comparison with previous uptake data of our lab.

First, the expression of SERT via immunoblot using anti-SERT (goat) antibody was checked. Two bands at a molecular weight of ~100 kDa and ~70 kDa representing the different cellular localization of SERT were observed (Fig.9). By means of a deglycosylation performance, the assumption of the two bands representing the different localization could be supported (Fig.10). Endo H digestion (sensitive for ER proteins) only indicated a sensitivity of the lower band by a reduction of the molecular weight about 2 kDa contrary to PNGase F digestion, which affected both bands. Based upon this observation, it could be illustrated that the upper band represents the transporter expression at the surface and the lower band the endoplasmic reticulum retention form of SERT. These results are almost congruent with a previous observation describing a molecular weight of 95 kDa and 60-66 kDa for glycosylated or unglycosylated form of SERT (Zhang and Rudnick, 2010). This fact results from the posttranslational modifications that took place during the transporter trafficking between the place of synthesize and the site of action at the plasma membrane, leading from an immature to a complex glycosylated protein.

Via confocal microscope the visualization of surface and intracellular pools of SERT in transiently YFP-hSERT transfected CAD cells was performed. Despite efficient transfection and thus expression of SERT, it was not possible to detect a clear tendency of its distribution nor after treatment neither without. Consequently, this investigation cannot provide support for the results and thus the focus was switched on other methods.

The redistribution of monoamine transporters between intracellular room and plasma membrane is known to regulate by therapeutic drugs, drug of abuse and multiple kinase interaction (Jayanthi et al., 2005; Zahniser and Doolen, 2001).

Here, the focus was placed upon both PKC and p38 MAPK activation, due to specific activators and their impact on SERT localization.

The effect of ß-PMA activation of PKC on SERT was demonstrated by considering and comparing the expression intensity of the two bands in immunoblots. YFP-hSERT cells were treated for 1 hour with 1 µM β-PMA, lysed and resolved by SDS-PAGE and immunoblotted for SERT by anti-SERT (goat). In comparison to untreated cells, the stimulation by β-PMA resulted in an obvious increase of ER retention form. Consequently, the data corroborates and provides a new argument for previous studies describing β-PMA treatment wreaking an activation of PKC followed by diminution of surface expression and 5-HT uptake of SERT (Ramamoorthy et al, 1998; Qian et al., 1997).

However, earlier studies have pointed out that the PKC-mediated reduction of SERT activity and surface density are caused by a rapid internalization. In relation to DAT, the activation of PKC is associated with both an increase of DAT endocytic rates and decrease in DAT recycling back to the plasma membrane and that an endocytic signal, conserved within SLC6 family, in the C-terminus of DAT is involved in this process (Loder and Melikian, 2003; Holton et al., 2005).

In this case, the focus was placed upon the ER retention form, which is increased due to β-PMA treatment and retrieved another possibility of a decreased surface density. Besides an enhanced translocation of SERT into the cell and associated endosomal/lysomal pathway, it is possible that an inhibition of trafficking of SERT within COPII causes the changes in the surface pool.

While molecular processes of a connection between protein retention and PKC activation are not well known, it was demonstrated that the GluR6 kainate receptor subunit trafficking is PKC dependent and mediates two mechanisms. Two phosphorylation sites in the C-terminus of Glu6 specific for PKC were found, whereby phosphorylation of either Ser846 or Ser868 mediated ER retention of Glu6 was associated with decreasing surface density. In addition, only phosphorylation of Ser846 increased endocytosis rates (Nishimura et al., 2010). Activation of PKC was stimulated by β-PMA like in the experiments of this thesis.

Thus, despite the difference of transporter family, it is estimated that similar effects of SERT phosphorylation by PKC could result in the retention of SERT in the ER. Phosphorylation of specific amino residues in the C-terminus of SERT can possibly lead to conformational changes, whereby the ER-export signal is not accessible for COPII subunit SEC24C and thus a translocation to the plasma membrane of SERT is not possible. A further possibility for ER retention of SERT due to β-PMA stimulation is that the activated PKC phosphorylates other kinases that negatively affect components of COPII assembling, which is essential for ER-export of proteins (Novick et al., 1981; Kaiser and Sche-

kman, 1990). To confirm this, further investigations are necessary to identify exact molecular processes that play a role in this correlation.

An oppositional effect on ER retention form was observed by anisomycin (p38 MAPK activator) stimulation in YFP-hSERT and transiently transfected CAD cells. 1h treatment of 1 µM anisomycin caused a clear reduction of unglycosylated SERT in comparison to the control. It is assumed that this effect is related to a higher or enchanced redistribution of SERT from the ER to the cell surface. This assumption is supported by previous reports of p38 MAPK activation affecting SERT catalytic function as well as trafficking (Samuvel et al., 2005; Jayanthi et al., 2005, Zhu et al., 2005). Specific p38 MAPK inhibitors (SB203580 and PD169316) decreased cell surface expression and transport capacity of SERT, while an upstream kinase (MKK3b) of p38 MAPK induced an increase of SERT surface expression and function (Samuvel et al., 2005). In addition, experiments with knock out mice showed a stress-induced activation of p38 MAPK, resulting in an enhanced translocation of SERT to the plasma membrane (Bruchas et al., 2011). However, regarding the p38 MAPK activation by anisomycin, no link could be established between the observed higher SERT activity and an elevation of the surface density of SERT (Zhu et al., 2005).

These different observations may be related to different time treatments, cell types or the expression level of SERT in the cell.

In contrast to other studies, which mainly worked with uptake and surface biotinylation experiments, my results are based upon immunoblots.

Surface biotinylation experiments conducted for a better evaluation of drug mediated trafficking via p38 MAPK or PKC were not successful despite multiple performances and different agents (data not shown). Nonetheless, with the help of *Image studio*, it was possible to quantify surface or ER retention expression of different independent immunoblots in the presence and absence of anisomycin. A significant reduction of the ER-form due to treatment could be observed in the treated cells, which displayed only 19,2 % of the control ER retention form. This data also supports the assumption of anisomycin-mediated reduction of immature SERT.

Furthermore, investigations were carried out to determine whether the opposite effects of the kinase activators on SERT ER retention form are time- and dose-dependent. It has already been demonstrated that β-PMA treatment reduces the surface expression of DAT in a time-dependent manner (Daniels and Amara, 1999) and the SERT uptake activity stimulation by anisomycin is dose- and time-dependent (Zhu et al., 2005).

In the performed experiment, both agents were used to treat samples of YFP-hSERT cells with 1 µM for different time periods (3-120 minutes), resulting in the observation that the ER retention form expression decreases with the duration of anisomycin treatment, reaching an almost total reduction after 60 minutes (Fig.15). This result of time dependence is

consistent with those of β-PMA treatment (Fig.17). Here, an increase of ER retention form could be detected, whereby a clear rise occurs after a duration of 60 minutes and longer.

Besides the time manner, a correlation between dose and transporter distribution was also reported in both cases, based upon western blot results. In case of anisomycin, a concentration of 0,5 µM for 60 minutes brought about a clear reduction of the lower band, unlike β-PMA, in which a concentration of 0,05 µM already had visible effects on distribution.

Despite these results showing the influence of SERT trafficking or its cellular distribution due to specific activation of kinases, the exactly molecular process is not known.

The similarity structure and affiliation of the monoamine transporters to one family prompted the next step to ascertain whether this mediated increase of surface expression or decrease of ER retention is specific for SERT, or if other transporters can be affected or regulated by this treatment. Thus far, it has been reported by uptake experiments that biogenic amine transporters are not equivalently sensitive to p38 MAPK activation (Zhu et al., 2005). In contrast to hNET, which showed a comparable effect by anisomycin treatment, hDAT only displayed a small SB203580-sensitive inhibition of anisomycin stimulated uptake (Zhu et al., 2005).

For this comparison, the focus was placed upon DAT. Immunoblot analysis of anisomycin stimulated DAT cell samples showed nearly no effect compared to SERT. Quantification of the immunoblots displayed that the ER retention form of SERT (control) is clearly reduced due to the treatment (80,8 %), in contrast to DAT, which showed a reduction of 5,6 % of the control ER retention form after analysis of five independent experiments (Fig.18).

Consequently, these results corroborate the suggestion that anisomycin only affects SERT and mediates by p38 MAPK activation a decrease of ER retention form of SERT associated with an enhanced trafficking of intracellular SERT to the surface.

At the moment, there is no specific evidence concerning how activated p38 MAPK influences SERT trafficking to the plasma membrane. SERT relies on SEC24C to exit the ER. The component of COPII exists in different isoforms, each of which binds to specific cargo molecules (Sucic et al., 2013). In contrast to SERT, its close relatives DAT (Sucic et al, 2011) and GAT1 (Farhan et al., 2004) are dependent upon SEC24D recruitment to exit the ER. Only GAT4 seems to rely on SEC24C like SERT (Sucic et al., 2013). These findings provide an argument for the collected results that only SERT is affected by anisomycin stimulation of p38 MAPK due to a possible association with SEC24C.

A study described that p38 MAPK influences COPII recruitment in cytosols from nocodazole treated HeLa cells by blocking the assembling (Wang and Lucocq, 2007). However, given that this observation does not refer to neurons or neuroamine transporters, it is

not necessarily the case that activated p38 MAPK also inhibit the assembling of COPII in connection with monamine tranporter trafficking.

Due to the fact that several components of COPII are known to be phosphoproteins, it is possible that p38 MAPK impacts SEC24C recruitment or phosphorylates SERT directly, resulting in a change of conformation that enables a rapid recognition of its ER-export signal by SEC24C. To confirm that anisomycin-mediated trafficking of SERT is SEC24C dependent, it is helpful to examine GAT4 for ansiomycin-mediated trafficking. In the case of an observed effect by treatment, SEC24C should play an important role in anisomycin-mediated surface trafficking; otherwise, another unknown component affects this redistribution of SERT.

Apart from the ER-export motif, the intracellular termini of monoamine transporters are generally very important for the regulation of function, trafficking or phosphorylation state. Regarding the N-terminus of transporters, a functional importance of the N-terminus of SERT in mediating action of amphetamine by threonine 81 could be shown (Sucic et al., 2010). In addition, the N-terminus of monoamine transporter seems to be involved in uptake and efflux mechanism based upon mutagenesis studies (Guptaroy et al., 2011) and a phosphorylation of threonine 53 in DAT influencing the regulation of DAT functions (Foster et al., 2012).

An in vivo phosphorylation assay (performed by Jae-Won Yang) indicated S52 and S62 as phosphorylation sites on SERT induced by anisomycin treatment and okadaic acid, which inhibits PP2A. However, I could not detect these phosphorylation sites by means of in vitro phosphorylation with recombinant p38 MAPK. This is possibly caused by the used construct, which was tagged with GST at the N-term of N-terminal SERT. Due to the fact that GST is larger than N-SERT, the tag might wreak a conformational change in the N-terminus of SERT, which prevents a modification by the kinase. Using a construct with GST or His tag at the C-terminus of N-SERT could be a possibility to avoid the rearrangement to a C-terminal protein.

Dephosphomimetic and phosphomimetic mutants were used to examine the possible role of both phosphorylation sites in regulating SERT surface expression or ER retention form due to anisomycin treatment. Immunoblotted samples indicated no impact in cellular distribution of SERT based upon the fact that dephosomimietc mutants of S52 and S62 as well as the double mutant still showed different cellular localization of SERT due to anisomycin. In addition, phosphomimetic S52 mutant showed no effect by anisomycin treatment.

These findings lead to the conclusion that S52 and S62 in the N-terminus of SERT are candidates for p38 MAPK-mediated phosphorylation, although these modifications have no implication in the ER retention of SERT. It is presumed that both phosphorylation sites

are relevant for the functional regulation of SERT. As already mentioned, the activation of p38 MAPK also mediates increased SERT activity by basal phosphorylation and PP2A recruitment (Liu and Hofmann, 2004). Interestingly, both identified phosphorylation sites were also found by stimulation with okadaic acid.

PP2A is known to interact with SERT, while its inhibitors, such as okadaic acid, promote SERT phosphorylation and a decrease of SERT activity as well as surface density (Zhu et al., 2005; Ramamoorthy et al., 1998; Qian et al., 1997). Accordingly, it is possible that both sites serve for phosphorylation sites of different kinases or phosphatases with opposing effects in SERT function.

To corroborate the assumption, the impact of okadaic acid as well as H_20_2, another p38 MAPK activator known to elevate 5-HT transport (Zhu et al., 2005), on SERT ER retention form was investigated. The immunoblot showed no clear differences in SERT expression in comparison to untreated wildtype SERT (Fig.21). Based upon this observation, it can be excluded that H_20_2 has an influence on p38 MAPK-mediated decrease of ER-resident, SERT despite its function of activating this kinase. In addition, given that okadaic acid treatment did not show an impact on localization expression of SERT, previous studies (Zhu et. al., 2005; Qian et al., 1997) could be confirmed. It is assumed that okadaic acid triggers SERT phosphorylation, i.e. p52 and p62 and decreased transport activity of SERT may be due to the disruption of the direct or indirect interaction between PP2A and the monoamine transporter. Furthermore, it was detected that PP2A or its inhibitors seem to have no influence in ER retention of SERT, based upon the western blot results.

In contrast to PKC induced internalization by β-PMA, whose activation also seems to influence a lower cell surface delivery or enhanced ER retention of SERT, okadaic acid only affects functional regulation and internalization of SERT.

Consequently, it is suggested that the N-terminus of SERT does not contribute to the trafficking of SERT to the cell membrane, but is possibly relevant for PP2A, PKC or p38 MAPK-mediated de-/phosphorylation of SERT resulting in changes of protein interactions or transporter conformation correlated to SERT activity.

Independent of binding regions for associated proteins and phosphorylation sites, studies have shown that a deletion of the C-terminus of SERT results in a reduction of its surface activity, which is possibly caused by a decrease of transporter density on the membrane (Nobukuni et al., 2009).

To clarify the role of the C-terminus in regulation and trafficking of SERT, in vitro phosphorylation was carried out to define specific phosphorylation sites for p38 MAPK and PKC. A GST fusion protein containing the C-terminus of SERT was generated and in vitro phosphorylation with p38 MAPK and PKC in accordance to 2.2.11 was performed. Via mass spectrometry analysis two phosphorylation sites in the C-terminus of SERT were

identified. Phosphorylated threonine were identified at residue 616 (p38 MAPK), which was previously identified in vivo and 603 (PKC). To investigate whether these sites are relevant for SERT regulation, site-directed mutagenesis were performed to create phosphomimic and dephosphomimic mutants of SERT, followed by treatment with anisomycin.
Wildtype SERT as well as T616D mutant showed a reduction of the retention form caused by anisomycin treatment in contrast to T616A. Quantification of several independent immunoblot experiments showed a significant tendency of a less decreased ER retention form for non-phosphorylatable mutant. These observations corroborate the assumption that p38 MAPK activation by anisomycin phosphorylates SERT at T616, leading to an increase of surface expression, i.e. a decrease of ER retention form. It provides an argument for a correlation between serotonin transporter phosphorylation and the regulation of SERT localization and also it is congruent to further publications about the C-terminus involvement in trafficking (Lau and Schloss, 2012; El-Kasaby et al., 2010).
Nonetheless, the findings about T616 are not consistent with previous functional results of uptake studies showing that T616 seems to play no role for p38 MAPK-dependent regulation of SERT activity (Soerensen et al., 2014). Uptake studies with hSERT in COS7 cells co expressed with p38 MAPK or treated with anisomycin showed no change compared to the control. A functional study of T616 mutants in the absence of an activated p38 MAPK showed a decreased maximal transport capacity, caused by a reduction of SERT surface expression that could not be confirmed by surface biotinylation (Soerensen et al., 2014).

Therefore, it is possible that the regulation of SERT activity or distribution is independent upon direct phosphorylation of T616 by p38 MAPK or that the different results arised from the diverse cells. The used CAD cells represent neuronal characteristics in contrast to COS7 cells in which cellular factors and protein interaction partners are absent (Soerensen et al., 2014).
Identified T603 phosphorylation site specific for PKC was also examined with generated mutants, followed by treatment wih β-PMA. The immunoblots showed no clear effect of increase ER retention form as a result of the treatment, and thus it was decided to focus on p38 MAPK-mediated phosphorylation of SERT (data not shown).

To summarize, one can state that the results provide an argument for the important role of the C-terminus in SERT trafficking, which requires phosphorylation of specific amino residue such as T616 to be successful. However, to ensure that phosphorylation of T616 by p38 MAPK influences the delivery of the transporter to the membrane, further investigations are necessary that advocate the assumption.

5 References

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6 Appendix

6.1 Abstract (German)

Posttranslationale Modifikationen von Proteinen spielen eine wichtige Rolle in verschiedenen molekularen Prozessen. Aufgrund der Existenz vieler Kinasen und Phosphatasen in Eukaryonten scheint die Phosphorylierung eine besondere Bedeutung für die Koordinierung des zellulären Netzwerkes zu haben.
Obwohl die genauen molekularen Abläufe noch unerforscht sind, wird die Aktivität, die Interaktion mit anderen Proteinen sowie die zelluläre Verteilung des Serotonintransporters (SERT) durch Phosphorylierung reguliert.

Der Serotonintransporter enthält Konsensusmotive für Kinasen wie die Proteinkinase C (PKC) und die p38 MAPK. Beide Kinasen sind bereits in Verbindung gebracht worden, die Aktivität und Oberflächenexpression des Transporters zu beeinflussen. Dennoch sind spezifische Phosphorylierungsstellen, die mit der Regulierung der Aktivität und des Transports verbunden sind, noch nicht genau beschrieben worden.

Aufgrund dessen war das Ziel, den Einfluss der Kinasenaktivierung auf die zelluläre Umverteilung von SERT zu untersuchen sowie spezifische Phosphorylierungsstellen zu identifizieren, die für die SERT-Funktion und -Oberflächenexpression bzw. ER-Retention wichtig sind.

Immunoblot-Analysen zeigten, dass die unglykosylierte Form von SERT als Folge der Behandlung mit dem p38 MAPK-Aktivator Anisomcyin in YFP-SERT stabil exprimierenden HEK293-Zellen reduziert wurde. Im Gegensatz dazu führte der Einsatz von β-PMA (PKC-Aktivator) zu einer erhöhten Expression der Retentions-Form.

Da Anisomycin keinen Effekt in Dopamintransporter exprimierenden Zellen zeigte, wird angekommen, dass die Anisomycin-vermittelte zelluläre Umverteilung spezifisch für SERT ist.

Desweiteren wurden p38 MAPK und PKC spezifische Phosphorylierungsstellen im C-Terminus von SERT via In vitro Phosphorylierung und Massenspektrometrie identifiziert. Obwohl durchgeführte In vivo Phosphorylierungsexperimente S52 und S62 als Anisomycin oder Okadasäure (Phosphatase 1/2 A Inhibitor) induzierte Phosphorylierungsstellen identifizierten, zeigten generierte phosphorylierungsresistente und phosphorylierte Mutanten der beiden Stellen das gleiche Anisomycin-vermittelte Expressionsmuster wie im SERT-Wildtyp.
Dagegen zeigte die phosphorylierungsresistente Mutante der identifizierten p38 MAPK-spezifischen Phosphorylierungsstelle Thr616 eine signifikante Expressionsreduzierung der unglykosylierten SERT Form durch Anisomycin.

Zusammenfassend lässt sich sagen, dass nur die Phosphorylierung des C-Terminus des Serotonintransporters Einfluss auf die zelluläre Umverteilung von SERT hat.

6.2 Curriculum Vitae

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