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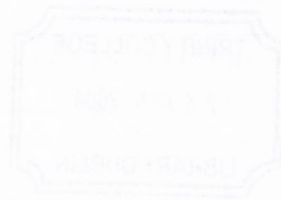
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Trafficking and Regulation of the Serotonin Transporter



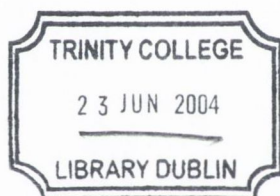
**A dissertation submitted to Trinity College Dublin for the degree of Doctor
of Philosophy**

by

Francesca Magnani

Department of Biochemistry Trinity College Dublin

March 2004

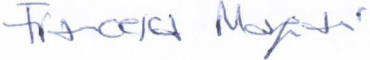


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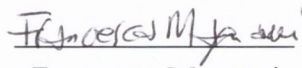
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Francesca Magnani

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Summary

The serotonin transporter (SERT) is responsible for the clearance of serotonin from the synaptic cleft. Together with the norepinephrine transporter and dopamine transporter, SERT is of great interest because it is the molecular target for many antidepressants, and substances of abuse, such as cocaine and MDMA (Ecstasy). Although the involvement of SERT in serotonin clearance has been clearly established for many years, our understanding of how the serotonin transporter is regulated *in vivo* is still fragmentary. The aim of my PhD project was to investigate the association of SERT with proteins and/or lipids that may regulate the trafficking and function of the serotonin transporter.

Three different cell lines were analyzed for my project, in order to find a heterologous expression system capable of producing high levels of SERT, functionally active in serotonin uptake and effectively localized on the plasma membrane. Protein expression levels were analyzed by Western blotting, transporter function by serotonin uptake assay, and cell surface localization both by confocal microscopy and cell surface biotinylation. The tetracycline-inducible TReX-SERT cell line yielded the highest levels of active SERT, and was therefore used for further experiments.

The SNARE protein syntaxin 1A (syn 1A) is implicated in the docking of synaptic vesicles with the plasma membrane during exocytosis of the neurotransmitter and more recently has been shown to interact with and regulate the SERT-related GABA and glycine transporters, and a number of ion channels. A preliminary study carried out in our laboratory using the GST-pulldown assay showed that SERT appears to form a complex with syn 1A *in vitro*. However, SERT-syn 1A interaction could not be shown *in vivo* by co-immunoprecipitation. I examined the functional consequences of the proposed interaction between SERT and syn 1A by co-expressing the two proteins in HEK cells. The co-expression of SERT and syn 1A in HEK cells resulted in a decrease of maximum velocity of serotonin transport, due to a decrease of SERT molecules at the cell surface. By means of confocal microscopy and cell surface biotinylation, I showed that the heterologous expression of syn 1A in HEK cells affected membrane protein trafficking to the cell surface. Such an effect was probably caused by the absence in HEK cells of some syn 1A-binding protein, necessary for the trafficking of the SNARE protein to the cell surface.

Membrane cholesterol had been shown to be essential for the binding of ligands to SERT. Because cholesterol is one of the major components of lipid rafts, during my PhD I have investigated whether SERT associates with lipid rafts. Rafts or lipid microdomains are discrete domains in bilayer membranes, enriched in cholesterol and sphingolipids, and play an important role in membrane protein function. I have shown that SERT requires the association with lipid rafts in order

to catalyse the transport of serotonin: the loss of interaction with lipid rafts is detrimental to SERT function, possibly due to conformational changes that mainly affect the translocation of substrate across the plasma membrane.

I have shown evidence that lipid rafts are also involved in the intracellular trafficking of SERT. Moreover, in rat brain SERT and syn 1A were both found associated with lipid rafts, whereas Munc 18-1, which inhibits the interaction of syn 1A with other interacting proteins, did not associate with rafts. These data indicate that in brain at least a fraction of SERT and syn 1A is spatially segregated from Munc 18-1. This may have important functional implications in the regulation of serotonergic neurotransmission, by allowing the efficient coupling of serotonin release with the reuptake of the neurotransmitter by SERT. Taken together, these findings suggest the existence of a novel mechanism of SERT regulation, based on lipid-protein interaction within plasma membrane lipid rafts.

N-glycosylation can promote protein association with lipid rafts. In order to investigate whether the N-glycans of SERT were involved in the association with rafts, I generated by site-directed mutagenesis three SERT mutants, bearing only one or none of the two N-glycosylation consensus site(s). Although reduced, all the transporter glycoforms produced by the glycosylation-deficient mutants were associated with lipid rafts and were able to transport serotonin. Thus, it appears that SERT association with rafts is not mediated by its glycan moiety.

Abbreviations

A	absorbance
BDNF	brain-derived neurotrophic factor
BET	betaine transporter
bFGF	basic fibroblast growth factor
BHK	baby hamster kidney
BSA	bovine serum albumin
CAS	confocal assistant
CFTR	cystic fibrosis transmembrane conductance regulator
CHO	chinese hamster ovary
CMC	critical micellar concentration
CNS	central nervous system
COP	coat protein
ConA	concanavalin A
CTxB	Cholera toxin B-subunit
DA	dopamine
DAT	dopamine transporter
DMEM	Dulbecco's modified Eagle's medium
DMJ	deoxymannojirimycin
DMSO	dimethylsulphoxide
dpm	disintegrations per minute
DTT	dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	ethylenediaminetetracetic acid
EGF	epidermal growth factor
EM	electron microscopy
EndoH	endoglycosidase-H
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FCS	foetal calf serum
FRET	fluorescence resonance energy transfer

GABA	γ -amino butyric acid
GAT	GABA transporter
GFP	green fluorescent protein
GLYT	glycine transporter
GPI	glycosylphosphatidyl-inositol
GST	glutathione S-transferase
HEK	human embryonic kidney
HEPES	N-(2-hydroxyethyl) piperazine-N'-2-ethanesulphonic acid
HLB	hydrophilic-lipophilic balance
HRP	horseradish peroxidase
5HT	5-hydroxytryptamine
IL	interleukin
LB	Luria-Bertani medium
LDL	low density lipoproteins
l_o	liquid-ordered phase
LSCM	laser scanning confocal microscopy
MAO	monoamine oxidase
M β C	methyl- β -cyclodextrin
MDCK	Madin-Darby canine kidney
MDMA	(3,4-methylenedioxy)-methamphetamine
MEM	minimal essential medium
MTT	3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide
NE	norepinephrine
NET	norepinephrine transporter
NO	nitric oxide
OD	optical density
P	pellet
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PK	protein kinase

PMA	phorbol 12-myristate 13-acetate
PMT	photomultiplier
PNGaseF	peptide N-glycosidase F
PP _i	protein phosphatase
PVDF	polyvinyl-difluoride
RBL	rat basophilic leukemia
RT	room temperature
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SERT	serotonin transporter
Sf9	<i>Spodoptera frugiperda</i> 9
SM	Sec1/Munc18-like proteins
SNAP 25	synaptosome associated protein of 25 kDa
v/t-SNARE	vesicles/target membranes-soluble N-maleimide sensitive factor attachment protein receptor
SSRI	selective serotonin reuptake inhibitor
SV	synaptic vesicles
Syn	syntaxin
TAT	taurine transporter
TBE	tris-boric acid-EDTA buffer
TBS	tris-buffered saline
TE	tris-EDTA buffer
TEMED	N, N, N', N'-tetramethylethylenediamine
TGN	<i>trans</i> -Golgi network
TLR	Toll-like receptor
T _m	melting temperature
TMD	transmembrane domain
TNF	tumor necrosis factor
TWB	tris-buffered saline/Tween 20
VMAT	vesicular monoamine transporter
UI	uninduced
UPR	unfolded protein response
WGA	wheat germ agglutinin

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Chapter 1

General introduction

1.1 Synaptic transmission

Synaptic transmission refers to the propagation of nerve impulses from one neuron to another. This occurs at a specialized cellular structure, first observed with light microscopy by Ramón y Cajal in 1879 (Ramon y Cajal, 1937) and later called the “synapse” by Sherrington (Sherrington, 1897). Synapses are interneuronal contacts, which can be electrical or chemical. Electrical synapses function by the propagation of electrical impulses and do not require an elaborate structural organisation of the plasma membrane and cytoplasmic organelles. These synapses, which are rare in mammals but common in lower vertebrates, are formed by the close apposition of the cell membranes at a gap junction, which maintain cytoplasmic continuity between the two neurons and provide a low-resistance pathway to the electric impulse.

At chemical synapses, nerve impulses are transmitted by the release of molecules called ‘neurotransmitters’. As a nerve impulse, or action potential, reaches the end of a presynaptic axon, molecules of neurotransmitter are released into the synaptic cleft, a gap of 20-40 nm. Neurotransmitters are a diverse group of chemical compounds ranging from simple amines, such as 5-hydroxytryptamine (5-HT), also known as ‘serotonin’, and amino-acids such as γ -aminobutyrate (Fig. 1.1), to polypeptides such as the enkephalins. Both classes of neurotransmitters are contained in vesicles: generally, small molecule transmitters are packaged in vesicles of 40-50 nm diameter, whereas neuropeptides are stored in large dense-core vesicles of 100-120 nm in diameter. However, cholinergic and serotonergic neurons contain dense-core vesicles where acetylcholine or serotonin respectively is stored together with a neuropeptide (Schwartz, 2000). The mechanisms by which neurotransmitters elicit responses in both presynaptic and postsynaptic neurons through receptors are extremely diverse, including both metabotropic and ionotropic receptors (Pauwels, 2000).

1.2 Serotonin (5HT)

5HT was isolated, identified and crystallized from platelet extracts in 1948 (Rapport et al., 1948a, b). This molecule was called 'serotonin' because it was isolated from serum and had a tonic effect on arteries. 5HT was later recognized as a neurotransmitter, when Twarog and Page showed that it is present in brain (Twarog and Page, 1953). Subsequently, it was shown that the blood pressure response is strongly influenced by the activity of the sympathetic nervous system. Such a cardiovascular function was attributed to serotonin and it was proposed that 5HT could have a role as a chemical mediator also in the autonomic nervous system (Brodie and Shore, 1957). Today, it is generally accepted that 5HT is important in several psychiatric disorders. Few biological agents have proven to have so many varied actions as serotonin, from transmission of nerve impulses to the formation of some intestinal tumors (Ahlman, 1985).

Neural 5-HT is produced in groups of neurons of midbrain and medulla oblongata, termed 'raphe nuclei', whose projections are found in the whole nervous system. This system was histologically described at the beginning of last century (Ramon y Cajal, 1995) and five decades later the anatomical architecture of the serotonergic projections was revealed (Dahlstrom and Fuxe, 1964).

As a neurotransmitter the ubiquity of 5HT in the central nervous system (CNS) is not only anatomical, but also phylogenetic: from primates to Chondroichtyes, the adult 5HT system is organized into two subsystems, a rostral division with cell bodies localized in the midbrain and rostral pons, providing five projections to the forebrain, and a caudal division, primarily located in the medulla oblongata with three descending projections to spinal cord and brainstem nuclei (Fig. 1.2; (Azmitia and Segal, 1978; Azmitia and Gannon, 1986; Tork, 1990)). In spite of this evolutionary stability, there are phylogenetic differences that can be summarized as follows: in higher mammals serotonergic neurons give rise to few collaterals and have a high proportion of myelinated axons; in lower

Fig. 1.1
Structures of several neurotransmitters.

Except for acetylcholine, all of these are amino acids (glycine and glutamate) or derived from the indicated amino acids. The three neurotransmitters synthesized from tyrosine, which contain the catechol moiety (blue highlight), are referred to as catecholamines. Serotonin is framed in yellow.

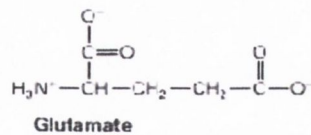
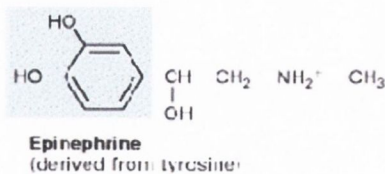
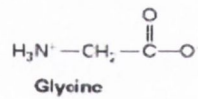
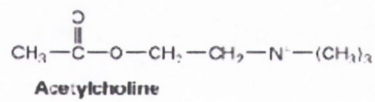
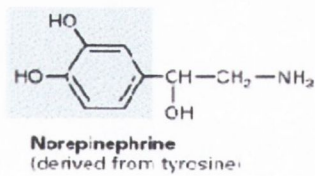
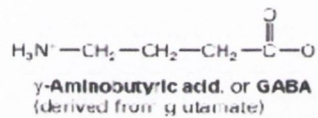
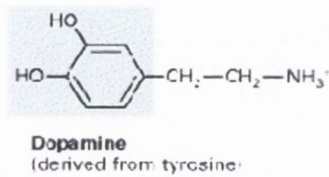
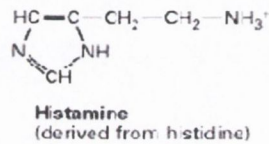
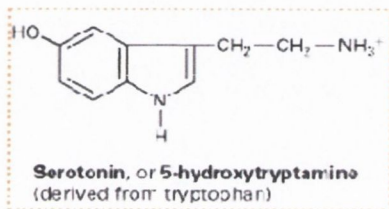
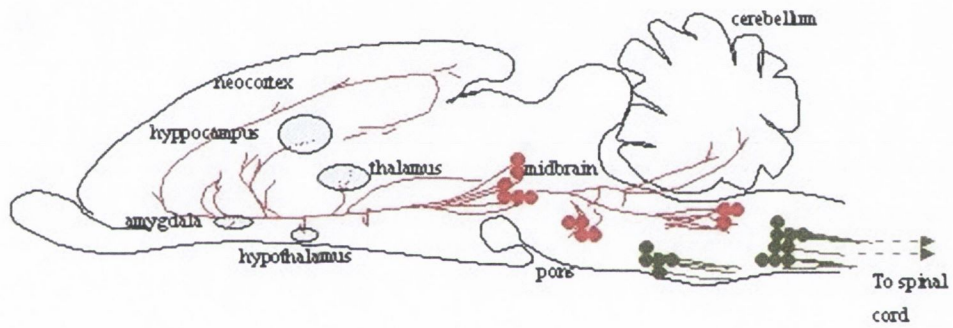


Fig. 1.2

Schematic representation of the serotonergic system in rat brain

Serotonergic neurons are mainly located in the raphe nuclei, along the midline of the brain stem. The caudal groups (B1-3, in green) send descending projections to the spinal cord. The groups B4-9 send ascending projections to the whole forebrain. (Modified from (Saper, 2000))



mammals the system is diffuse, highly branched and unmyelinated (Tork, 1990; Jacobs and Azmitia, 1992).

The serotonergic system is an early forming component of the neural circuitry. In rat embryos the serotonin transporter (SERT)-mRNA is detected in neural crest cells, and then throughout the sensory pathway from the CNS to sensory nerves and their target organs, suggesting that 5HT may be a regulator of peripheral synaptogenesis (Hansson et al., 1999). The formation and integration of neural networks have been shown to depend on the action of serotonin acting through multiple 5HT-receptor subtypes and through the release of the astroglial protein S-100 β (Nishi et al., 1997). Moreover, depletion of 5HT during synaptogenesis has been shown to decrease synaptic density and elicits learning deficits in the adult (Mazer et al., 1997).

1.3 Synthesis of 5HT

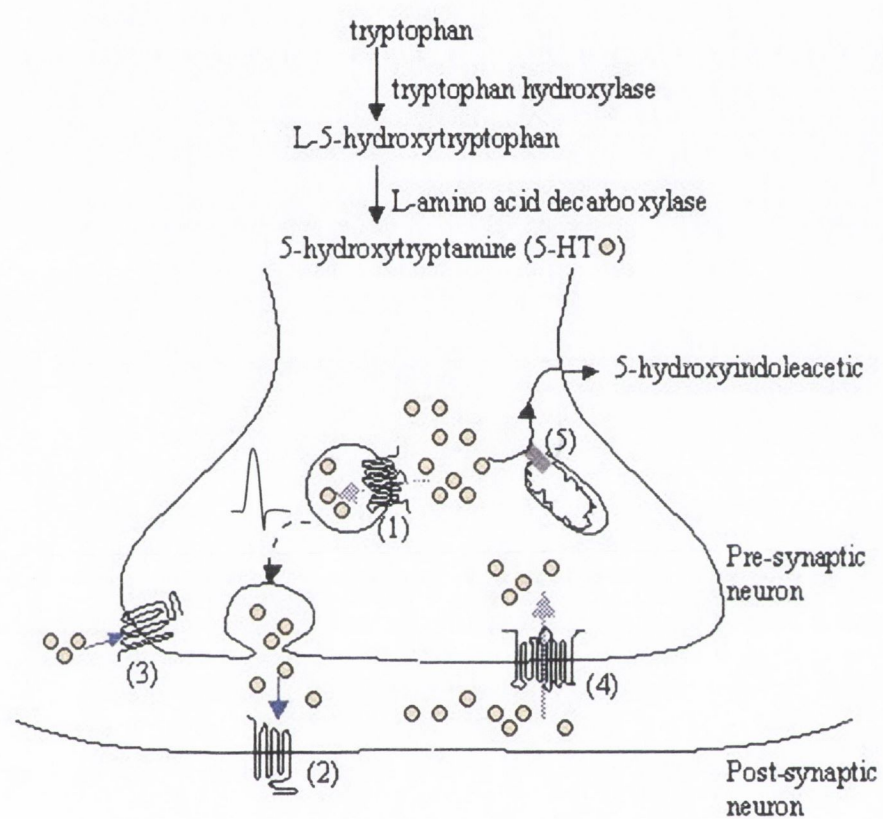
5HT is not exclusive to brain, and the bulk of 5-HT is found in the blood stream. Despite this fact, in brain 5HT must be produced *in loco* because it cannot permeate the blood-brain barrier. 5HT is synthesized from the amino acid tryptophan in two steps catalysed by the enzymes tryptophan hydroxylase and aromatic L-amino acid decarboxylase (Fig. 1.3). Tryptophan availability is the rate-limiting factor: bound to albumin in the serum, tryptophan reaches neurons crossing the blood-brain barrier via active transport. Insulin facilitates the transport of tryptophan essentially by increasing the affinity of the transport system for tryptophan (Tagliamonte et al., 1976; Cangiano et al., 1983).

As outlined in Fig. 1.3, once synthesized, cerebral 5HT is stored in vesicular pools by the vesicular monoamine transporter (VMAT) and can be released when the neuron fires. 5HT is then taken back into the serotonergic neuron cytoplasm (re-uptake) by the serotonin transporter. Here 5HT can be restored into vesicles or be degraded by monoamine oxidases (MAO), enzymes found in a wide variety of neurons such as dopaminergic neurons, and also in

Fig. 1.3

5HT synthesis and synaptic transmission

5HT is synthesised from the amino acid tryptophan. Two enzymes are needed for this reaction: tryptophan hydroxylase, the rate-limiting step, and L-amino acid decarboxylase. Once produced, 5-HT is stored into synaptic vesicles by vesicular transporters (1). An action potential arriving at the terminal of a presynaptic axon elicits the aperture of Ca^{++} -channels resulting in Ca^{++} -influx, which in turn causes the exocytosis of the synaptic vesicles. The released 5-HT diffuses in the synaptic cleft and bind to 5-HT receptors on the postsynaptic membrane (2). The further release of neurotransmitter is regulated by autoreceptors on the terminal from which the 5-HT is released (3). 5-HT is removed from the synaptic cleft by diffusion (non-specifically) and by the serotonin transporter (SERT) on the membrane of nerve terminals (4) and glial cells (not indicated). After re-uptake, 5-HT can be stored again in vesicles or degraded by monoamine oxidases (MAO) located on the outer mitochondrial membrane. MAO converts 5HT to 5-hydroxyindoleacetaldehyde, which is in turn oxidized by an aldehyde dehydrogenase to form 5-hydroxyindoleacetic acid.



non-neuronal cells (endothelial, astrocytes; (Levitt et al., 1982; Fitzgerald et al., 1990)).

Once serotonin is released into the synapse in response to a depolarising stimulus, it triggers pre- and post-synaptic responses through the activation of 5HT-receptors. Lack of selective agents, alternative splicing, and transcript editing contribute to the difficulty of characterizing and organizing a nomenclature for the 5HT-receptors. However, to date seven distinct families of 5HT-receptors have been identified (5HT₁₋₇). These are all G-protein coupled receptors, except for the class 5HT₃, which is a ligand-gated ion channel. Subclasses have also been described for several of these and to date at least 14 subclasses have been cloned (reviewed in (Pauwels, 2000)).

1.4 SERT

As already mentioned, at a molecular level serotonin neurotransmission, and in general monoamine neurotransmission, is dynamically regulated by a diverse set of macromolecules including biosynthetic enzymes, pre- and post-synaptic receptors, and transporters.

SERT is a member of the Na⁺/Cl⁻-dependent family of neurotransmitter transporters (Worrall and Williams, 1994; Blakely and Bauman, 2000) and is responsible for the clearance of 5HT from the synaptic cleft. The transport of serotonin is energized by a Na⁺/Cl⁻ gradient and will be described in section 1.8. Neurotransmitter reuptake has several important consequences. Firstly, levels of transmitter in the synaptic cleft are reduced faster than can be achieved by simple diffusion, exerting a fine-tuning of neurotransmission. Secondly, transmitter can be recycled for another round of release once it is transported back across the presynaptic membrane and into synaptic vesicles.

Together with the NE transporter (NET) and DA transporter (DAT), SERT is of great interest because it is the molecular target for many antidepressants, and substances of abuse (see section 1.9). Although the involvement of the transporter in 5HT clearance had been known for several

decades, progress in understanding SERT structure and regulation has been limited, largely due to difficulties associated with its purification. Recently, by means of expression and homology-based cloning (Blakely et al., 1991; Hoffman et al., 1991; Ramamoorthy et al., 1993), SERT has been revealed to be a member of a large gene family comprised of carriers for other neurotransmitters, osmolytes, and nutrients.

1.4.1 Cloning of SERT

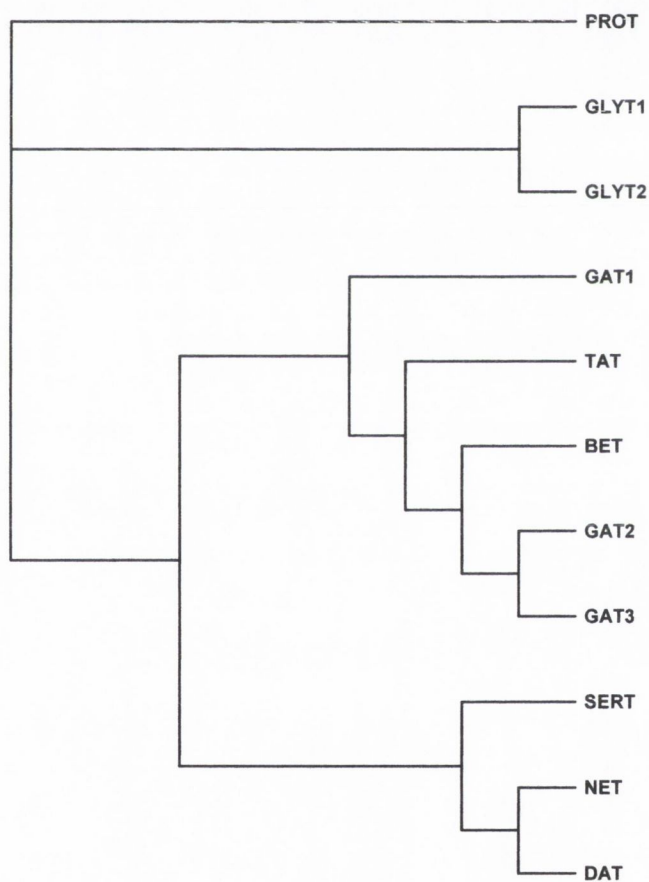
SERT was first cloned from rat brain (Blakely et al., 1991) and a rat basophilic leukaemia cell line by homology screening (Hoffman et al., 1991). These rat SERT cDNAs encode a GABA transporter (GAT) and NET homologue of 630 amino acids (Ramamoorthy et al., 1993) with a predicted size of ~68 kD and 50% identity to human NET (Fig. 1.4). SERT has been cloned from many organisms, i.e. *H. sapiens*, that shares 92% sequence identity with the rat SERT (Ramamoorthy et al., 1993), *D. melanogaster* (Corey et al., 1994; Demchyshyn et al., 1994), *Mus musculus* (Chang et al., 1996), *Ovis aries* (Padbury et al., 1997) *Bos taurus* (Mortensen et al., 1999a), *Macaca mulatta* (Miller et al., 2001), and *C. elegans* SERT (Ranganathan et al., 2001). SERT sequences fall into the same family of NET, DAT, and a large number of related Na⁺-dependent transporters, including among others carriers for glycine, taurine, proline, creatine, and betaine transporters (Fig. 1.4). The L-glutamate transporters belong to a distinct gene family unrelated to the SERT subgroup and do not exhibit a requirement for extracellular Cl⁻.

1.4.2 Structure of SERT

SERT, NET, and DAT are most closely related, defining a subgroup characterized by similarity in sequence and pharmacological properties. At the primary amino-acid level approximately 20% of SERT and NET residues are

Fig. 1.4
Amino acid sequence relationship between some members of the Na⁺ (and Na⁺/Cl⁻) dependent transporter gene family

On the basis of amino acid identities it is possible to distinguish distinct subfamilies: the subfamily of biogenic amine (SERT, NET, and DAT); the subfamily of GABA (GAT), betaine (BET), and taurine (TAT) transporters; and the subfamily of glycine (GLYT) transporters. The phylogenetic tree was constructed using default parameters of the program ClustalX.



absolutely conserved, with particularly high conservation evident in transmembrane domains (TMDs) 1–2 and 5–8 (Fig. 1.5; (Blakely et al., 1991)). It is likely that these conserved sites are involved in common properties such as Na⁺ and Cl⁻ binding and/or in defining the structure required for substrate translocation across the plasma membrane (Barker et al., 1994; Barker and Blakely, 1996; Barker et al., 1998; Adkins et al., 2001). One feature that separates the SERT subgroup from the other family members is high-affinity recognition of cocaine and amphetamines, and, for SERT and NET, of tricyclic antidepressants. Sequence alignment shows that N- and C-termini are poorly conserved and thus, potentially involved in the regulation of each carrier. Indeed, chimera studies in which these domains are swapped between SERT and NET reveal no alteration in substrate or antagonist selectivity (Blakely et al., 1993), suggesting that N- and C-termini are not involved in ligand recognition. A similar conclusion can be reached for the GABA transporters: removal by mutagenesis of both GAT termini fails to alter transport properties (Bendahan and Kanner, 1993). It has been shown that SERT-external loops are responsible for maintaining the conformation of the transporter (Tate and Blakely, 1994; Stephan et al., 1997; Chen et al., 1998; Smicun et al., 1999).

The cytoplasmic N- and C-termini of SERT contain five potential PKC-phosphorylation sites (Blakely et al., 1991; Hoffman et al., 1991; Ramamoorthy et al., 1993), a large extracellular loop between TMD-3 and -4 with multiple N-linked glycosylation sites (Tate and Blakely, 1994), suggesting an extracellular localization of this domain. Indeed, this prediction has been confirmed by site-directed chemical labelling (Chen et al., 1998).

Hydropathy profiling of SERT shows that it contains probably twelve transmembrane domains (TMDs), a topology proposed for other cloned Na⁺-dependent transporters (Fig. 1.6). Due to the lack of recognizable signal sequences, the N-terminus is predicted to be cytoplasmic and the even number (12) of putative TMDs places the C-terminus in the cytoplasm as well (Lesch et al., 1993a). However, the actual topology of the neurotransmitter transporters is still uncertain. For the glycine (Olivares et al., 1997) and GABA transporters (Bennett and Kanner, 1997) there is evidence that the topology deviates from the

Fig. 1.5

Sequence alignment of rat monoamine transporters NET, DAT, and SERT

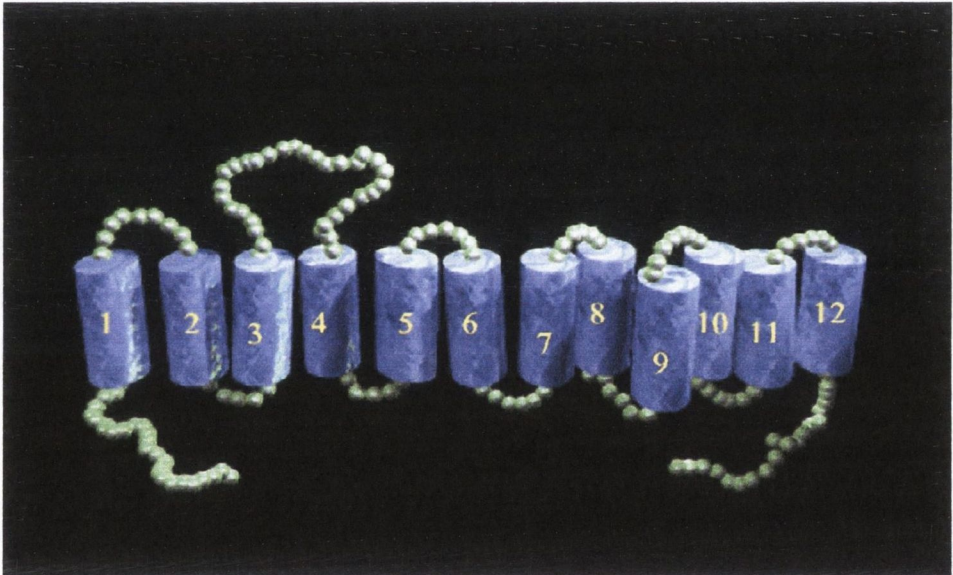
Asterisks mark amino acids identical in all transporters; the blue boxes indicate putative transmembrane domains. Gaps were introduced to optimise identical sequence position. Multiple sequence alignment was obtained using default parameters of ClustalW. SWISS-PROT accession numbers are Q63380, P23977, and P31652, respectively.

[illegible]

Fig. 1.6

Structural model of Na/Cl dependent neurotransmitter transporters

The twelve proposed membrane spanning domains are shown as cylinders representing α -helices. Extracellular loops are shown at the top. N-terminal and C-terminal sequences and intracellular loops are at the bottom. Figure reproduced from <http://www.emory.edu/CHEMISTRY/justice/research/>.



theoretical model. In fact, the putative first TMD may not span the membrane completely and it has been proposed that the TMD1 may undergo conformational changes, consequently switching position, in and out the plasma membrane. For these same transporters the loop connecting TMD-1 and -2 is proposed to be intracellular, whereas the loop between TMD-2 and -3 is glycosylated *in vivo* and thus it is probably extracellular (Bennett and Kanner, 1997; Olivares et al., 1997; Lopez-Corcuera et al., 2001).

The use of cloning as an approach for studying SERT has defined the functional transporter as a single protein subunit, competent for transport activity as well as drug recognition. However, Kilic and Rudnick have shown by co-immunoprecipitation that SERT exists at least as a dimer (Kilic and Rudnick, 2000). Other data on putative native stoichiometries are becoming available, thanks to the development of non-invasive techniques. By means of green fluorescent protein (GFP) derivatives and fluorescence resonance energy transfer (FRET), hSERT has been shown to exist as an oligomer in the membrane of living cells and can also heteromerize with the rat GABA transporter 1 (Schmid et al., 2001). More recently, the N-glycans of SERT have been shown to be required for the homo- and hetero-oligomerization of the transporter (Ozaslan et al., 2003).

1.4.3 Histological distribution of SERT

The cloning of SERT cDNAs has provided means to define the tissue and cellular distribution of transporter gene expression. Analysis of SERT distribution by Northern blot hybridisation of rat and mouse RNAs shows a single SERT mRNA species present in brainstem, midbrain, lung, spleen, gut, and adrenal gland (Blakely et al., 1991; Hoffman et al., 1991; Chang et al., 1996). *In situ* localization of SERT mRNA in rodent and human brain shows prominent expression in the serotonergic neurons of the median and dorsal raphe nuclei (Blakely et al., 1991; Austin et al., 1994). However, Bengel and colleagues have shown that the highest concentration of SERT binding sites were found outside

the raphe complex, particularly in the substantia nigra, globus pallidus, and superior colliculi (Bengel et al., 1997b). Interestingly, in the cerebral areas reached by the serotonergic ascending pathways, the concentration of SERT mRNA was shown to be affected by chronic treatment with antidepressants (Lesch et al., 1993b). This finding suggests the existence of a connection between the regulation of SERT at the level of gene activation, or transcript stability, and the therapeutic action of antidepressants that usually occurs only after 2-3 weeks of treatment (see section 1.4.5).

In situ hybridization studies are consistent with a single gene encoding the hSERT, localized to chromosome 17q11.1-17q12 (Ramamoorthy et al., 1993). However, unlike rat and mouse, where a single mRNA species occurs, at least three different RNAs are observed in human placenta and lung, beginning at exons 1a, 1b or 2. The polyadenylation signal appears to be highly degenerate in humans, perhaps leading to alternate usage of additional polyadenylation sites (Heils et al., 1995).

At the cytological level, more than 90% of SERT immunoreactivity is found in serotonergic axons and axon terminals (Pickel and Chan, 1999). SERT is also expressed in astrocytes, in glioma-derived cell lines (Kubota et al., 2001), in platelets (Lesch et al., 1993a), pulmonary endothelium (Qian et al., 1995), mast cells (Chen et al., 2001a), and immortalized B lymphocytes (Mossner et al., 2000). The serotonin uptake carrier is the means by which platelets acquire 5-HT, since they do not synthesize it (Blakely et al., 1997). The existence of identical SERTs in brain and peripheral cells justifies the frequent use of human platelet SERT as a diagnostic tool for psychiatric disease. Even though the primary amino-acid sequences of CNS and peripheral SERTs appear identical, the possibility of tissue-specific post-translational modifications cannot be excluded.

1.4.4 Transport of 5HT

According to the proposed model for multisubstrate uptake process named 'alternating-access model', single Na^+ and Cl^- ions, and a protonated 5HT

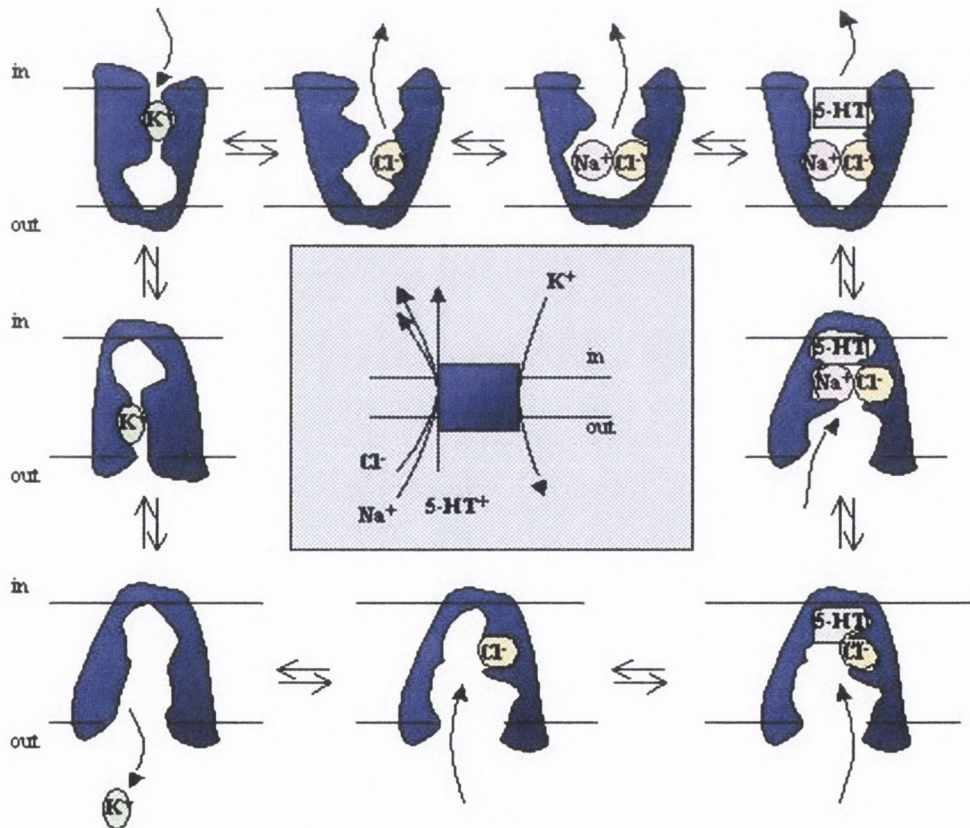
molecule bind to the transporter, forming a quaternary complex, which then undergoes a conformational change to release the neurotransmitter and the ions into the cytoplasm. Subsequently, a single intracellular K^+ ion is countertransported and promotes the reorientation of the unloaded carrier for another transport cycle. Intracellular K^+ accelerates 5HT influx, presumably by supplying energy and thus facilitating a conformational change required for the reorientation of the unoccupied 5HT binding sites (Fig. 1.7; reviewed by (Rudnick and Clark, 1993)). A consequence of neurotransmitter uptake driven by transmembrane ion gradients is that the transporter can work in reverse mode (efflux of 5-HT) if ion gradients are reversed and/or membranes are depolarised (Levi and Raiteri, 1993).

The alternating-access model is currently under scrutiny. In this model the transport of serotonin is electroneutral ($5HT^+ : Na^+ : Cl^- : K^+ = 1:1:1:1$). However, substrate-induced and substrate-independent currents have been empirically measured both for mammalian (Mager et al., 1994) and insect SERTs (Galli et al., 1997). To explain these data it has been postulated that under particular circumstances SERT can function as a 5HT-permeable channel. Once the neurotransmitter has been released in the synaptic cleft, synapses are exposed to high 5HT concentrations. Isolated Retzius cells of the leech *Hirudo medicinalis* produce serotonin, which is stored in synaptic vesicles at a concentration of about 250 μM (Bruns and Jahn, 1995). Synaptic transmitter is released in discrete packages called 'quanta' and it has been estimated that at most synapses of the CNS each action potential releases 1-10 quanta of neurotransmitter (Kandel and Siegelbaum, 2000). Therefore, it is likely that, once the transmitter has been released, the synapse is exposed to 5HT concentration of 1-2 mM, far above the micromolar range of serotonin necessary in a standard assay to saturate SERT. Petersen and DeFelice observed that SERT mediated 5HT transport continued to increase even at 0.3 mM serotonin (Petersen and DeFelice, 1999). Thus, it is possible that channel permeation could be an efficient and rapid mechanism to regulate neurotransmission of 5HT. Nevertheless, the entrance of the SERT into a channel mode was observed to occur with low probability and therefore does not appear to represent an obligatory step in transport (Lin et al., 1996).

Fig. 1.7

Mechanism of 5HT transport: alternating-access model

The central box summarizes the driving forces for 5HT transport: Na^+ and Cl^- are co-transported inward with the cationic form of 5HT, while K^+ is countertransported out from the cytoplasm. The influx of Na^+ and Cl^- and the efflux of K^+ occur downhill their concentration gradients, allowing the accumulation of 5HT. The binding in sequence of Cl^- , 5-HT $^+$ and Na^+ permits a conformational change of SERT (upper right corner). Following the release of 5HT $^+$, Na^+ and Cl^- into the cytosol, the transporter undergoes another conformational change due to the binding of K^+ , thus completing the transport-cycle. The stoichiometry for each cycle of transport is 5HT $^+$: Na^+ : Cl^- : K^+ = 1:1:1:1. Modified from (Rudnick and Clark, 1993).



1.4.5 Pharmacological profile

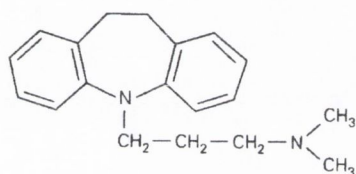
Mood, emotion, and cognition are modulated by the serotonergic raphe system, which appears to be involved in the pathogenesis of psychiatric disorders, like those of the affective spectrum. Indeed, stress, steroids, alcohol and drugs induce 5HT imbalances with consequent alteration of brain architecture that could result in mental disorders (Azmitia and McEwen, 1969; Boadle-Biber et al., 1989; Zhou et al., 1991; Azmitia et al., 1995; Sari et al., 2001). SERT is the pharmacological target for several substances of abuse and a variety of therapeutic antidepressants, and changes in 5HT uptake have been observed in human brain and platelets in depression and suicide (for a review, see (Mann et al., 2001)).

The sensitivity to tricyclic antidepressant is shared by SERT and NET, but not by DAT (Hoffman et al., 1991; Pacholczyk et al., 1991; Bannon et al., 1994). For unknown reasons, tertiary amine tricyclics, such as imipramine, are more potent at SERT as compared to the NET, on which secondary amine tricyclics, such as desipramine, are more effective. Selective serotonin reuptake inhibitors (SSRI), such as paroxetine and fluoxetine, are highly selective antagonists for SERT. Although their chemical structures differ from the one of tricyclic compounds, they are effective antidepressants, supporting the hypothesis that alterations in serotonergic neurons could lead to affective disorders (Fig. 1.8; (Fuller and Wong, 1990)).

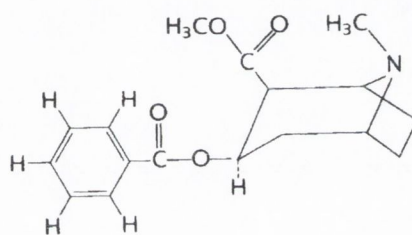
Cocaine is an inhibitor of 5HT, NE, and also DA transport, leading, therefore, to a distinct spectrum of behavioural alterations. In addition to cocaine, other drugs of abuse including p-chloroamphetamine, fenfluramine, and (3,4-methylenedioxy)-methamphetamine (MDMA, also known as 'ecstasy') also are inhibitors (at low doses) of serotonin uptake. Substituted amphetamines can, at higher doses, additionally cause efflux of 5HT through the transporter, working in a reverse mode (Rudnick and Wall, 1992). Cocaine and fluoxetine can inhibit the release of 5HT induced by substituted amphetamine (Berger et al., 1992).

Fig. 1.8
SERT reuptake inhibitors

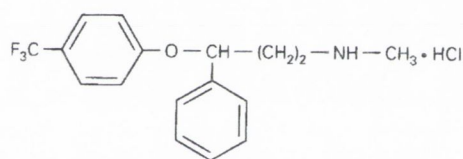
Tricyclic antidepressant drugs, such as imipramine (top), take their name from the three rings molecular structure. They inhibit the uptake of both serotonin and norepinephrine. Fluoxetine (bottom) is a SSRI. These molecules are amongst the most widely used antidepressants, which also include a third class of drugs, the MAO inhibitors (not shown).



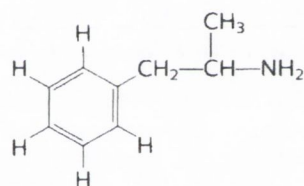
Imipramine



Cocaine



Fluoxetine HCl



Amphetamine

Interestingly, it has been shown that long-term administration of antidepressants that are specific SERT antagonists markedly decreases the levels of serotonin transporter density in rat brain after 21 days of SSRI administration (Benmansour et al., 1999). The long-term effect of SSRI has been shown to cause a much stronger inhibition of 5HT clearance *in vivo* compared to the immediate inhibition of SERT (Benmansour et al., 2002). Such delayed effects may contribute to adaptive mechanisms, not due to SERT gene downregulation, which underlie the therapeutic efficacy of SSRI. However, this picture is still unclear, as discrepancies are present regarding the effect of SSRI on SERT-mRNA levels (Lesch et al., 1993b; Benmansour et al., 2002). As pointed out by Benmansour and coworkers, *in vivo* studies of antidepressant can yield inconsistent results due to methodological issues (e.g. drug administration; (Lesch et al., 1993b; Benmansour et al., 2002)). For such reasons, to date the most clear data about SERT modulation come from more defined systems, such as cell lines and transgenic mice.

1.4.6 Organization of SERT gene

The human and murine SERT genes have been identified on chromosome 17 and chromosome 11, respectively (Gregor et al., 1993; Ramamoorthy et al., 1993). Both are composed of 14 exons spanning more than 30 kb, with conservation of the exon/intron organization and, to a lesser extent, the 5'-flanking regulatory sequences (Lesch et al., 1994). All the transmembrane domains but the putative TMD11 are uninterrupted by intron/exon boundaries, placing each of these TMDs in a separate exon. A highly conserved TATA-like motif and several potential binding sites for transcription factors including AP1 (subject to phorbol esters regulation), AP2, AP4, SP1, a NF- κ B and CRE-like motifs are present in the promoter region across species (Heils et al., 1998). The role of these transcription factor motifs in the regulation of the human and murine SERT gene expression has been studied using reporter gene fusion constructs of 5'-flanking sequences (Heils et al., 1995; Bengel et al., 1997a). The data described in those

studies indicate that the SERT promoter is active in cells that constitutively express SERT, the information contained within ~2 kb of 5'-flanking sequence is sufficient to confer its cell-specific expression, the promoter responds to cAMP/PKA and PKC induction, and the expression of the SERT gene is regulated by a combination of positive and negative cis-acting elements operating through a basal promoter unit defined by a TATA-like motif.

In immortalized serotonergic raphe neurons, RN46A, three cis-acting, cell specific, activating elements and a silencer were identified. A fourth activating element was found to be active in both RN46A cells and in the non-neuronal serotonergic cell line, JAR, which also possess an alternative promoter within the intron 1 (Mortensen et al., 1999b). Transcriptional activity of the hSERT gene promoter is modulated by a polymorphic repetitive element (5-HTTLPR; (Heils et al., 1996)). The 5-HTTLPR is located approximately 1 kb upstream of the SERT gene transcription initiation site in a GC-rich region and is composed of 14-16 repeat elements with the consensus sequence CCCCCCTGCACCCCCCAGCAT. Comparison of the SERT gene promoter between different species revealed that the 5-HTTLPR is "foreign" DNA possibly of viral origin and unique to humans and non-human primates including *Macaca mulatta* (rhesus monkeys) (Lesch et al., 1997). The number of 5-HTTLPR polymorphisms has been progressively increasing over the years, arriving to 14 for the Japanese population (Delbruck et al., 1997; Nakamura et al., 2000). 5-HTTLPR-dependent variation in functional SERT expression is a potential genetic susceptibility factor for affective disorders. Indeed, association studies support the hypothesis that allelic variation in the SERT gene may contribute to susceptibility for unipolar, bipolar disorder, and to anxiety-related personality traits (Lesch et al., 1996; MacKenzie and Quinn, 1999). Moreover, the response to SSRI drugs, which is not homogeneous within populations, has been related to allelic variation in the SERT gene (Kim et al., 2000).

1.4.7 Regulation of SERT

Given the physiological and pathological role played by SERT, it seems important to understand how this transporter is regulated. Function and regulation of SERT have been analyzed mainly through the expression of the transporter in non-neuronal cells. This has allowed the study of acute changes in 5HT uptake, which occur in minutes to hours, as opposed to long-term changes that manifest after days or weeks. Acute changes in serotonin clearance are more likely to originate from local variations in 5HT concentration, cell surface distribution of SERT, interactions with regulatory proteins or reversible post-translational modifications. Here follows a discussion on the acute regulation of SERT by protein kinases, protein phosphatases, calcium, growth factors, cytokines, and serotonin autoreceptors. The effects of these stimuli is summarized in table 1.1.

1.4.7.1 Serine/Threonine protein kinases

NET, DAT and SERT have been shown to rapidly redistribute from the cell surface to the cytoplasm upon activation of protein kinase C (PKC; (Apparsundaram et al., 1998; Daniels and Amara, 1999)). PKC is a family of serine/threonine kinases, consisting of at least twelve isoforms, having distinct enzymatic properties and cellular localization. The majority of PKC isoforms are endogenously stimulated by the second messenger diacylglycerol, and can be stimulated by phorbol esters, such as PMA (Way et al., 2000). Activation of PKC with phorbol esters causes a dose-dependent down-regulation of SERT, due to internalisation of the transporter (Anderson and Horne, 1992; Qian et al., 1997; Ramamoorthy et al., 1998). As already mentioned, the analysis of the amino-acid sequences of the cloned human (Ramamoorthy et al., 1993) and rat (Blakely et al., 1991; Hoffman et al., 1991) SERTs reveals five potential sites of phosphorylation by PKC. Nevertheless, Sakai and coworkers have shown that mutagenesis of phosphorylation consensus sites has no effect on the 5HT uptake regulation by PKC (Sakai et al., 1997), thus suggesting that PKC might regulate SERT via an indirect mechanism. Similar results have been obtained also for the human dopamine transporter (Chang et al., 2001). Once SERT has been internalized, it is

Table 1.1 Summary of acute stimuli reported to regulate clearance of 5HT.

The stimuli include protein kinases (PK), protein phosphatases (PP), calcium and calcium-binding proteins, serotonin autoreceptors (5HT-R), and cytokines. + upregulation, - downregulation.

Stimulus	Regulation	Reference
PKC	-	(Apparsundaram et al., 1998; Daniels and Amara, 1999)
PKA	no agreement	(King <i>et al</i> , 1992; Ramamoorthy <i>et al</i> , 1998)
NO/PKG	+	(Miller and Hoffman, 1994)
Ca ⁺⁺ /Calmodulin	+	(Nishio et al., 1979; Ikegami et al., 1991; Ramamoorthy et al., 1998)
Tyrosine kinases	+	(Helmeste and Tang, 1995; Prasad et al., 1997)
PPs	+	(Sakai et al., 1997; Ramamoorthy et al., 1998; Bauman et al., 2000)
5HT _{1B}	+	(Daws et al., 2000Mlinar and Corradetti, 2003)
IL-1 β , TNF α , INTs	+	(Ramamoorthy et al., 1995; Mossner et al., 1998; Morikawa et al., 1998)
IL-4	-	(Mossner et al., 2001)

not clear whether it is recycled or degraded. In this regard, more data are available for the dopamine transporter. Degradation appears to occur upon PKC activation when the transporter is expressed in MDCK cells (Daniels and Amara, 1999), whereas in PC12 cells DAT is recycled (Melikian and Buckley, 1999): the discrepancy of these results may be due to the use of different cell lines and/or to the fact that MDCK cells, that do not endogenously express DAT, may lack some essential component for the traffic of DAT to the recycling pathway. In rat whole brain and synaptosomes, the heavy chain of tetanus toxin has been shown to exert an acute down-regulation of 5HT uptake: the effect of the toxin is elicited through translocation of PKC isoforms, PLC γ 1- and trkA-activation (Gil et al., 1998; Gil et al., 2000; Najib et al., 2000).

Putative consensus sites for PKA and PKG phosphorylation are also predicted in the amino-acid sequence of SERT. However, transporter regulation by PKA and PKG is much less evident than for PKC. Moreover, the effect of PKA on SERT expression may reflect a cell-specific sensitivity of SERT to second messengers. In rat PC12 cells and C33-14-B1 mouse fibroblasts increasing levels of cAMP levels have been reported to down-regulate SERT activity (King *et al*, 1992). Conversely, in human embryonic kidney (HEK)-293 cells, PKA activators enhance hSERT phosphorylation (Ramamoorthy *et al*, 1998), but fail to acutely down-regulate 5-HT uptake. In a rat basophilic leukemia cell line (RBL 2H3), which expresses the serotonin transporter, the activation of the A3-adenosine receptor increases SERT activity by elevating the intracellular levels of nitric oxide, which in turn increases the levels of cyclic GMP with consequent activation of PKG (Miller and Hoffman, 1994). It is not known whether PKG phosphorylates either SERT itself or some interacting proteins.

When the action potential arrives at the axon terminal, voltage-dependent Ca⁺⁺-channels open and the concentration of intracellular Ca⁺⁺ increases, triggering the release of neurotransmitter through the fusion of vesicles with the presynaptic membrane. Does Ca⁺⁺-influx affect SERT? Some evidence suggest that PKC is involved in Ca⁺⁺-modulation of SERT. Nishio and colleagues have shown that in platelets, concanavalin A (ConA) decreases 5-HT uptake (Nishio et al., 1979). The lectin, ConA, causes a rise of intracellular concentration of Ca⁺⁺,

first triggering calcium release from intracellular stores, then elevating Ca^{++} influx (Ikegami et al., 1991). ConA also stimulates PKC activation and, indeed, PKC inhibitors block the ConA-mediated reduction of 5-HT uptake (Ikegami et al., 1991).

The findings concerning the regulation of SERT by the Ca^{++} -binding protein calmodulin are conflicting. In JAR and BeWo cells, that endogenously express the SERT, calmodulin antagonists inhibit 5HT, mainly by decreasing SERT V_{max} (Jayanthi et al., 1994). Ramamoorthy and coworkers show that in HEK cells intracellular Ca^{++} and calmodulin do not modulate SERT phosphorylation (Ramamoorthy et al., 1998), but unfortunately the authors do not show whether calmodulin and/or calcium downregulates the transporter, as shown for cell lines that endogenously express the transporter.

1.4.7.2 Tyrosine protein kinases and growth factors

Many membrane proteins, including growth factor receptors and ion channels, are reversibly modified by tyrosine phosphorylation. Although for neurotransmitter transporters, the attention is mainly focused on Ser/Thr kinases, Helmeste and Tang report that in platelets inhibitors of tyrosine kinases induce an acute decrease in 5HT uptake (Helmeste and Tang, 1995). Conversely, JAR cells treated for more than four hours with a tyr-kinase inhibitor show an increase in the density of SERT and in the basal levels of the SERT mRNA (Prasad et al., 1997). Accordingly with this report, EGF has been shown to increase SERT expression in JAR cells via EGF-receptor by tyrosine phosphorylation (Kekuda et al., 1997).

Other growth factors appear to regulate SERT, such as the basic fibroblast growth factor (bFGF) (Kubota et al., 2001) and the brain-derived neurotrophic factor (BDNF), which is a specific growth and differentiation factor for serotonergic neurons (Mossner et al., 2000).

1.4.7.3 Protein phosphatases

If protein kinases play a role in SERT regulation, either via a direct or indirect mechanism, their counterparts, protein phosphatases (PPs), are likely to modulate serotonin transport as well. Indeed, SERT has been shown to be downregulated by PKC activators, as well as PP inhibitors (Sakai et al., 1997; Ramamoorthy et al., 1998). More recently, Bauman and colleagues have shown that at the cell surface SERT forms stable complexes with the catalytic subunit of PP2A (Bauman et al., 2000). Interestingly, the addition of serotonin is shown to reduce SERT phosphorylation by PKC (Ramamoorthy et al., 1998), suggesting that the substrate might stabilize the SERT/PP2A complex, leading to the retention of the transporter at the cell surface.

1.4.7.4 Cytokines

A number of cytokines, including interleukin (IL)-1 β (Ramamoorthy et al., 1995), IL-4 (Mossner et al., 2001), tumor necrosis factor (TNF)- α (Mossner et al., 1998), and interferon- α/γ (Morikawa et al., 1998), have been shown to regulate SERT. SERT is upregulated following an exposure of 3-4 hours to IL-1 β and interferons, whereas TNF- α requires 24 hours to enhance SERT function. IL-4, an anti-inflammatory cytokine of CNS, down-regulate SERT by acting preferentially on alleles with the long 5-HTTLPR promoter.

1.4.7.5 Presynaptic autoreceptors

Presynaptic autoreceptors are activated by the same neurotransmitter that is released from the neuron on which the autoreceptors are localized. In most cases their activation results in a negative feedback loop resulting in the inhibition of neuron firing and/or transmitter synthesis or release. Since autoreceptors and

transporters are present on the same neurons, autoreceptors provide another potentially important mechanism of neurotransmitter transporters. Despite this fact, only a few reports are available on the cross-talk between serotonin autoreceptors (5HT-R_{1A,1B,1D}) and SERT. Whereas conflicting studies are present for 5HT-R_{1A,1B}, the autoreceptor 5HT-R_{1B} has been shown to regulate SERT activity in the hippocampus. Cyanopindolol, a 5HT-R_{1B} inhibitor, inhibited the clearance of serotonin with a potency equivalent to that of the SSRI fluvoxamine (Daws et al., 2000). Moreover, the release of 5HT mediated by MDMA is inhibited by a selective 5HT-R_{1B} antagonist (Mlinar and Corradetti, 2003). Inhibitors of other 5HT autoreceptors did not elicit an effect on 5HT clearance.

1.4.8 Cellular localization of SERT

To carry out clearance of extracellular 5HT, SERT must be inserted into plasma membrane in an active form. This may require targeting the transporter to specific plasma membrane regions, but not others. For example, the homologous rDAT has been shown by electron microscopy (EM) to localize in the plasma membrane of varicosities, terminals and dendritic spines (Nirenberg et al., 1996). Light microscopy and EM observations with SERT antibodies are consistent with DAT studies (Raisman et al., 1980; Schroeter et al., 1997). Notably, sorting mechanisms appear to be cell-type dependent: in fact, non-neural polarized cells, such as MDCK cells, stably expressing SERT, NET and DAT, present SERT and NET within the baso-lateral plasma membrane (considered equivalent to neuronal dendrites and soma), whereas DAT is sorted to the apical one (equivalent to the axon; (Gu et al., 1996)). These data suggest the existence of an active mechanism to insert or exclude SERT and homologues from specific domains of the plasma membrane. Two mechanisms seem possible for the targeting of SERT to specific areas of the cell surface: association of SERT to proteins involved in vesicular trafficking (see section 1.5) and/or association of the transporter with lipid rafts (section 1.6).

1.5 Membrane trafficking

Eukaryotic cells contain membrane-bound compartments that are connected by vesicular intermediates. Fusion of membranes must obey certain restrictions that prevent uncontrolled intermixing of membranes that could lead to the loss of compartmental organization. Proper targeting of vesicles is critical to the sorting of proteins newly made in the rough endoplasmic reticulum and of proteins internalised from the cell surface.

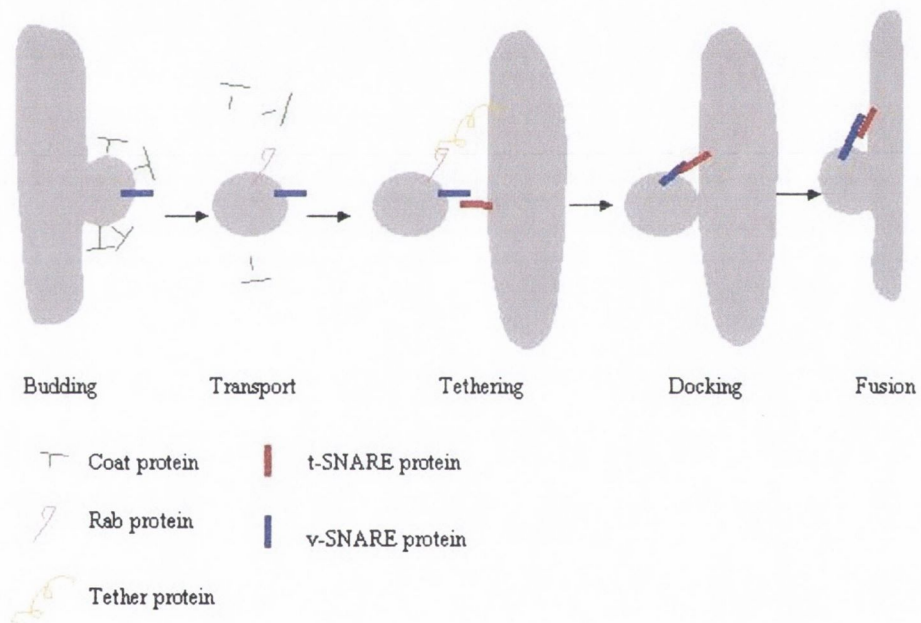
The first step for understanding the molecular basis of synaptic transmission has been through the purification of synaptic vesicles (SV), first from Torpedo electric organ (Carlson et al., 1978), then from rat brain (Huttner et al., 1983). Once the proteins associated with the SV had been identified, the plasma protein syntaxin was discovered because of its interaction with the SV Ca^{++} -binding protein synaptotagmin (Bennett et al., 1992)). After this, protein-protein interaction has been used successfully to identify other components of the so-called SNARE (soluble N-maleimide sensitive factor attachment protein receptor)-complex.

Membrane trafficking is mediated by transport vesicles and can be subdivided into several steps: budding, transport, tethering, docking and fusion (Fig. 1.9). Coated vesicles mediate the movement of membranes and their cargo between different compartments. These vesicles have a distinct coat, a complement of proteins that are necessary for the budding and the release from the donor membrane to occur. There are two well-characterized types of coated vesicles - clathrin-coated and coatomer-coated. The major protein component of clathrin-coated vesicles is clathrin itself, consisting of three large and three small polypeptide chains that together form the so-called 'triskelion'. Coatomer-coated vesicles consist in part of a large protein complex called 'coatomer', comprising seven individual coat-protein subunits (COPs). Fully formed vesicles usually lose their coat soon after their emergence from the membrane (Rothman, 1994). The plasma membrane of most cells has morphologically and biochemically distinct invaginations called 'caveolae', whose coat form through the oligomerization of a

Fig. 1.9

Schematic representation of vesicular trafficking

Separate steps are assumed to exist in vesicular trafficking of membranes: budding from the donor membrane, transport, tethering, docking and fusion with the acceptor membrane. Each of these steps involves distinct interactions between vesicle proteins and proteins located on the target membrane.



structural coat protein called caveolin (Fra et al., 1995; Monier et al., 1995; Sargiacomo et al., 1995).

Recently, another set of proteins has been described, called 'tethering' proteins, as new components of the targeting machinery. The tethering factors probably interact with Rab proteins (belonging to the Ras superfamily of GTPases) and SNAREs to generate targeting specificity (Waters and Pfeffer, 1999). A common feature of many proteins involved in tethering is their propensity to form highly extended, coiled-coil structures (Pfeffer, 1999). Although this mechanism is not known in detail yet, tethering has been shown to be SNARE-independent and a reversible process, and may allow fusion partners an opportunity to sample and, possibly, to reverse inappropriate encounters (Cao et al., 1998).

The first selective recognition of vesicle and target membrane takes place during docking, and it involves formation of a complex between specific evolutionary conserved transmembrane proteins that reside on vesicles (v-SNAREs) and target membranes (t-SNAREs). So far, 21 SNARE proteins have been found in yeast and over 30 in mammals (Chen et al., 2001b). Binding of v- and t-SNAREs induces a conformational change and leads to membrane fusion. Complex formation is accompanied by changes in the protein structure, which probably drives membrane fusion. Structural studies suggest that SNAREs form a SDS-resistant four-helix bundle of which one helix is supplied by a v-SNARE and the other three by t-SNAREs (Poirier et al., 1998). Each protein contributes to the bundle formation with a domain of about 60 amino acids (called the 'SNARE motif'). The formation of this helix bundle may help to overcome the energetic barrier for lipid bilayer fusion. The best-characterized SNARE complex is the one involved in the exocytosis of synaptic vesicles. Recently, Chen and coworkers have proposed that the SNARE-complex assembles in two stages: in presence of ATP, synaptobrevin (also known as VAMP) binds to SNAP (soluble N-maleimide sensitive factor attachment protein)-25 (Chen et al., 2001b). Upon Ca^{++} -dependent triggering syntaxin 1 (syn 1) is thought to dissociate from an inhibitory partner, munc 18-1, and to 'zipper' into a tight SDS-resistant complex

with SNAP-25 and VAMP, which allows a rapid fusion of the vesicle with the plasma membrane (Fig. 1.10; (Wu et al., 1999)).

The elegant SNARE hypothesis (Rothman and Warren, 1994) postulates that pairing between a v-SNARE and a t-SNARE is the primary molecular interaction underlying the specificity of vesicle targeting as well as lipid bilayer fusion. This hypothesis is supported by a minimal artificial system (Weber et al., 1998), and *in vivo* studies (Gaisano et al., 1996). Nevertheless, SNAREs implicated in diverse fusion reactions can form v- and t-SNARE complexes in solution that probably never exist in the cell. Every cell expresses a large number of SNARE proteins that are localized to particular membrane compartments (Gaisano et al., 1996), suggesting that the fidelity of vesicle trafficking might be in part determined by specific SNARE pairing. However, several observations demonstrate that SNAREs can pair promiscuously (Grote and Novick, 1999), suggesting that the information for membrane compartment organization is not encoded in the inherent ability of SNAREs to form complexes. Moreover, other proteins have been shown to be important in membrane docking or tethering (Cao et al., 1998). Thus, if the minimal machinery is defined as the set of proteins sufficient to reproduce *in vitro* the fidelity of vesicle targeting, docking and fusion as *in vivo*, then SNAREs do not appear to be sufficient to specify vesicle targeting.

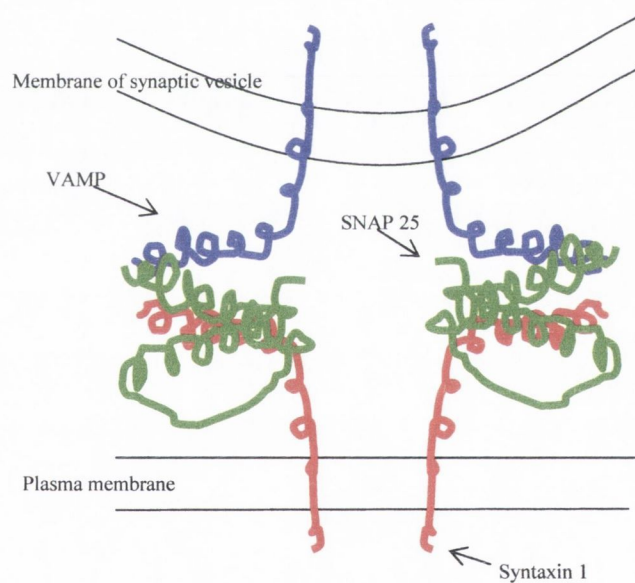
In the past few years, several studies have linked the SNARE protein syntaxin 1A to more complex functions. As will be discussed in Chapter 4, syn 1A has been shown to interact and regulate a number of ion channels and also neurotransmitter transporters, such as GLYT_s, GAT1, NET and SERT. These findings suggest that syn 1A is involved not only in the initiation of the neurotransmission, but also in modulating its duration.

1.6 Lipid microdomains ('rafts')

Fig. 1.10

Schematic model of the SNARE complex at the presynaptic membrane

Docking and fusion of membranes require interactions between specific sets of vesicular- and target-SNAREs. VAMP/synaptobrevin and the calcium-binding protein synaptotagmin (not shown) are located on the vesicle membrane, and syntaxin 1 and SNAP-25 are located on the presynaptic membrane. VAMP, syntaxin 1 and SNAP-25 form a ternary complex consisting of four α -helices, one each from VAMP and syntaxin 1, and two from SNAP-25. This bundle of α -helices brings the two membranes in close apposition and promotes their fusion.



Since the early 1970s, cell membranes have been considered as a neutral two-dimensional solvent for membrane proteins (Singer and Nicholson, 1972). However, over the past few years many reports have been providing evidence that lipids are not randomly distributed, but that there is a structure in their organization within the plasma membrane. Studies in model lipid bilayers showed that lipids could exist in different phases (reviewed in (London and Brown, 2000)). Depending on the composition of the bilayer and on the temperature, lipids can be in a gel, liquid-ordered (l_o) and liquid-disordered states (as in the Singer-Nicholson model). Lipids in the gel phase have little lateral mobility. The gel phase exists below the melting temperature (T_m) of constituent lipids: the higher the T_m value, the more stable the gel phase. Saturated acyl chains, such as sphingolipids, confer a high T_m . In contrast, glycerophospholipids tend to be *cis*-unsaturated and thus have low T_m . Similarly to the gel phase, in the liquid-ordered phase l_o saturated hydrocarbonic acyl chains of lipids are tightly packed. However, this phase is characterized by a rapid lateral diffusion, closer to what observed for the loosely packed lipids in the liquid disordered phase. Importantly, cholesterol promotes the formation of l_o domains in artificial bilayers (Silvius et al., 1996). It is believed that cholesterol interacts with saturated acyl chains of sphingolipids, near their headgroups, promoting their self-association in tightly packed detergent-resistant clusters that also contain cholesterol (McIntosh et al., 1992). This model has been confirmed at least partially in cell membranes: the exoplasmic leaflet contains lipid microdomains, enriched in sphingolipids and cholesterol, segregate from glycerolipids. Such lipid microdomains, also known as 'rafts', resist solubilization at 4°C in non-ionic detergents, such as Triton X-100 (Brown and London, 1998; Rietveld and Simons, 1998). The segregation of proteins into lipid microdomains is lost after depletion of cholesterol or sphingolipids (Keller and Simons, 1998).

Rafts contain few transmembrane proteins, but have concentrated certain classes of lipid-anchored proteins, including extracellular glycosylphosphatidylinositol-anchored (GPI) proteins. Although a GPI-anchor is required for the association with rafts, N-glycans appear to act as an apical sorting

signal on GPI-anchored protein, transmembrane and secretory proteins (Benting et al., 1999).

Initially, lipid rafts were viewed as part of a sorting mechanism for some membrane proteins during signal transduction (Simons and Ikonen, 1997), a role that they are likely to play in the activation of T-cells (Montixi et al., 1998; Puertollano and Alonso, 1998; Viola et al., 1999). More recently, lipid rafts have been also involved in sorting proteins in vesicular transport and in organizing molecules involved in docking and fusion of vesicles (Chamberlain et al., 2001; Ikonen, 2001; Lang et al., 2001).

1.7 Aim of the study

The involvement of SERT in 5HT clearance has been clearly established for many years, and information regarding the molecular mechanisms of substrate transport and transport inhibition has been growing over the past decade. However, our understanding of how the serotonin transporter is regulated *in vivo* is still fragmentary. SERT is targeted to portions of the axolemma near sites of 5HT storage and release (Zhou et al., 1998; Pickel and Chan, 1999), and Blakely and coworkers suggested that “SERT is organized in a macromolecular complex in the plasma membrane that may serve to locate reuptake activity near release sites” (Blakely et al., 1998). The aim of this work is to investigate the association of SERT with proteins and/or lipids, which may form such macromolecular complexes for the targeting and regulation of the serotonin transporter.

Firstly, the association of SERT with the SNARE protein syntaxin 1A is investigated. Syn 1A is implicated in the docking of synaptic vesicles with the plasma membrane during exocytosis of the neurotransmitter. Furthermore, it has been shown to interact with and regulate a number of ion channels as well as the SERT-related GABA and glycine transporters.

A second line of work is to investigate if SERT is segregated into lipid rafts microdomains, and whether this association may serve to sort the transporter to specific plasma membrane regions and/or to regulate the clearance of 5HT.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Antibodies

Anti-FLAG M2 (Mouse, Sigma), anti-C SERT (ST-C20, Goat, SantaCruz Biotechnology), anti-N SERT (Rabbit, courtesy of Dr. C. Tate), anti-syntaxin 1 (HPC1, Mouse, SantaCruz Biotechnology), anti-syntaxin 1 (Rabbit, Calbiochem), anti-syntaxin 2, 3 and 4 (Rabbit, Courtesy of Prof. R. Jahn), anti-actin (Mouse, Sigma), anti-Cholera Toxin-B subunit (Rabbit, Sigma), anti-streptavidin-horseradish peroxidase (HRP, Amersham), anti-mouse, -goat, -rabbit HRP conjugated (Sigma), anti-mouse, -rabbit, -goat FITC-conjugated (IgG (H+L) AffiniPure F(ab')₂ Fragment, raised in Donkey, Jackson ImmunoResearch).

2.1.2 Plasmids

The pcDNA3 expression vector was obtained from Luke O'Neill's laboratory. pFLAG-CMV-5b was obtained from Sigma. The pSERT-FLAG plasmid was obtained from Dr. Jana Haase (Biochemistry Department, Conway Institute, UCD, Belfield, Dublin, Ireland). The pTLR4-FLAG plasmid was obtained from Marta Muzio (DRO-Oncology, Pharmacology Department-Pharmacia Corporation, Nerviano, Italy). pcDNA3-Syn 1A and pcDNA3-munc 18-1 were a kind gift from Dr. A. Geerlings (Centro de Biologia Molecular Severo Ochoa, Facultad de Ciencias, Universidad Autonoma de Madrid, Madrid, Spain)

2.1.3 Other materials

5-Hydroxy[G- ³ H]tryptamine creatine sulphate	Amersham Pharmacia
5-Hydroxytryptamine	Sigma

Agarose	Promega
Ampicillin	Sigma
BES	Sigma
Blasticidin	Invitrogen
Bovine Serum Albumin	Sigma
Brij 58	Sigma
Cholera toxin-B subunit	Sigma
Complete TM Protease Inhibitor Cocktail	Boehringer Mannheim
DMSO	Sigma
Donkey serum	Sigma
Dry fat milk	Marvel
Dulbecco's Modified Eagle's Medium	Sigma
Ecoscint TM	National Diagnostic
Fetal Calf Serum	Sigma
Folin-Ciocalteu Reagent	Merck
GeneJuice TM Transfection Reagent	Novagen
Geneticin	Promega
Glass cover slips	BDH
Glycerol	Sigma
Hygromycin	Roche
Imipramine	Sigma
L-glutamine	Sigma
Luminol	Sigma
Lysozyme solution	Sigma
Microscope slides	BDH
Minimal Essential Medium Eagle	Sigma
NeutrAvidin	Pierce
N-Glycosidase F	Boehringer Mannheim
Paraformaldehyde	Sigma
Para-p-phenylenediamine	Sigma
Paroxetine	SmithKline Beecham
p-coumaric acid	Sigma
Penicillin/Streptomycin solution	Sigma

Phosphate buffered saline	Oxoid
Poly-L-lysine	Sigma
Protogel™	National Diagnostic
Puromycin	Clontech
PVDF membrane	Sigma
Qiagen™ Plasmid Maxi Prep kit	Qiagen
QuikChange™ Site-Directed Mutagenesis Kit	Stratagene
Restriction enzymes	New England Biolabs
Sulfo-NHS-Biotin	Pierce
TEMED	Sigma
Tetracycline	Sigma
Tet-system approved Fetal Calf Serum	Clontech
Triton X-100	Sigma
Trypsin/EDTA	Sigma
Tryptone	Difco
Tween 20	Sigma
Tween 80	Sigma
X-Omat LS photographic film	Sigma
Yeast extract	Difco
Zeocin	Invitrogen

All other reagents were obtained from Sigma.

2.2 Methods

2.2.1 Cell culture

HEK-293 cells were grown in Minimal Essential Medium Eagle (MEM) containing Foetal Calf Serum (FCS 10% v/v), glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml) (complete MEM) at 37°C in a humidified atmosphere with 5% v/v CO₂ on 10 cm tissue culture plates.

The cell lines 293/IMI270, BHK/CytTS, and 293/TREx-SERT were obtained from Dr. Chris Tate (MRC Laboratory of Molecular Biology, Cambridge, UK; (Tate et al., 2003)).

293/IMI270 cells are derived from 293-EBNA (Invitrogen). This cell line expresses the EBNA-1 antigen that is essential for the episomal replication of the plasmid. The 293/IMI270 cells stably transfected with the plasmid pCGT270, expresses rSERT fused to the *E. coli* thioredoxin (TrxA) gene, followed by an in-frame histidine tag (His₁₀) and a FLAG tag (rSERT-TrxA-His₁₀-FLAG fusion protein). The 293/IMI270 cells were grown in complete MEM medium containing 0.25 mg/ml geneticin, 0.5 mg/ml hygromycin, and 1 µg/ml imipramine.

The stable cell line BHK/CytTS was made in suspension-adapted baby hamster kidney (BHK) cells using the temperature-inducible expression system pCytTSTM (Cytos Biotechnology). BHK/CytTS cells stably express an rSERT-His₁₀-FLAG fusion protein. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing FCS (10%, v/v), puromycin (10 µg/ml) at 37°C, 5% v/v CO₂ on 10 cm tissue culture plates. In this inducible system protein expression starts by decreasing the temperature below 34°C. The best expression level has been shown to be at 29°C (Boorsma et al., 2000). Therefore the induction of SERT-FLAG was carried out by placing the cells at 29°C, unless otherwise stated, in a humidified atmosphere with 5% v/v CO₂.

TREx-SERT cells are derived from HEK293 cells stably expressing the TetR repressor protein that turns off SERT-FLAG expression (TREx-293TM system, Invitrogen). The parental cell line is stably transfected with an rSERT-His₁₀-FLAG construct. The TREx-SERT cells were grown in DMEM containing Tet-system approved-FCS (10% v/v), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), blasticidin (5 µg/ml), zeocin (200 µg/ml) - complete DMEM. SERT-FLAG expression was induced by adding tetracycline (1 µg/ml, if not otherwise specified)

Confluent cells were split 5-8 fold/ 2-3 times a week in the following way. The growth medium was removed and the cell monolayer was washed with pre-warmed Ca^{++} -PSA (137 mM NaCl, 5.4 mM KCl, 3 mM NaHCO_3 , 5.5 mM glucose, 109 μM $\text{CaCl}_2 \cdot \text{H}_2\text{O}$). Ca^{++} -PSA was removed and 0.5 ml of trypsin/EDTA solution (5 g/l of porcine trypsin and 0.2% EDTA in 0.9% NaCl) was added into each plate. Cells were detached after a few minutes under gentle rocking. Trypsin was inactivated by adding pre-warmed complete medium. Cells were carefully resuspended by pipetting up and down several times and then seeded down at the appropriate dilution in fresh sterile plates.

For cryopreservation, cells were suspended in ice-cold dimethylsulphoxide (DMSO, 10% v/v) diluted in appropriate complete medium, and aliquoted in cryotubes. The cells were transferred to -80°C overnight allowing them to freeze slowly and then placed in a liquid nitrogen container.

Cells were defrosted from liquid nitrogen storage by rapid thawing in appropriate pre-warmed serum-free medium. Cells were then centrifuged at 1000 g for 5 minutes: the supernatant was discarded and the pellet resuspended in complete medium. The cells were then seeded at different dilutions in a 6 well-plate.

2.2.2 Determination of DNA concentration

The concentration of DNA was measured using the fluorophore PicoGreen (Molecular probes) using a fluorimeter (Wallac-1420 Multilabel Counter). PicoGreen fluoresces only upon binding to double-stranded DNA at the fluorescein wavelength (excitation 480 nm, emission 520 nm). Concentrations of DNA samples were calculated calibrating the readings from standard DNA samples of known concentrations.

2.2.3 DNA agarose gel electrophoresis

Agarose electrophoresis was carried out using a minigel electrophoresis apparatus. Agarose (1% w/v) gels were made up in TBE buffer (0.09 M Tris base, 0.09 M Boric acid, 0.5 M EDTA, pH 8.0). DNA samples were mixed with 3 x stopmix solution (5% w/v glycerol, 0.125% w/v bromophenol blue, 1 % v/v TBE), loaded onto the gel, and run initially at 60 V, then, once they have passed the wells, at 100-120 V. The gel was stained in ethidium bromide (25 μ M) and documented in a UVP ultra-violet trans-illuminator using the software Grab-IT 2.0.

2.2.4 Preparation and transformation of ultra-competent *E.coli* cells

Competent cultures of *E. coli* were generated using the Inoue method as described in (Sambrook and Russell, 2001). Competent cells were stored in aliquots at -80°C until needed, up to six months. Transformation of competent cells was carried out as described in (Sambrook and Russell, 2001).

2.2.5 Analytical preparation of plasmid DNA from *E.coli* cells

Plasmid DNA from bacteria was prepared as described in (Holmes and Quigley, 1981), with some modifications. TY-ampicillin medium (3 ml) was inoculated with a single colony from the transformation plate and incubated overnight at 37°C . Cells from 2 ml of the overnight culture were harvested by centrifugation at full speed in a bench minifuge for 2 minutes at RT. The supernatant was discarded. Cells were vortexed for 15 seconds. Solution C (0.5 ml) was added to the cells and briefly mixed: solution C was prepared freshly from solution A (50 mM Tris-HCl, pH 8.0, 20 % w/v sucrose) and solution B (10% v/v Triton X-100, 50 mM EDTA) at 1:1 ratio. Lysozyme solution (10 μ l) was added and the tube was incubated in a waterbath for 90 seconds. The mixture was centrifuged in a bench minifuge at full speed for 15 minutes at 4°C . The supernatant was transferred to a new minifuge tube. Isopropanol (0.7 ml, -20°C) was added, the solution was mixed and then incubated at -20°C for 20 minutes. The plasmid DNA was precipitated by centrifugation for 30 minutes at 4°C . The

supernatant was carefully removed, the pellet was air dried for 20-30 minutes and then resuspended in 20 µl of TE buffer (10 mM Tris-HCl pH 8.0, containing 1 mM EDTA).

2.2.6 Large scale plasmid purification

TY-ampicillin medium (100 ml) was inoculated with overnight culture (250 µl) from selected transformants and incubated overnight at 37°C with shaking (250 rpm). Plasmids were extracted and purified from the culture using the Qiagen Endotoxin-Free™ maxi preparation plasmid purification kit and suspended in TE buffer. The Qiagen protocol is based on the optimised alkaline lysis (Birnboim and Doly, 1979), followed by binding of plasmid DNA to Qiagen Anion-Exchange Resin under specific low salt and pH conditions. Impurities such as RNA, protein, and dyes are removed by a medium salt wash. Plasmid DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation. All steps were carried out according to the manufacturer's instructions.

2.2.7 Glycerol stocks

800 µl of a selected transformant was mixed to 80% v/v glycerol (1 ml, sterile) in a sterile cryotube and stored at -80°C.

2.2.8 Transient transfection of mammalian cells

Cells were plated 48 hours before transfection and incubated at 37°C, 5% CO₂ to give 60-70% confluence at the day of transfection. 3 µl (for a 35 mm dish) or 10 µl (for a 10 cm dish) GeneJuice™ transfection reagent was added dropwise into 100 µl (for a 35 mm dish) or 250 µl (for a 10 cm dish) serum free medium in a sterile tube, vortexed and incubated at room temperature (RT) for 5 minutes. DNA was then added into the mix in a ratio 1 µg DNA: 3 µl GeneJuice, mixed gently, and incubated at RT for 15 minutes. The GeneJuice/DNA mixture was

added dropwise to the cells in the complete growth medium and the cells were incubated for 24-48 hours at 37°C, 5% CO₂.

2.2.9 Preparation of cell lysates

The growth medium was removed and the cell monolayer was washed twice in PBS. Cells were then lysed at 4°C in RIPA buffer (1% w/v Triton X-100, 0.1% w/v SDS, 1x Complete™ Protease Inhibitor cocktail in PBS) for 20-30 minutes under gentle shaking. The lysate was then transferred into a minifuge tube and centrifuged at 4°C for 20 minutes at 20,800 g in an Eppendorf Centrifuge 5417 R. The supernatant was transferred into a fresh tube and stored at -20°C.

2.2.10 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using the discontinuous Laemmli system (Laemmli, 1970) using an ATTO electrophoresis unit with 5% v/v stacking gel and either 10%, 12%, or 15% v/v resolving gel.

Protein samples were added to 1/3 volume of 3x SDS-gel loading buffer, containing 125 mM DTT and mixed carefully. The samples were boiled at 100°C for 3-5 minutes. Samples were loaded into the gel along with a molecular weight marker. The gel was run in running buffer (0.025 M Tris-base, 0.19 M glycine, 0.2% w/v SDS) at 80 V through the stacking gel and then at 120 V through the resolving gel for approximately 1.5-2 hours.

Following electrophoresis, the gel was equilibrated in transfer buffer (0.025M Tris base, 0.19 M glycine, 0.1% w/v SDS and 20% v/v methanol) for 15 minutes. The proteins were transferred onto poly-vinyl-difluoride (PVDF) membrane, briefly pre-soaked in methanol, washed twice in ddH₂O, and then equilibrated in transfer buffer for 5-10 minutes. Transfer was carried out using a BioRad tank at a constant current of 160 mA for 1.5 hour or at 40 mA overnight.

2.2.11 Western blot analysis

Following transfer, the blot was blocked for 1 hour at RT or overnight at 4°C in blocking buffer (3% w/v BSA, 0.5% w/v milk powder, 0.5% v/v Tween 20 in TBS). The blot was incubated in primary antibody diluted in blocking buffer for 1-2 hours at RT or overnight at 4°C under constant shaking. The blot was washed for 10 minutes four times in TWB (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% v/v Tween20). The blot was then incubated with an appropriate secondary antibody horseradish peroxidase (HRP)-conjugated diluted in blocking buffer for 1 hour at RT under constant shaking. The blot was washed as above.

Blots were developed using enhanced chemiluminescence (ECL) detection system, prepared mixing an equal amount of solution 1 (2.5 mM luminol, 90 μ M p-coumaric acid, 100 mM Tris pH 8.5) and solution 2 (0.02% v/v hydrogen peroxide, 100 mM Tris pH 8.5). Following oxidation, luminol (3-aminophthalhydrazine) is in an excited state, which then decays to its ground state by emitting a photon. Enhanced chemiluminescence is achieved by performing the oxidation of luminol by the horseradish peroxidase in presence of chemical enhancers, such as phenols (e.g. p-coumaric acid). This has the effect of increasing the intensity and the time of light emission. The maximum light emission is at λ_{428} .

The light emitted is detected by exposing the blot, placed on the underside of an acetate sheet, to photographic film (Kodak X-Omat LS). The film was then developed and dried in an automated developer (RGII, Fuji x-ray film processor).

2.2.12 Markwell protein assay

Protein concentration of cell lysates was assayed using a modification (Markwell et al., 1978) of the Lowry method. A standard protein solution, BSA (1 mg/ml) was diluted to 0-100 μ g/ml. Solution C was freshly prepared by mixing 100 volumes of solution A (2% w/v NaCO_3 , 0.4% w/v NaOH, 0.16% w/v Sodium Potassium Tartrate, 1% w/v SDS) and 1 volume of solution B (4% w/v $\text{Cu}^{\text{II}}\text{SO}_4$).

ddH₂O was added to standards and samples to reach a final volume of 0.5 ml, then mixed with 1.5 ml of solution C and allowed to stand at RT for 10 minutes. 150 µl of Folin-Ciocalteu reagent (diluted 1:2 with ddH₂O) was added to each tube, mixed and incubated at RT for 45 minutes. The absorbance was measured at 660 nm using a microplate spectrophotometer (Molecular Devices™).

2.2.13 5HT uptake assay

Cells were plated on poly-L-lysine coated (0.1mg/ml) 24 well plates 3 days before experiments. If plasmid transfection was required, mock-transfected cells were included in the experiment to determine the amount of non-specific binding. At assay, the medium was removed by aspiration and the cells were washed twice with 0.5 ml per well of TB buffer (10 mM Hepes, 150 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂) pre-warmed at 37°C. A saturation curve was used to determine the $V_{\max}$ and K_m of 5HT transport. In general, six different concentrations of serotonin were chosen in a range between 0 and 10 µM. The specific activity of [³H]-5HT was diluted with unlabelled 5HT. Cells were incubated in TB for 15 minutes. The assay was started with the incubation of [³H]-5HT (0.4 ml per well) for 6 min (2 minutes for TReX-SERT cells) at RT and promptly stopped by aspiration followed by three washes with ice-cold TB containing 1 mM Paroxetine to inhibit 5HT transport. For the leucine and glycine uptakes described in section 2.2.13, cells were incubated with 20 nM [³H]-leucine or 2 µM [³H]-glycine for 6 minutes. The assay was stopped by aspiration followed by three washes with ice-cold TB. Then, 2% w/v SDS was added (0.5 ml per well) and the radioactivity from lysates was quantified by liquid scintillation counting. Each data point was measured in triplicate. Non-specific uptake was defined as the uptake in presence of 10 µM Paroxetine and this was subtracted from the total to obtain the specific uptake. To calculate the exact amount of radioactive substrate three controls for each concentration were measured.

The ³H isotope was measured using a Packard Tricarb 2100 TR liquid scintillation analyser. The cocktail used was the commercial scintillant,

Ecoscint™, for aqueous samples. Analysis of [³H] 5-HT uptake was carried out using the software SigmaPlot™.

2.2.14 Immunofluorescence for laser scanning confocal microscopy (LSCM)

Patching experiments were carried out as described by Harder and Simons (1999). Briefly, cells were grown on sterile glass cover-slips (treated with 0.1 µg/ml poly-L-lysine) in 6 well-plates. If required, cells in DMEM medium were incubated with 5 µg/ml CTxB at 4°C for 10 min. Cells were washed twice with DMEM and incubated with anti-CTxB (1:500) for 10 minutes at 37°C. The cells were then placed on ice, washed with ice-cold PBS, and fixed with 4% (w/v) paraformaldehyde in PBS pH 7.5 for 10 minutes at RT. The fixative was quenched by incubating cells with 50 mM NH₄Cl for 10 minutes. Fixed cells were incubated with blocking solution (0.2% (v/v) Triton X-100, 5% (v/v) donkey serum in PBS) for 1 hour at RT, followed by incubation with anti-FLAG M2 antibody (mouse, 1:1000, Sigma) diluted in blocking solution overnight at 4°C. Cells were washed with PBS and incubated with the anti-mouse FITC- and anti-rabbit TexasRed-conjugated antibody (donkey, Jackson ImmunoResearch), diluted in blocking solution for 1 hour at RT. After washing, the cells were mounted in 0.02% (w/v) sodium azide, 50% (v/v) glycerol, 2 µg/µl para-p-phenyldiamine in PBS. Samples were imaged using either a MRC-2400 BioRad LSCM or a Zeiss LSM510 laser scanning confocal microscope.

Although only a 5-10% of FITC emission spectrum overlaps with the TexasRed emission spectrum, this 'bleed-through' would generate a mistaken colocalization. Therefore, for the MRC-2400 BioRad microscope the bleed-through between the two fluorochromes was avoided by electronics exclusion of the FITC-signal from the output of the TexasRed-signal. Each digital mixer displays and mixes the signal from the PMTs according the following equation:

$$\text{Mixer } x = a\% \text{ PMT1} + b\% \text{ PMT2} + c\% \text{ PMT3}$$

Usually PMT1 collects the emission signal from TexasRed whereas FITC-emission is collected by PMT2. By default, mixer A displays 100% of PMT1 and mixer B displays 100% of PMT2 (a third mixer, C, is present for far red fluorescence signal, whose output is collected by PMT3). By excluding any contribution from PMT2 in mixer A, only the output generated by the excitation of TexasRed is obtained, with no contribution from the green fluorochrome. For the LSM510 microscope, the same operation was achieved using the option 'multitracking'.

Post-acquiring processing of the BioRad pic.files was carried out using the software Confocal Assistant (CAS), written by Todd Breljie and available in the Internet as free downloadable software (e.g. Biorad-website at <ftp.genetics.biorad.com>). Files obtained with the Zeiss microscope were handled using the Zeiss LLSM Image Browser version 3, downloaded from the Carl Zeiss website www.zeiss.co.uk.

2.2.15 Isolation of non-ionic detergent-insoluble membrane fractions

Isolation was carried out using a protocol slightly modified from the Brown-Rose protocol (Brown and Rose, 1992). At confluence, cells were washed twice with ice-cold PBS. If required, the cells were incubated with 3 µg/ml CTxB for 10 minutes at RT. The cell monolayer was washed three times with PBS and cells were lysed with 1 ml of TNE buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1x Complete™ Protease Inhibitor cocktail) containing the indicated concentration of non-ionic detergent, for 30 minutes at 4°C. The lysates were homogenised with 15 strokes of a Dounce homogenizer (Thomas Scientific, USA), transferred into a minifuge tube and centrifuged at 4°C for 10 minutes at 830 g in an Eppendorf Centrifuge 5417 R. Supernatants were transferred into a fresh minifuge tube. Pellets (P₁) were resuspended in 1 ml of solubilization buffer (50 mM Tris pH 8.8, 5 mM EDTA, 1% SDS) and stored at -20°C. 500 µl of cell supernatant was mixed with an equal volume of 80 % w/v sucrose in TNE buffer, transferred into a ultracentrifuge tube, then carefully overlaid with 1 ml of 30% w/v sucrose in TNE buffer and finally with 0.5 ml of 5% w/v sucrose in TNE

buffer. The tube was placed in a cooled AH650 swinging-bucket rotor and centrifugation was carried out in a Discovery100-Sorvall ultracentrifuge at 4°C, for 17 hours at 134,400 g. Eight fractions were collected from the top to the bottom of the gradient. The remaining pellet (P₂) was resuspended following centrifugation in 500 µl of solubilization buffer and stored at -20°C.

2.2.16 Cholesterol depletion

Cells were washed twice with PBS and then incubated with the appropriate amount of Methyl- β -Cyclodextrin (M β C, Sigma) for 30 minutes at 37°C. The cell monolayer was washed three times with buffer and immediately processed for the indicated assay as described. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out using the procedure described by Carmichael *et al.* (Carmichael *et al.*, 1987). Cholesterol content of cells was measured enzymatically using the Infinity Cholesterol Reagent (Sigma), according to the manufacturer's protocol.

2.2.17 Cell surface biotinylation of subcellular fractions

Cells were washed three times with ice-cold PBS⁺⁺ (PBS containing 0.1 mM CaCl₂ and 1 mM MgCl₂) and incubated in ice-cold 1.5 mg/ml Sulfo-NHS-Biotin/PBS⁺⁺ (Pierce) at 4°C for 30 minutes with gentle agitation. Cells were washed three times with ice-cold quench buffer (100 mM glycine in PBS⁺⁺), and incubated in quench buffer for 30 minutes at 4°C with gentle shaking. The cell monolayer was rinsed three times with ice-cold PBS⁺⁺ and then lysed in RIPA buffer, if not otherwise specified. Aliquots of lysates were incubated overnight at 4°C with equilibrated NeutrAvidin beads (60 µg protein/50 µl beads; Pierce), with end-over-end mixing. The supernatant (unbound fraction 'in', corresponding to cytoplasmic proteins) was saved in a fresh tube. The beads were washed three times in lysis buffer. Bound proteins ('out', corresponding to cell surface proteins) were eluted by boiling the beads in SDS-sample buffer.

2.2.18 Membrane preparation and N-glycosidase F treatment

Membrane were prepared as described in (Tate et al., 2003). Briefly, cells were grown to confluence in 10 cm Petri dishes and were washed three times in PBS. 2 ml of PBS was added to the cell monolayer, and cells were scraped off and pelleted (5 minutes at 1,000 g). The pellet was resuspended in 0.1 x PBS containing protease inhibitors (1x Complete™ Protease Inhibitor cocktail) and passaged 7 times through a 26 gauge needle. Membranes were pelleted at 14,000 g for 10 minutes, 4°C, washed once with ice cold PBS containing protease inhibitors and then resuspended in a final volume of 1 ml. Protein concentration was determined and membranes were aliquoted in fresh minifuge tubes.

Membranes were mixed with an equal volume of 2x deglycosylation buffer (0.4% w/v SDS, 2% v/v Nonidet P-40, 20 mM EDTA, 2% v/v mercaptoethanol, 2x Complete™ Protease Inhibitor cocktail, 2x PBS). A volume equivalent to 50 mg membrane proteins was incubated with 1 unit of N-glycosidase F and incubated overnight at 37°C. SDS sample buffer was added and samples were analyzed by SDS-PAGE.

2.2.19 Site directed mutagenesis

Site directed mutagenesis of pSERT-FLAG was carried out using the QuikChange Site-Directed Mutagenesis kit following the manufacturer’s instructions. Two oligonucleotide primers, each complementary to opposite stands of the plasmid and containing the desired mutation, are extended by PCR using PfuTurbo DNA polymerase. This polymerase replicates both plasmid strands with high fidelity. The cycling parameters of the amplification reactions were:

Segment	Cycles	Temperature	Time (min)
1	1	95°C	1
2	16	95°C	1
		55°C	1
		68°C	15
3	1	4°C	5

The PCR product is treated with Dpn I endonuclease, which is specific for methylated DNA and thus digest the parental (non-mutated) DNA template. The Dpn I treated DNA is then used to transform *E.coli*.

Chapter 3 Results

Characterization of SERT expression in three different mammalian systems

3.1 Introduction

SERT has been studied from native sources e.g. synaptosomes and platelets (Graham et al., 1992; Launay et al., 1992). The possibility to express heterologously SERT has facilitated the study of the transporter function and regulation. SERT has been successfully expressed transiently and stably in several mammalian cells: HeLa cells (Blakely et al., 1991), COS cells (Hoffman et al., 1991) and HEK293 cells (Schloss and Betz, 1995).

To date little information is available on the cellular trafficking of SERT. Laser scanning confocal microscopy is a powerful tool, which combined with the use of appropriate markers, offers the possibility to study the subcellular distribution of proteins. However, previous attempts performed in our laboratory to immuno-detect the transporter by confocal microscopy using a SERT-specific antibody (ST C-20, Santa Cruz Biotechnology) in HEK293 cells had been unsuccessful (Loane, 2000). Therefore, it was necessary to investigate the detection of a tagged form of the serotonin transporter (e.g. FLAG), for which a monoclonal antibody was commercially available.

A collaboration with Dr. Chris Tate (MRC-Laboratory of Molecular Biology, Cambridge, UK) has allowed the testing of several cell lines stably expressing the fusion protein SERT-FLAG. Three different stable cell lines were analyzed for this project, in order to find a heterologous expression system capable of producing high levels of SERT-FLAG, functionally active in serotonin uptake and effectively localized on the plasma membrane. One of the tested cell lines, 293/IMI270, constitutively expressed SERT-FLAG, whereas in the stably transfected BHK/CytTS and 293/T-REx cell lines the expression of SERT-FLAG is cold- and tetracycline-inducible respectively.

The three cell lines were analysed for protein expression levels by Western blotting, for transporter function by [³H]-5HT uptake assay, and for cellular localization of SERT by confocal microscopy.

3.2 Results

3.2.1 Characterization of 293/IMI270 cell line

The 293/IMI270 cell line was constructed using a plasmid carrying the EBV (Epstein-Barr virus) origin of replication and an rSERT-TrxA-His10-FLAG fusion protein (Tate et al., 2003). The plasmid was introduced in 293-EBNA (Invitrogen) cells expressing the EBNA-1 antigen necessary for the episomal replication of the retroviral vector. The selection of cells carrying the plasmid was accomplished by growing the cells in medium containing the antibiotic hygromycin (0.5 mg/ml).

To verify whether 293/IMI270 cells expressed detectable amounts of SERT, Western blotting analysis was carried out on cell extracts. In parallel, cell extracts from the parental cell line HEK293, which does not express the transporter, were used as a negative control. In 293/IMI270 cell extracts the monoclonal anti-FLAG antibody detected three bands (Fig. 3.1). The higher molecular weight form has already been described for SERT and other neurotransmitter transporters (Olivares et al., 1994; Tate and Blakely, 1994; Qian et al., 1995), and are likely to represent non-specific aggregates (Baker, 1997). Two polypeptides with an apparent molecular mass of 85,000 and 70,000 were also detected. As shown by N-glycan removal with peptide:N glycosidase F, these two peptides correspond respectively to the fully and partially glycosylated form of SERT (Tate et al., 2003).

The functional expression of SERT in 293/IMI270 cells was analyzed by [³H]-5HT uptake assay. SERT displayed saturation kinetics with a $V_{\max}$ of 29.9 ± 1.9 pmol/min/ 10^6 cells and a K_m of 1024 ± 138 nM (Fig. 3.2).

Activation of PKC with phorbol esters is known to cause a dose-dependent down-regulation of SERT due to internalization of the transporter, which does not appear to be followed by degradation (Qian et al., 1997). A 35-40% maximal reduction of serotonin uptake had been observed after 30 minutes of treatment with phorbol esters, followed by a 10% recovery at longer incubation times. In order to verify whether the tagged serotonin transporter expressed by

Fig. 3.1
Expression of SERT-FLAG fusion protein in 293/IMI270 cells

Cell extracts from HEK cells and 293/IMI270 cells were prepared as described in Methods. Equal amounts of protein samples were loaded into the wells of a 10% SDS-polyacrylamide gel and transferred onto PVDF membrane. The membrane was probed with the polyclonal anti-CSERT antibody (ST C-20; 1:2000). Arrows indicate the fully glycosylated form and the partially glycosylated form of SERT. The band at the top of the gel corresponds to SDS-resistant aggregates.

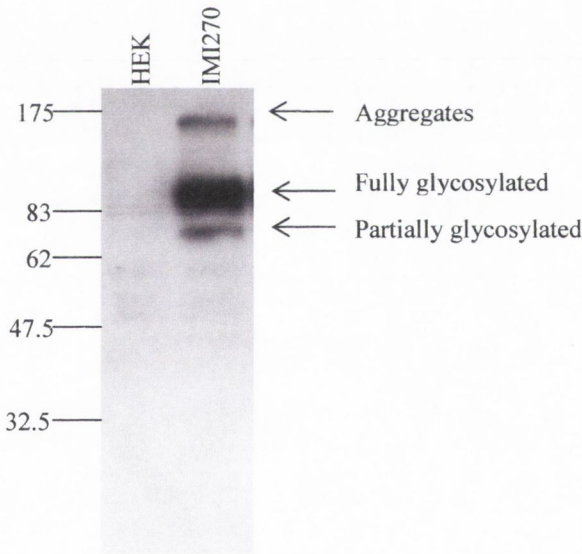
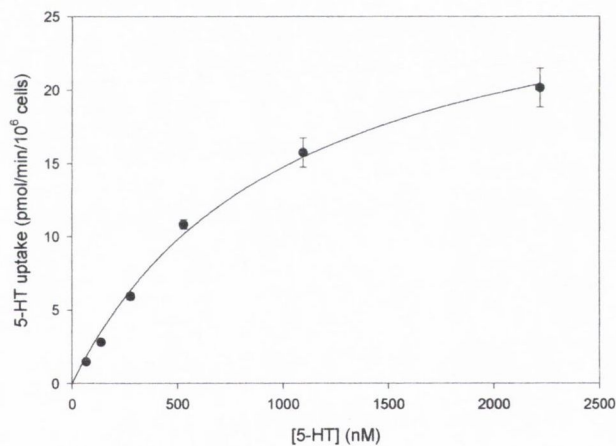


Fig. 3.2
Specific uptake of [³H]-5HT into 293/IMI270 cells

293/IMI270 were seeded onto poly-L-lysine coated 24-well plates and grown for 48 hours prior to the assay as described in Methods. Non-specific uptake was defined as the uptake in presence of 10 μ M Paroxetine and subtracted from the total uptake as described in Methods. Each data point was assayed in triplicate in each experiment. The graph below is representative of three independent experiments that yield similar results. The plot was graphed using the non-linear regression equation of the software SigmaPlot™.



293/IMI270 cells retained the responsiveness to PKC activation, the sensitivity of SERT to the phorbol ester PMA was tested. Cells were washed twice with pre-warmed TB buffer supplemented with 10 mM glucose. Due to the photosensitivity of the compound, incubation with 1 μ M PMA, diluted in DMSO, was carried out for the appropriate time at 37°C in the dark. In parallel, cells treated with the vehicle alone, DMSO, were used as control. Cells were then incubated with 2 μ M 5HT for 6 minutes and the assay was carried out as described in Methods. 293/IMI270 cells exhibited in average a 20-30% reduction of [3 H]-5HT uptake in presence of 1 μ M PMA. The peak of down-regulation was reached after 10-15 minutes from the start of the treatment, followed by a progressive and total recovery of the [3 H]-5HT uptake after 30 minutes of treatment (Fig. 3.3). This was in contrast with a previous study where the recovery of 5HT uptake to control values was only partial (~10%; (Qian et al., 1997)). The mechanism of SERT redistribution upon PKC activation is not yet known. However, it is possible that the addition of a FLAG tag at the C-terminus of SERT alters the intracellular retention of the transporter following PMA treatment, by inhibiting its degradation and/or by enhancing its recycling to the cell surface.

PMA has been shown to affect also Na⁺-dependent glycine uptake, whereas does not appear to alter leucine uptake (Qian et al., 1997). In agreement with this previous study, the incubation of 293/IMI270 cells with 1 μ M PMA for 15 minutes reduced not only serotonin uptake, but also glycine uptake via endogenous transport, whilst endogenous leucine transport was not affected (Fig. 3.4).

Taken together these data suggest that the addition of a FLAG tag at the C-terminus of SERT does not appear to affect its re-uptake function, but it may affect the retention of the transporter upon internalization.

SERT expression in heterologous systems has been observed to exert a toxic effect on cell viability (Grisshammer and Tate, 1995; Qian et al., 1997). It had been hypothesized that this might be due to uptake of serotonin present in the

Fig. 3.3
Regulation of 5HT-transport by PKC activation in 293/IMI270 cells

293/IMI270 cells were grown in poly-L-lysine coated 24 well-plates. Cells were incubated for the indicated time with 1 μ M PMA diluted in DMSO. In parallel, control cells were treated with an equal volume of DMSO. The experiment was assayed in triplicate; each data point was expressed as percentage of control and plotted as mean value $\pm$ SE. The graph shown is representative of three independent experiments.

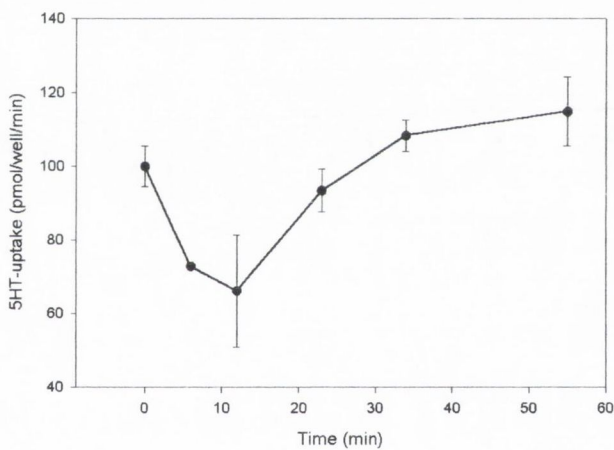
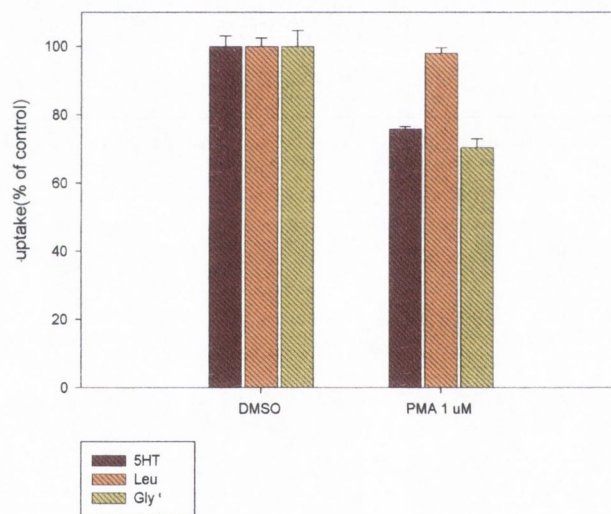


Fig. 3.4

Regulation of serotonin, glycine and leucine uptake by PMA in 293/IMI270 cells

293/IMI270 cells were seeded in poly-L-lysine coated 24 well-plates. 48 hours later cells were incubated with 1 μ M PMA for 15 minutes or with an equal amount of vehicle, DMSO, as a control. Cells were then incubated with 2 μ M 5HT, 1 μ M glycine and/or 20 nM leucine, and the uptake assays were carried out as described in Methods. The experiments were assayed in triplicate and repeated twice with similar results.



serum added to the cells' growth medium, which may in turn result in a negative selection against cells expressing higher levels of transporter. The reasons for this to happen are not fully understood. Transport of serotonin is coupled to a movement of Na^+ , K^+ , and Cl^- ions. Therefore, it is possible that the heterologous expression of a high number of SERT molecules would create an osmotic imbalance by altering the electrochemical gradients of these ions. In the attempt to avoid such a detrimental effect, the 293/IMI270 cell line were propagated in the constant presence of the SERT inhibitor imipramine. Due to the high affinity of imipramine for SERT, to carry out an experiment cells were plated into the appropriate plate format, grown for 3 days in absence of the antidepressant and then quickly rinsed three times with TB buffer prior to the experiment in order to facilitate imipramine's removal. The importance of propagate 293/IMI270 cells in growth medium including imipramine was evident when the $[^3\text{H}]\text{-5HT}$ uptake activity was measured in 293/IMI270 cells grown for 3, 12, or 21 days in absence of the antidepressant. Cells were counted prior to the assay and 5HT uptake was expressed per million of cells. Deprivation of imipramine for 12 or 21 days caused a progressive decrease in $[^3\text{H}]\text{-5HT}$ uptake compared to the control (3 days of deprivation), mainly by affecting the velocity of transport, whereas the substrate affinity was only slightly affected (Fig. 3.5).

The cellular distribution of SERT in 293/IMI270 cells was investigated by means of confocal microscopy imaging. SERT, detected by the anti-FLAG antibody, was localized mainly on the plasma membrane with a punctate distribution. In order to rule out the possibility that this staining pattern could be an artefact generated by the fixative, two different fixation methods were compared: acetone 100% (v/v) for 10 minutes at -20°C , and paraformaldehyde 4% (w/v) for 10 minutes at RT (Fig. 3.6, left and right panel, respectively). The patched distribution of SERT along the plasma membrane was retained in both cases.

As shown above, PMA treatment down-regulates $[^3\text{H}]\text{-5HT}$ uptake activity of SERT expressed in 293/IMI270 cells (Fig. 3.3). Specifically, PKC activation by phorbol esters has been shown to result in the reduction of 5HT transport velocity (Qian et al., 1997). By labelling cell surface proteins with an impermeant biotin

Fig. 3.5

Stability of [^3H]-5HT uptake in imipramine-deprived 293/IMI270 cell line

293/IMI270 cells were grown in absence of imipramine for 3 (▼), 12 (·) and 21 (○) days. Two days prior to the 5HT-uptake assay, cells were seeded onto poly-L-lysine coated 24-well-plates. 5HT uptake assay was carried out as described in Methods. Each data point was assayed in triplicate and plotted as mean value $\pm$ SE. Data were fitted to the Michaelis-Menten equation using the non-linear regression equation of the software SigmaPlotTM. V_{max} values for the data are, respectively, 29.5 ± 1.3 , 21.5 ± 0.7 , and 8.6 ± 0.7 pmol/min/ 10^6 cells. K_m values are, respectively, 987 ± 95 , 988 ± 66 , and 463 ± 99 nM. The experiment was performed twice, yielding similar results.

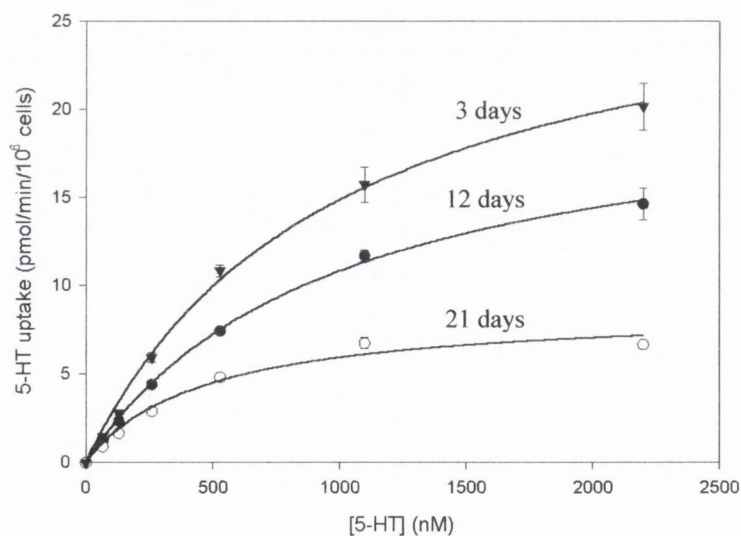
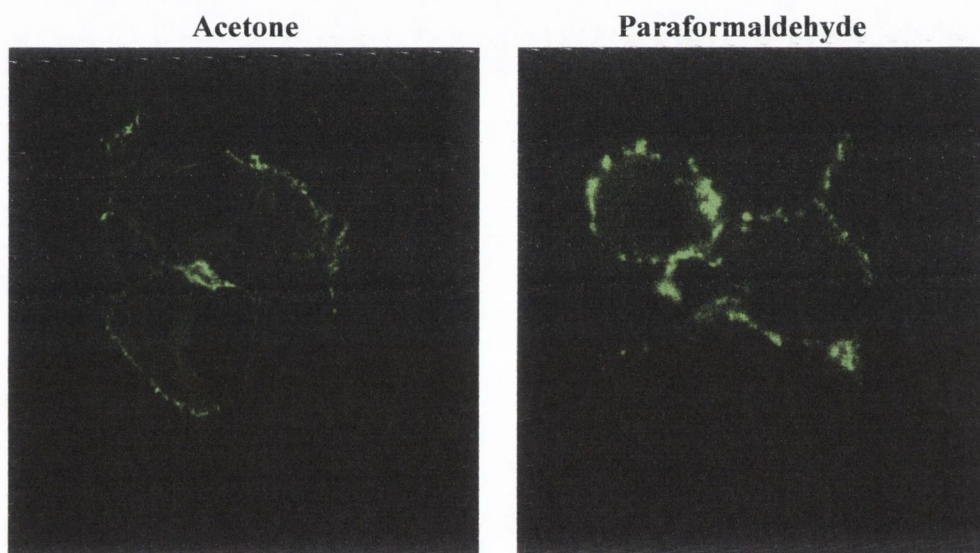


Fig. 3.6

SERT-FLAG cellular localization in 293/IMI270 cells

293/IMI270 cells were fixed as described in Methods using cold acetone for 10 minutes at -20°C (right), or 4% w/v paraformaldehyde for 10 minutes at RT (left). The serotonin transporter was detected using the monoclonal anti-FLAG antibody (1:1000). Samples were imaged using a MRC-1024 laser scanning confocal microscope (BioRad).



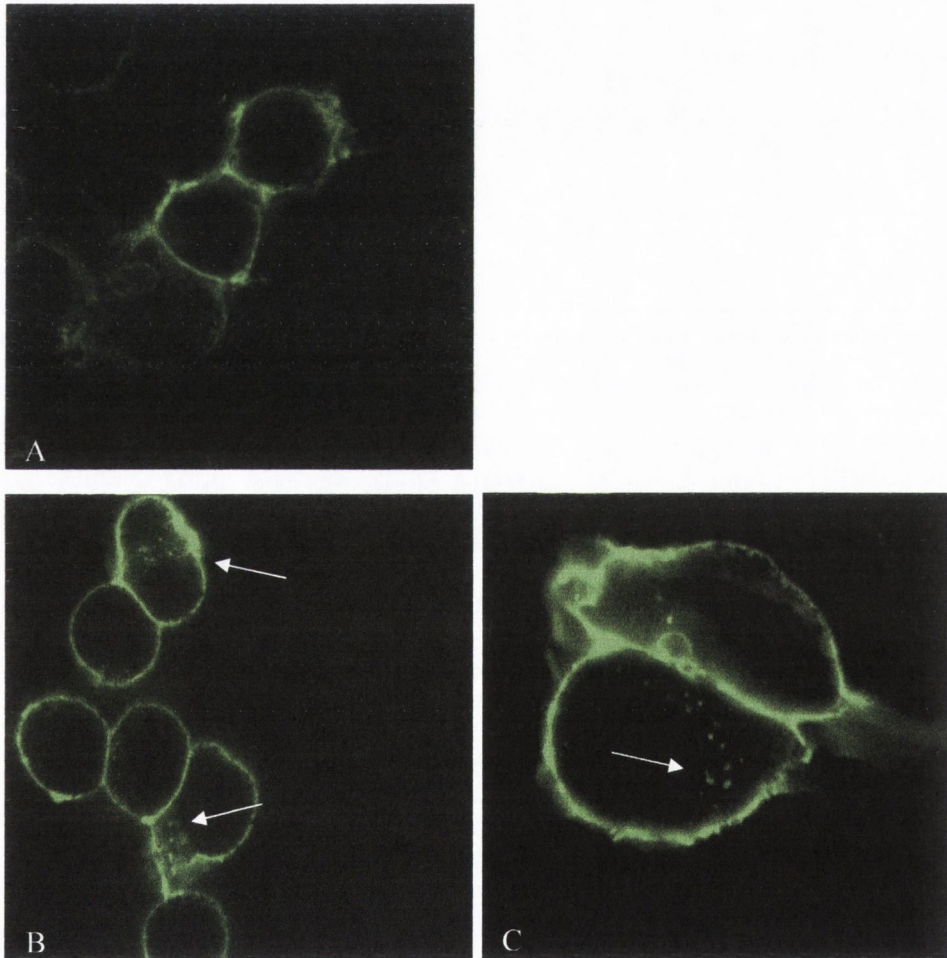
derivative, these authors showed that upon treatment with PMA the transporter was internalized. In the present study, confocal microscope imaging of cells treated with PMA appears to corroborate such a mechanism for transporter downregulation. Following incubation with PMA or the vehicle DMSO for 15 minutes at 37°C in the dark, 293/IMI270 cells were fixed with acetone and processed for cytochemistry as described in Methods. In PMA-treated cells, SERT-immunopositive intracellular vesicles of about 50-80 µm were evident (Fig. 3.7 B and C, arrows). In order to detect SERT-containing vesicles the power of the laser needed to be raised from 3% to 30%. However, using the same settings, control cells exhibited no vesicular intracellular labelling and SERT-immunopositivity was detected mainly at the plasma membrane (Fig. 3.7 A).

Unfortunately, the 293/IMI270 cell line did not represent an ideal model system. Even in presence of imipramine, the number of cells expressing SERT dramatically decreased to 20-30% after approximately 10-12 passages in culture compared to cells derived from early passages. Therefore, some event, independent of 5HT transport, but due to the expression of the transporter, appears to affect cell growth. Cells were still able to grow in selective medium and cell-death was not apparent. It is thus likely that cells that expressed very low levels of serotonin transporter progressively overgrew cells with higher SERT expression levels. In spite of the fact that the classical model of 5HT transport predicts zero net charge movement, currents associated with the rat SERT have been described (Mager et al., 1994). Particularly, rat- and *Drosophila*-SERT display 5HT- and inhibitors-independent currents (named 'leakage' currents) (Mager et al., 1994; Galli et al., 1997). It is possible that the presence of leakage currents may disturb the osmolarity of 293/IMI270 cells, with affecting the viability of cells expressing high levels of SERT. Consequently, SERT expression in this cell line progressively decreased and experimental results obtained for 5HT uptake and confocal microscopy became less reproducible.

Fig. 3.7

Internalization of SERT upon treatment with PMA in 293/IMI270 cells

293/IMI270 cells were incubated with DMSO (A) or 1 μ M PMA (B, C) for 15 minutes. Cells were fixed with acetone as described in Methods and stained with the monoclonal anti-FLAG antibody (1:1000). Arrows indicate intracellular vesicles containing SERT. C) Cells treated with 1 μ M PMA, shown at higher magnification.



In order to overcome problems generated by the constant presence of high levels of SERT, the use of inducible expression systems for the serotonin transporter was investigated.

3.2.2 Characterization of BHK/CytTS cell line

The pCytTS system uses the Sindbis virus replicase stably expressed in tandem with the SERT-FLAG construct on the same RNA (Tate et al., 2003). The replicase, positioned upstream of SERT coding sequence, is inactive at 37°C and becomes active at 29-35°C. At permissive temperature, the replicase copies the negative strand RNA and, after second strand synthesis, multiple copies of SERT mRNA are generated using the subgenomic promoter located upstream from SERT.

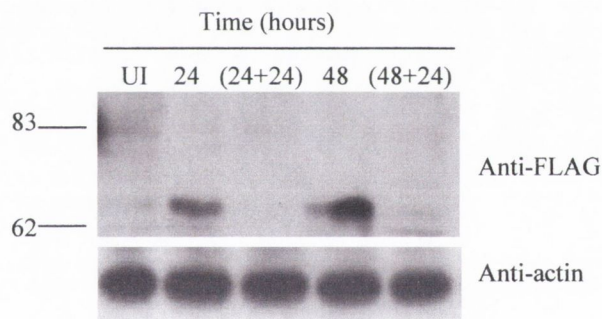
First, SERT expression-level in BHK/CytTS cells was tested by Western blotting. Decreasing the temperature to 29°C, at which the replicase becomes active, induced the expression of SERT-FLAG. In uninduced cells, SERT was weakly detected with the anti FLAG antibody probably due to a 'leaky' basal expression (Fig. 3.8, top panel). In fact, practical difficulties were experienced in keeping the temperature at 37°C during the propagation of the cells. After 24 hours of induction at 29°C a 75 kDa SERT-immunopositive band, corresponding to the partially glycosylated form of the transporter (Tate et al., 2003), was detected (Fig. 3.8). No fully glycosylated transporter could be detected, even after 48 hours at permissive temperature. The hypothesis that the induction at 29°C was preventing complete glycosylation of SERT, possibly by inhibiting the ER to Golgi trafficking, was tested. Following induction for 24 or 48 hours, BHK/CytTS cells were left to recover for an additional 24 hours at 37°C. This attempt to rescue the processing of the SERT was unsuccessful, resulting in the undetectability of SERT-FLAG, possibly due to protein degradation.

Confocal microscopy imaging confirmed the presence of a few SERT-FLAG positive elements in uninduced cells (Fig. 3.9, left panel). After 24 or 48

Fig. 3.8

Expression of SERT-FLAG fusion protein in BHK/CytTS cells

BHK/CytTS cells were seeded onto 10 cm Petri-dishes and grown at 37°C. SERT-expression was induced by placing cells at 29°C for 24 or 48 hours. In the attempt to improve SERT post-traslational processing, cells were placed back at 37°C for an additional 24 hours after induction (indicated as 24+24 and 48+24). In parallel, uninduced cells (UI) were grown for 48 hours at the non-permissive temperature. Cell extracts were analyzed by Western blotting. The blot was probed with the monoclonal anti-FLAG antibody (1:1000), and subsequently washed and reprobed with the monoclonal anti-actin antibody (1:5000) to confirm equal protein loading onto the gel.



hours of induction at 29°C the number of SERT-expressing cells appeared to be increased (Fig. 3.9, middle and right panel, respectively). The use of a cell-surface marker showed that in these cells SERT did not localize to the plasma membrane of cells (Tate et al., 2003).

In addition, the transporter glycoform expressed by CytTS cells correspond to a transporter decorated with a high-mannose glycan-moiety, sensitive to the action of the endoglycosidase EndoH (Tate et al., 2003). The high-mannose core of N-glycoproteins is co-translationally added to the polypeptide in the ER. Further modifications of N-glycans to a complex-type, EndoH-insensitive, occur in the Golgi stack, prior to the delivery of the protein to the plasma membrane. Thus, the glycosylation status of SERT expressed by CytTS cells suggested that the transporter was not efficiently localized to the cell surface. To further investigate this possibility, cell surface biotinylation was performed on induced and uninduced CytTS cells as described in Methods. Briefly, cells were incubated in ice-cold PBS⁺⁺ in absence or presence of the impermeant biotin, Sulfo-NHS-Biotin. Cells were lysed and cleared lysates were incubated with NeutrAvidin beads to specifically bind biotinylated material. Therefore, the unbound fractions represented the intracellular portion of protein, not exposed at the cell surface. The beads were boiled in 3x SDS sample buffer to elute bound proteins, representing the fraction of proteins exposed at the cell surface. Fig. 3.10 shows that the transporter was not detected at the cell surface (top panel). In order to confirm that no intracellular material had been biotinylated, the blot was reprobed for the cytosolic protein actin (fig. 3.10, middle panel). Finally, the blot was stripped and reprobed with Streptavidin-HRP to confirm that the NeutrAvidin beads effectively bound most of the biotinylated proteins.

Functional assays revealed the presence of 5HT saturable uptake in uninduced BHK/CytTS cells, with a $V_{\max}$ of 7.8 ± 0.5 pmol/min/ 10^6 cells and a K_m of 266 ± 86 nM. Over 48 hours of induction, these values progressively dropped to a $V_{\max}$ of 5.8 ± 1.1 pmol/min/ 10^6 cells and a K_m of 159 ± 35 nM (Tate et al., 2003). The presence of serotonin uptake in uninduced cells appears to be due not

Fig. 3.9

SERT-FLAG cellular localization in BHK/CytTS cells

BHK/CytTS cells were seeded onto poly-L-lysine coated slides in 6-well plate and were grown at 37°C for 24 hours. Cells were induced at 29°C for the indicated time or left at 37°C for an additional 48 hours (uninduced cells, UI). Cells were then fixed with acetone and labelled using the monoclonal anti-FLAG (1:1000).

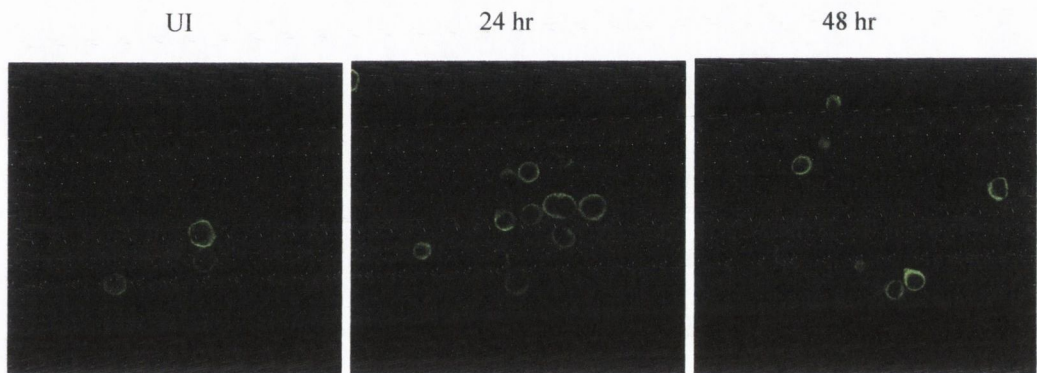
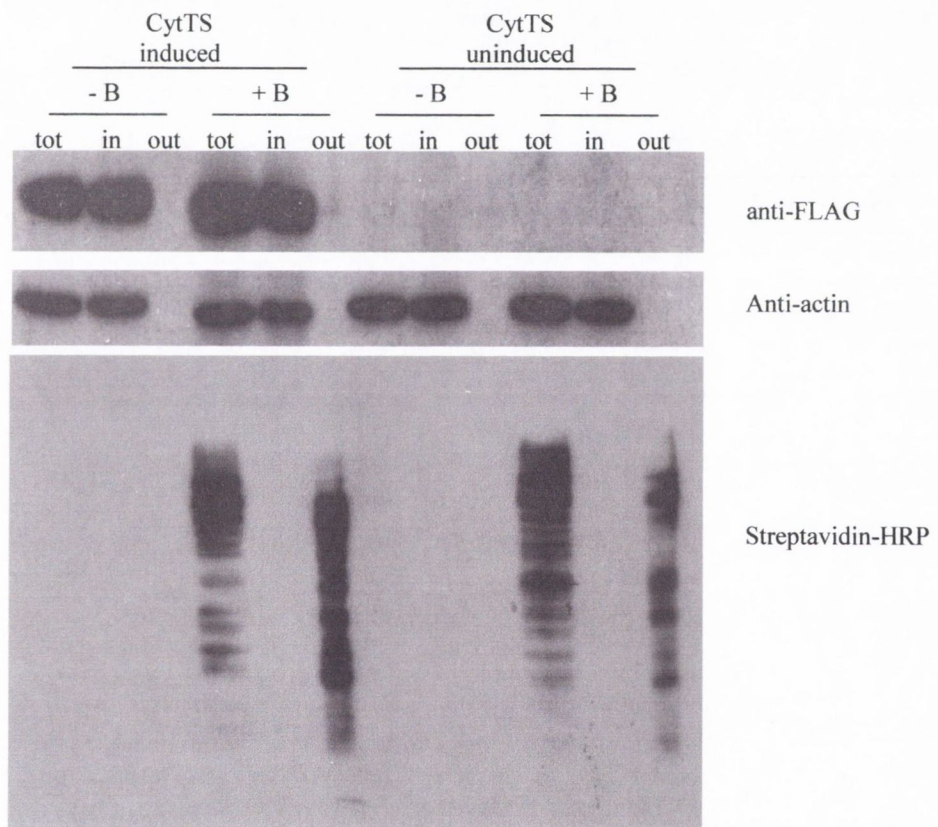


Fig. 3.10

SERT-FLAG expressed by BHK/CytTS cells does not localizes at the cell surface

CytTS cells were incubated in ice-cold PBS⁺⁺ in absence (-B) or presence of Sulfo-NHS-Biotin (+B) and cell surface biotinylation was performed as described in Methods. An aliquot of cleared lysates (designated 'total'), unbound fractions (intracellular, 'in') and bound proteins (cell surface, 'out') were loaded onto a 10% poly-acrylamide gel. The blot was probed with the monoclonal anti-FLAG antibody (1:1000), and then stripped and reprobed sequentially with the monoclonal anti-actin antibody (1:5000) and with Streptavidin-HRP (1:500).



only to the aforementioned leakage expression of SERT-FLAG, but also to the presence of an endogenous serotonin transporter in BHK cells (Jana Haase, unpublished data).

In conclusion, the BHK/CytTS cell line failed to produce detectable levels of fully matured transporter. SERT was not efficiently delivered to the cell surface but was mostly retained intracellularly, possibly due to the low temperature required for the induction of SERT expression that may inhibit the transporter intracellular trafficking from the ER to the Golgi apparatus.

3.2.3 Characterization of 293/TREx-SERT cell line

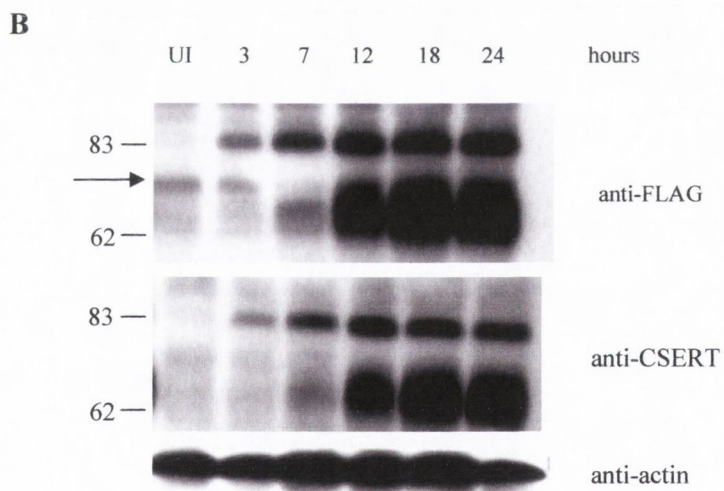
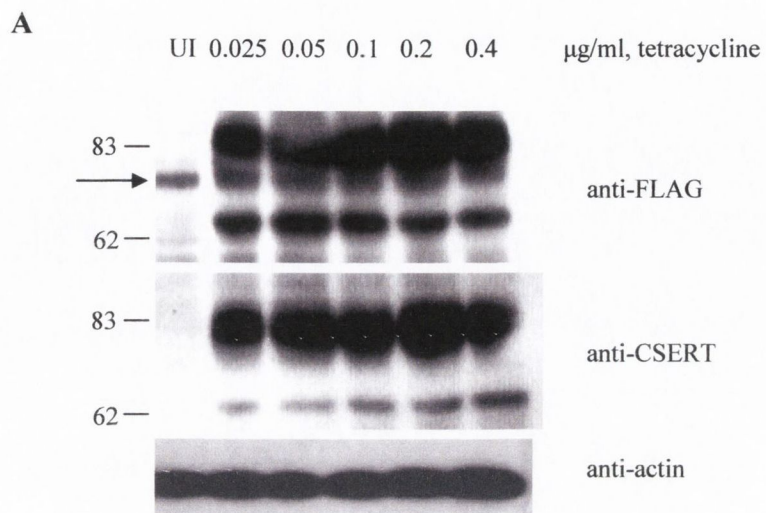
The T-REx system (Invitrogen) relies on the presence of a bacterial tetracycline-repressor protein (TetR), which binds to the Tet-operator engineered into the CMV promoter region, inhibiting transcription. 293/TREx cells (Invitrogen) are 293HEK cells stably expressing TetR. 293/TREx cells are stably transfected with SERT-His10-FLAG to generate the 293/TREx-SERT cell line (hereafter referred to as TREx-SERT). The fusion protein is transcribed under the control of a CMV promoter followed by two Tet-operator sites (Tate et al., 2003). In the absence of tetracycline, the constitutively expressed TetR turns off the expression of the SERT. Expression of the SERT is induced by the addition of tetracycline to the growth medium.

Protein expression was analysed with respect to tetracycline-induction, by analyzing the expression levels obtained with various tetracycline concentrations and at different time-points of induction. SERT was strongly expressed after 24 hours of induction with 0.025 µg/ml of tetracycline, corresponding to only one fifth of the tetracycline concentration recommended by the manufacturer (Fig. 3.11, A). Time-dependence of induction was tested as well. The partially glycosylated transporter was first detected after 3 hours of induction with 1 µg/ml of tetracycline (Fig. 3.11, B). The fully glycosylated transporter of M_r 83,000 was

Fig. 3.11

Expression of SERT-FLAG fusion protein in TREx-SERT cells

TREx-SERT cells were seeded onto a 6-well plate. A day later cells were treated with the indicated concentration of tetracycline for 24 hours (A) or with 1 $\mu\text{g/ml}$ tetracycline for the indicated times (B). Samples were analyzed by Western blotting. Blots were probed with the monoclonal anti-FLAG antibody (1:1000, top panel), stripped and reprobed with the polyclonal anti-CSERT antibody (1:2000, middle panel), directed against the C-terminus of SERT. The band marked with an arrow represented non-specific binding of the anti-FLAG antibody. Blots were reprobed for actin to verify equal protein loading (bottom panel).



detected after 7 hours of induction. The peak of expression appeared to be at 18 hours post-induction.

Laser scanning confocal microscopy was used to analyze the subcellular distribution of SERT upon induction of expression. After 3 hours of induction with 1 $\mu\text{g/ml}$ tetracycline, FLAG-immunopositivity was evident, mainly in perinuclear position, which morphologically resembles the Golgi apparatus (Fig. 3.12, B). After 7 hours, and even more strongly after 12 hours, the transporter localized at the cell surface. Uninduced cells did not express a detectable amount of SERT (Fig. 3.11 and 3.12). Cell surface biotinylation confirmed that the transporter expressed by TREx-SERT cells is localized to the cell surface, even though the majority of it was found in intracellular fractions (Fig. 3.13).

The functionality of SERT expressed in TREx-SERT cells was investigated by [^3H]-5HT uptake assays. Uninduced cells displayed a saturable [^3H]-5HT uptake, with V_{max} of 3.2 ± 0.3 pmol/min/ 10^6 cells and K_m of 329.3 ± 125.3 nM. However, the overnight induction of cells with 1 $\mu\text{g/ml}$ tetracycline elevated by more than 20-fold the specific [^3H]-5HT uptake (Fig. 3.14; V_{max} of 73.6 ± 8.1 pmol/min/ 10^6 cells and K_m of 1367.5 ± 419.8 nM). Notably, the affinity for the substrate appeared to decrease upon increase of the level of SERT expression. This observation will be further discussed at the end of this chapter.

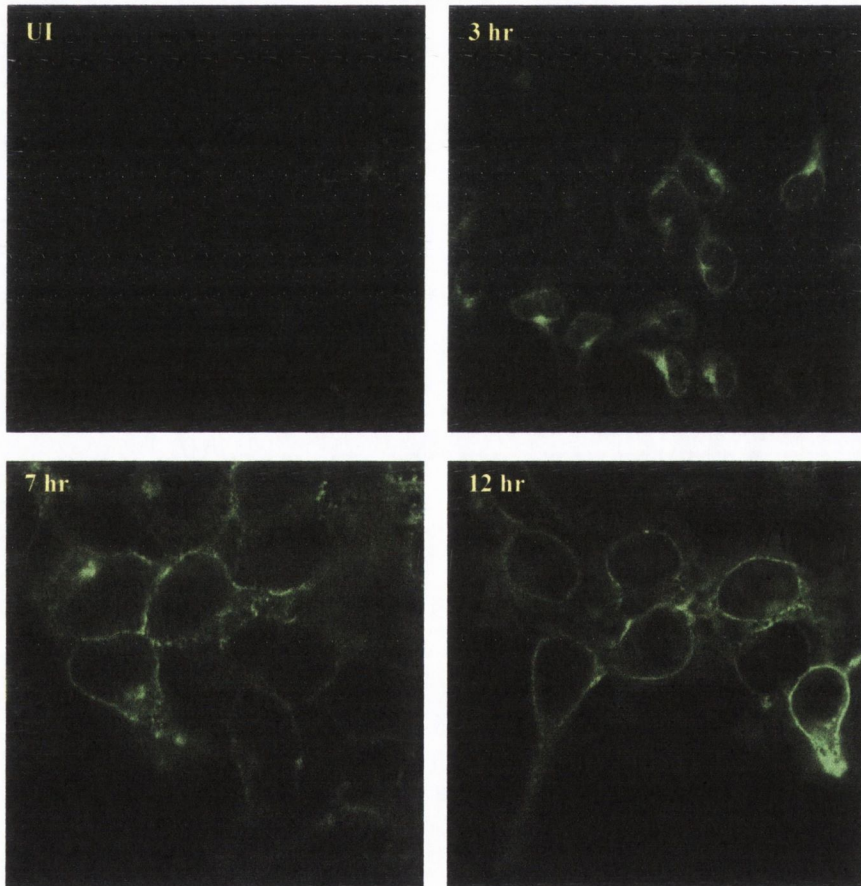
Responsiveness to PMA-downregulation of SERT expressed in TREx-SERT cells was examined. High levels of SERT expression could potentially mask the internalization of the transporter from the cell surface to the cytoplasm induced by PMA. Therefore, in the attempt to decrease the levels of SERT expression, cells were induced with only 10 ng/ml tetracycline. The induction of TREx-SERT cells with 10 ng/ml tetracycline was shown to elicit the half maximal induction of measurable SERT expression obtained with 1 $\mu\text{g/ml}$ tetracycline (Tate et al., 2003). 24 hours later, cells were incubated with 1 μM PMA for various time as indicated in Fig. 3.15. Single-point 5HT uptake assay showed that SERT was down-regulated upon PMA incubation. According to previous studies (Qian et al., 1997), maximum inhibition of uptake in TREx-SERT cells was

Fig. 3.12

SERT-FLAG subcellular localization in TREx-SERT

TREx-SERT cells were seeded onto poly-L-lysine coated slides in 6-well plates. 24 hours later cells were induced with 1 $\mu\text{g/ml}$ tetracycline for the indicated time (A) or with various amounts of tetracycline for 24 hours (B). Cells were then fixed with acetone and immunostained with the monoclonal anti-FLAG antibody (1:1000).

A



B

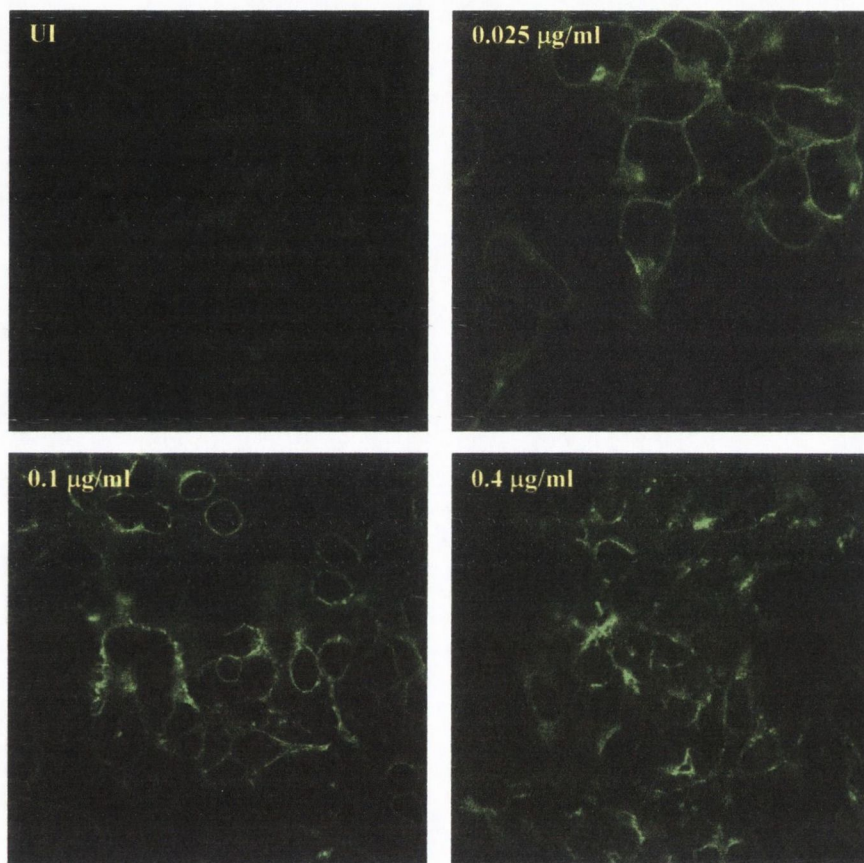


Fig. 3.13

Cell surface expression of SERT in TREx-SERT cells

Cells were seeded onto a 6-well plate and after 24 hours were induced with 1 µg/ml tetracycline. 24 hours later, cell surface biotinylation was carried out as described in Methods. An aliquot of cleared lysates (designated ‘total’), unbound fractions (intracellular, ‘in’) and bound proteins (cell surface, ‘out’) were loaded onto a 10% poly-acrylamide gel. The blot was probed with the monoclonal anti-FLAG antibody (1:1000), and then sequentially stripped and reprobed with the monoclonal anti-actin antibody (1:5000) and with Streptavidin-HRP (1:500).

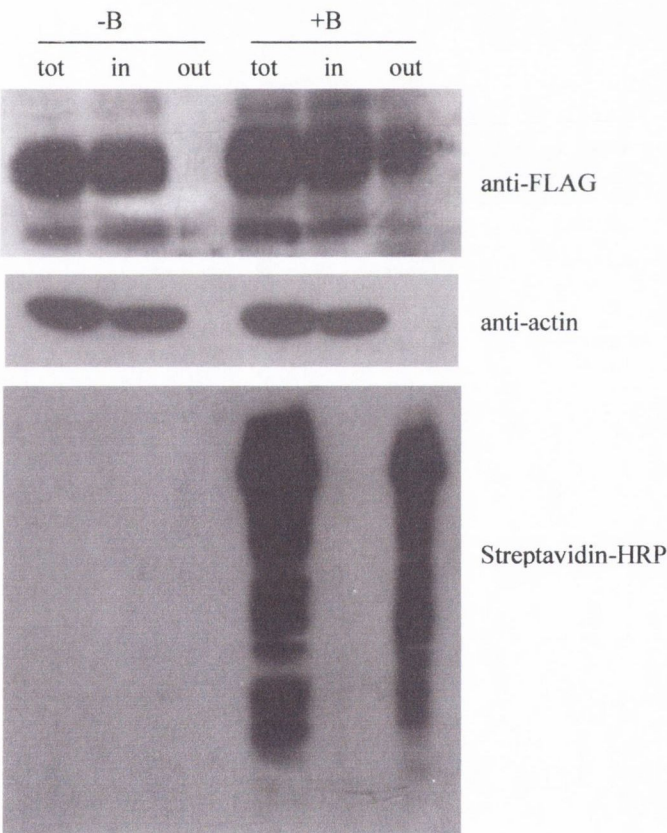


Fig. 3.14
Specific uptake of [³H]-5HT into TREx-SERT cells

TREx-SERT cells were seeded in poly-L-lysine coated 24 well-plates and grown overnight in presence of 1 $\mu\text{g/ml}$ tetracycline. Uninduced cells displayed a V_{max} of 3.2 ± 0.3 pmol/min/ 10^6 cells, a K_m of 329.3 ± 125.3 nM. Cells induced overnight with 1 $\mu\text{g/ml}$ tetracycline showed V_{max} of 73.6 ± 8.1 pmol/min/ 10^6 cells, a K_m of 1367.5 ± 419.8 nM. Non-specific uptake was defined as the uptake in presence of 10 μM Paroxetine and subtracted from the total uptake as described in Methods. Each data point was assayed in triplicate in each experiment and the experiment was repeated three times with similar results. The plot was graphed using the non-linear regression equation of the software SigmaPlot™.

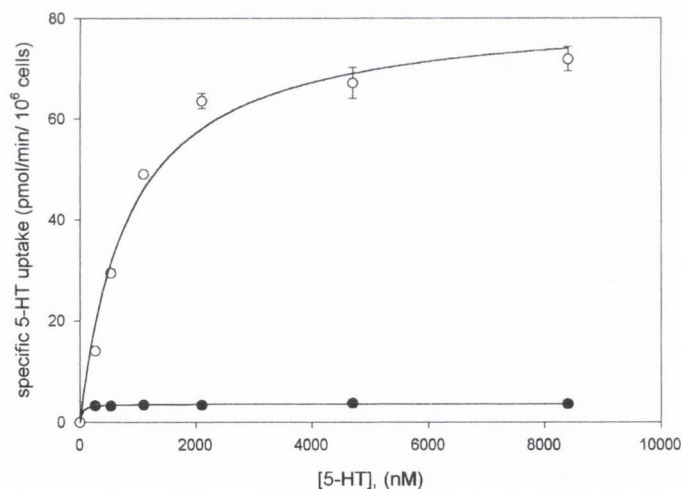
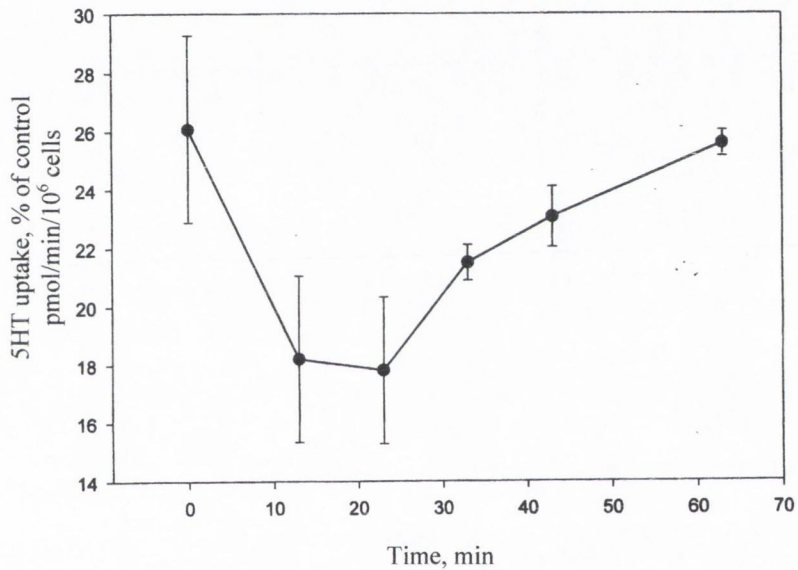


Fig. 3.15

Regulation of 5HT transport by PKC activation in TREx-SERT cells

TREx-SERT cells were grown in poly-L-lysine coated 24-well plates. Induced cells were incubated for the indicated time with 1 μ M PMA diluted in DMSO. In parallel, control cells were treated with an equal volume of DMSO. The experiment was assayed in triplicate; each data point was plotted as mean value $\pm$ SE. The graph shown is representative of two independent experiments.



observed at 20-25 minutes incubation. However, as seen before for the 293/IMI270 cell line, a complete recovery to control value was observed at later time points.

Taken together these results show that the TREx-SERT cell line expresses high levels of functional serotonin transporter.

3.3 Discussion

Since its cloning in 1991 (Blakely et al., 1991; Hoffman et al., 1991), heterologous expression of SERT has been the tool of choice to perform functional and structural studies. In order to broaden the understanding of the function of this pharmacologically important protein, several heterologous expression systems have been investigated over the years. Although the serotonin transporter has been extensively studied for more than 20 years, very little is known of its structure and regulation.

Due to initial difficulties in detecting rSERT by confocal microscopy in our laboratory, the expression of a FLAG-tagged SERT in stable transfectants was investigated. For this purpose, three different cell lines were characterized to test their suitability for studying the regulation of SERT.

293/IMI270 constitutively expressed a functional rSERT as shown by [^3H]-5HT uptake, which is almost exclusively localized on the plasma membrane. Unfortunately, a serious pitfall of this cell line was the poor growth rate displayed. After a few passages in culture, only 20-30% of the cell population expressed SERT. The attempt to clonally select 293/IMI270 cells to circumvent this problem, was not successful (Tate et al., 2003). SERT is known to be not well tolerated by cells when expressed at high levels. The reason for this was thought to be the flux of ions that is coupled to the transport of 5HT, which may alter the osmolarity of SERT-expressing cells, with negative consequences for their viability. However, even with the inclusion of a SERT inhibitor in the growth medium, the increase of number of passages in culture appears to result in a positive selection for 293/IMI270 cells that expressed low levels of transporter, possibly favoured by their higher rate of growth.

A similar negative selection towards SERT expressing cells had been described by Qian and colleagues (Qian et al., 1997): they hypothesized that the presence of 5-HT in the complete growth medium could be detrimental to the viability of cells heterologously expressing SERT, even though they too have failed to reduce the toxicity by growing cells in presence of a SERT antagonist. Therefore, the ion flux coupled with 5HT transport is not the sole cause that

affects the viability and growth rate of these cells. An explanation of such a reduced viability may be offered from the study of SERT-associated currents (Mager et al., 1994; Galli et al., 1997). In fact, even though the current model of SERT transport-cycle is electroneutral, both rat- and drosophila-SERT exhibit currents. Two of these currents are associated with the substrate transport and are antidepressant-sensitive. A third current ('leakage' or 'constitutive' current) has been measured in absence of serotonin and is sensitive to SERT-inhibitors. It is thus possible that this leak current may generate an electrolytic imbalance, which is detrimental to cell viability.

The decreased expression of SERT after a few passages in cell culture enhanced considerably the difficulties in obtaining reproducible results. For this reason the 293/IM1270 cell line was not considered suitable to carry out subsequent experiments.

The production over the past few years of inducible systems of protein expression has considerably helped the study of cytotoxic proteins. Two different inducible expression systems were tested. The first one was the BHK/CytTS cell line, where the expression of SERT was cold-induced by decreasing the temperature from 37°C to 29°C. The system relies on a retroviral replicase, which yields a logarithmic production of the protein of interest. However, BHK/CytTS cells expressed very low levels of functional SERT. These cells also displayed a significant basal expression of SERT as shown by [³H]5-HT uptake, Western blotting and confocal microscope imaging. It is likely that such a basal expression could be due to a leak in the cold inducible system, caused for example by the difficulty in keeping cells at 37°C during the time required for their propagation. Moreover, the presence of specific 5HT uptake has been measured in BHK cells (Jana Haase, unpublished results), suggesting that these cells may express an endogenous serotonin transporter.

Even though after induction SERT was strongly expressed, the protein produced was the result of an incomplete post-translational processing. High levels of unglycosylated and partially glycosylated transporter were produced, possibly as a consequence of the low temperature (29°C) used to induce the cells. Cell surface biotinylation experiments showed that SERT was not present at the

cell surface of BHK/CytTS cells. As it will be further discussed in Chapter 6, glycosylation is important for folding of glycoproteins. Thus, it is possible that the unglycosylated transporter may accumulate in the endoplasmic reticulum as unfolded material and not be delivered to the plasma membrane. The attempt to overcome this problem by increasing the temperature of induction to 33°C did not improve the expression of functional SERT (Tate et al., 2003). Therefore, the BHK/pCytTS cell line was not used for further experiment.

The second inducible expression system examined was the TREx-SERT cell line, where SERT expression was turned on by the addition of the antibiotic tetracycline to the growth medium. The electrophoretic analysis and the 5HT uptake assay showed that the serotonin transporter produced by these cells is a glycosylated protein located at the cell surface and is functional. Thus, this system of expression overcomes the negative effect on cell viability shown by the non-inducible 293/IMI270 stable cell line. Moreover, the tetracycline inducible system has the advantage to allow the modulation of protein expression levels by varying the concentration of antibiotic used (e.g. 10 ng/ml of tetracycline are enough to achieve half-maximal 5HT uptake (Tate et al., 2003)). One of the problems raised by the overexpression of a given protein is that the high concentration of such a protein can impair the observation of ongoing regulatory processes. The possibility to fine-tune protein expression in the TREx system could be critical in studying SERT regulation. On the other hand, for structural studies it is fundamental to achieve high levels of expression, in order to isolate the protein of interest for the production of 2D- or 3D-crystals. When sodium butyrate is added to the growth medium together with tetracycline, SERT expression levels are 5-times enhanced (Jana Haase, personal communication). This is perhaps caused by the ability of butyrate to inhibit the enzyme histone deacetylase, thus abrogating transcriptional repression (Kruh, 1982). The exploitation of trichostatin A, a more specific and potent histone deacetylase inhibitor (Yoshida et al., 1995; Condreay et al., 1999), to augment the production of SERT in TREx-SERT cells, may yield enough active protein for crystallization.

Table 3.1 lists 5-HT uptake values for the three cell lines. Notably, the K_m appears to increase with the expression levels of functional SERT. A similar variation in substrate affinity has been recently observed in *Xenopus* oocyte expressing SERT (Ramsey and DeFelice, 2002). The authors propose that the affinity for substrate and inhibitors of the serotonin transporter may be sensitive to the expression level and that SERT may interact with an unknown endogenous factor of limited abundance that governs the interaction of the transporter with substrate and ligands. Additional comments to this hypothesis will be presented in Chapter 7.

In conclusion, the TREx inducible system produced high levels of functional transporter and was therefore used to investigate novel mechanisms of SERT regulation, presented in the following chapters. The introduction of a tag at the C-terminus of SERT did not appear to alter the re-uptake function of the transporter, even though it may alter SERT downregulation upon PKC activation. Prior to the start of this project, the attempt to detect SERT by confocal microscopy using the commercial antibody anti-CSERT had been unsuccessful. The production of SERT-FLAG by TREx-SERT cells eventually allowed the immunocytochemical detection of the transporter. However, during the course of this project the detection of SERT using the anti-CSERT antibody, directed to the transporter C-terminus, was finally achieved (mainly by modifying the fixation conditions, data not shown). In addition, an antibody against the transporter N-terminus became available (anti-NSERT), which recognizes both the native- and the denatured-serotonin transporter.

Table 3.1: Summary of [3H]-5HT uptake for different mammalian cell line.
 The table is compiled using my own data presented in this study (293/IMI270 and TREx-SERT cells) and data obtained by Dr. Jana Haase (BHK/CytTS cells).

	$V_{\max}^1$ (pmol/min/ 10^6 cells)	K_m^1 (nM)
293/IMI270	29.9 ± 1.9	1024 ± 138
BHK/CytTS uninduced	7.8 ± 0.5	266 ± 86
induced (48h, 29°C)	5.8 ± 1.1	159 ± 35
293/T-Rex uninduced	3.2 ± 0.3	329.3 ± 125.3
induced (1 µg/ml tetracycline)	73.6 ± 8.1	1367.5 ± 419.8

¹ 5-HT uptake assays were carried out as described in Methods. Data were fitted to the Michaelis-Menten equation using the non-linear regression equation of the software SigmaPlot™. $V_{\max}$ and K_m values given are the mean $\pm$ SEM of 2-4 independent experiments.

Chapter 4 Results

Heterologous expression of syntaxin 1A impairs SERT trafficking to the plasma membrane

4.1 Introduction

4.1.1 Syntaxin 1A

Syn 1A was the first t-SNARE to be cloned and for long time syn 1A had been considered specific to neural tissues. However, Martin-Martin and colleagues have found that syn 1A is also expressed in peripheral blood lymphocytes and neutrophils, mainly in the membranes of cytoplasmic granules in human resting neutrophils (Martin-Martin et al., 1999).

Syn 1A consists of a long N-terminal domain, forming a bundle of three anti-parallel α -helices (Ha, b, c), followed by a coiled coil region (SNARE motif or H3 domain) and a C-terminal transmembrane domain. In the absence of VAMP 2 and SNAP 25, the syn 1A N-terminal domain is folded back onto the SNARE motif to form a four-helix bundle resembling the SNARE complex. This conformation of syntaxin 1A is referred as the 'closed' conformation, as opposed to the 'open' conformation when the SNARE complex forms in presence of SNAP 25 and VAMP. In the open conformation of syn 1A the N-terminal domain is extended from the SNARE motif. The structure of the cytoplasmic portion of syn 1A has been solved in solution by NMR (Fernandez et al., 1998), and the structure of the domain participating in the SNARE complex has been solved for syn 1A, synaptobrevin-II and SNAP-25B (Sutton et al., 1998). It has been proposed that exocytotic events are regulated by switching of syn 1A between the closed and open conformation, since the interaction of syn 1A with its specific binding partners, VAMP 2 and SNAP-25, can be regulated this way.

However, syn 1A and its regulatory proteins seem to exert more complex functions, which are not only limited to the vesicle docking/fusion events. Syn 1A has been shown to bind and to stabilize the inactivated state of N- and Q-type

Ca^{++} -channels in the mammalian central nervous system (Sheng et al., 1994; Bezprozvanny et al., 1995). Therefore, it is possible that synaptic vesicles may be tethered to the presynaptic membrane until the formation of a v/t-SNARE pair relaxes the inactivated Ca^{++} -channel. The docked complex would then respond to a depolarising signal and allow a localized Ca^{++} -flux. However, Ca^{++} -channels that are bound to syn 1A but are not engaged in a docked state would remain refractory to a depolarising signal.

Syn 1A has also been shown to physically interact and regulate the cystic fibrosis transmembrane conductance regulator (CFTR)-channel both in *Xenopus* oocytes and epithelial cells that normally express these proteins. The interaction is blocked by Munc 18-1, a syn 1A-binding protein (Naren et al., 1997).

4.1.2 Syntaxin 1A and neurotransmitter transporters

More recently, attention was focused on the interplay between syn 1A and neurotransmitter transporters: syn 1A has been shown to interact with the N-terminal cytoplasmic domain of GAT 1 and to down-regulate GABA uptake by reducing V_{max} (Beckman et al., 1998; Deken et al., 2000). However, in hippocampal neurons syn 1A acutely increases GAT 1 surface expression (Horton and Quick, 2001). Thus, it is possible that syn 1A regulate GAT 1 in two distinct ways: by facilitating the insertion of the transporter into the plasma membrane and by inhibiting the transport of GABA. Furthermore, in primary cells the PKC-modulation of GAT 1 is exerted via syn 1A: in fact, GAT 1 mutants that fails to interact with syn 1A also become insensitive to PKC-dependent regulation (Beckman et al., 1998).

Analogous results have been obtained for the glycine transporters: both GLYT 1 and 2 co-immunoprecipitate with syn 1A in COS cells and in rat brain tissue. The interaction results in decreased glycine uptake, an effect reversed by coexpression of Munc18-1 (Geerlings *et al*, 2000). The same group has also shown that syn 1A is necessary for the arrival of GLYT 2 to the plasma

membrane, but it is not involved in its retrieval from the cell surface, during the endocytotic internalization mediated by PKC (Geerlings et al., 2001).

The interaction between syn 1A and Munc 18-1 (also known as n-sec1 in *S. cerevisiae*, unc18 in *C. elegans*, ROP in *D. melanogaster*) is intriguing. On the basis of work on mammalian cells, it has been suggested that the syn 1A/Munc18-1 interaction regulates neurotransmitter secretion. Syn 1A binds to Munc18-1 in a closed conformation, which is incompatible with the binding to SNAP-25 and synaptobrevin. In fact, the disruption of the closed conformation abolishes the ability of syn 1A to bind to Munc18-1 and inhibits the secretion of neurotransmitter in PC12 cells, suggesting that this conformation represents an essential intermediate in exocytosis (Dulubova et al., 1999). In adrenal chromaffin cells, Fisher and colleagues expressed a Munc 18-1 mutant with reduced affinity for syn 1A, which specifically modified the kinetics of post-fusion events of synaptic vesicles, consistent with an acceleration of fusion pore expansion (Fisher et al., 2001). Therefore, Munc18-1 is likely to function in a late stage in the membrane fusion process.

Thus, syn 1A appears to interact with and regulate a number of ion channels as well as the SERT-related GABA and glycine transporters. Whether regulated serotonin transporter trafficking and/or its activity are similarly affected by syn 1A interaction was unknown at the outset of this project. For this reason the role of syn 1A in regulating the serotonin transporter was investigated. Preliminary work was carried out in our laboratory in order to examine whether syn 1A interacts with SERT. By GST-pull down assay, syn 1A was shown to interact with the N-terminal domain of SERT. The first TMD of SERT was required for syn 1A interaction, and the binding was enhanced by high ion concentration, suggesting that formation of the complex depends on hydrophobic interactions. The *in vivo* interaction between SERT and syn 1A coexpressed in HEK cells or brain extracts could not be shown by coimmunoprecipitation in our laboratory. The heterologous expression of syn 1A and SERT in HEK cells resulted in a $V_{\max}$ reduction of 5HT transport (Haase et al., 2001). Changes in the maximum transport capacity could indicate either changes in the number of

transporter molecules at the cell surface or changes in the turnover rate of SERT. In order to discriminate between these two possibilities, the effect of syn 1A-heterologous expression in HEK cells on SERT subcellular distribution was examined.

4.2 Results

4.2.1 Syn 2 is the plasma membrane-associated syntaxin isoform endogenously expressed by HEK cells

Of the about 18 syntaxin isoforms identified so far, syn-1, -2, -3, and-4 have been shown to localize on the plasma membrane of cells. Syn 2-4 are ubiquitous, whereas syn 1A is mainly present on the plasma membrane of neurons. Little or no information was available regarding plasma membrane associated-syntaxin isoforms in HEK cells (the parental cell line of the TReX-SERT cells). For this reason, the endogenous expression of membrane associated-syntaxin isoforms in total cell extracts from HEK cells was investigated. As shown in Fig. 4.1, immunoblotting with antibodies specific for each of the four syntaxins identified syn 2 as the only plasma membrane associated-syntaxin isoform endogenously expressed in HEK cells. Syn 1A, 3, and 4 were not expressed in detectable amounts in HEK cells.

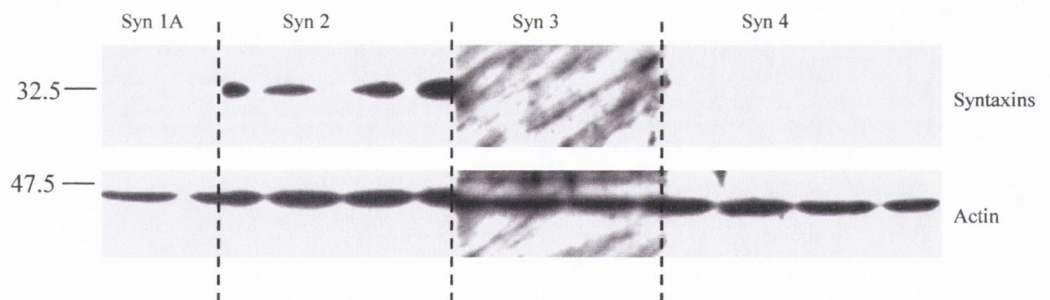
4.2.2 Effect of syn 1A heterologous expression on SERT cellular distribution

Using confocal microscopy the subcellular localization of syn 1A and SERT was investigated. SERT was primarily found on the plasma membrane of TReX-SERT cells (Fig. 4.2A, left panel), but homogenously distributed in the cytoplasm and plasma membrane of transiently transfected HEK cells (Fig. 4.2A, right panel). The different distributions of SERT between TReX-SERT cells and transiently transfected HEK cells is most likely due to higher expression levels, i.e. greater number of SERT molecules per cell, obtained by transient transfection that may slow down SERT maturation and its trafficking to the cell surface (see Chapter 6). The coexpression of SERT and syn 1A resulted in a rather dramatic change of the transporter's distribution. Both SERT and syn 1A concentrated in spheroidal intracellular structures with little apparent plasma membrane staining,

Fig. 4.1

Syn 1A is not immunodetected in HEK cell extracts.

Total cell extract was prepared from HEK cells. An equal volume of cell lysate was loaded into the wells of a 15 % polyacrylamide gel. Proteins were transferred onto a PVDF membrane as described in Methods. Following blocking, the membrane was cut into four pieces and each piece was immunoprobed with the monoclonal syn 1A- (1:2000), or the syn 2-, syn 3-, or syn 4-polyclonal antibody (1:500). Endogenous syn 2 was detected as a band of M_r 35,000. Blots were then reprobed with anti-actin monoclonal antibody (1:5000, bottom panel), showing equal protein loading.



either in TReX-SERT cells or in HEK cells (Fig. 4.2C). The localization of syn 1A in these unknown intracellular structures appeared not to be dependent on SERT. In fact, even in the absence of SERT, syn 1A was detected in such intracellular structures, whose number and size varied (1-3 μm ; Fig. 4.2B).

The optical sectioning along the z-axis of the sample was used to build a three-dimensional projection of the SERT- and syn 1A-immunofluorescent signal. Fig. 4.3A shows a stack of 14 z-sections collected with a 1 μm -step and below the corresponding three-dimensional projection (Fig. 4.3B). The scanning along the z-axis of SERT- and syn 1A-immunopositive cells revealed that some of the bigger structures appeared as tubular structures folded onto themselves. In HEK cells syn 1A and SERT extensively colocalized and were strongly detected in these intracellular structures. Thus, syn 1A appeared to affect the trafficking of SERT, causing the transporter to accumulate intracellularly in structures of unknown identity.

4.2.3 Syn 1A expression alters post-translational processing and cell-surface expression of SERT

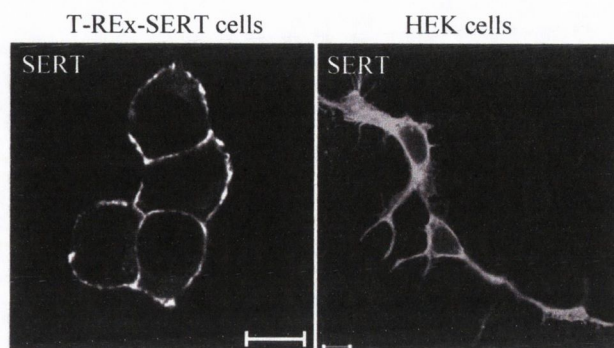
The subcellular distribution of SERT in the presence or absence of syn 1A was also investigated by cell surface biotinylation. The cotransfection of SERT and increasing amounts of syn 1A causes a reduction of fully glycosylated transporter with a concomitant increase of the partially glycosylated transporter (Fig. 4.4A). As shown in Fig. 4.4B, by increasing the expression levels of syn 1A the fraction of fully glycosylated SERT found at the cell surface was reduced compared to mock-transfected cells. SERT maturation was affected by the lowest amount of transfected pSyn 1A (0.15-0.3 μg DNA/ 32 mm dish). The observed effect on SERT subcellular distribution was thus unlikely to be an artifact due to overexpression of syn 1A *per se*. Similarly to the findings obtained by confocal microscopy, syn 1A was highly enriched in the intracellular fractions. However, by elevating the amount of transfected syn 1A-cDNA, syn 1A-immunoreactive bands were increasingly detected also at the cell surface.

Fig. 4.2

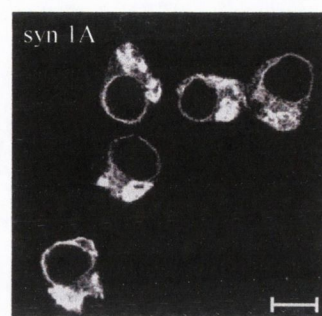
Subcellular localization of SERT and syn 1A.

Cells were seeded onto lysine-coated slides in a 6-well plate. A) T-REx-SERT (left panel) and HEK cells (right panel) were fixed after either induction with 1 $\mu\text{g/ml}$ tetracycline or transfection with pSERT-FLAG (0.3 μg DNA/ well). Cells were immunolabelled with the polyclonal anti-NSERT antibody (1:500). B) HEK cells were transfected with pSyn 1A (0.3 μg DNA/ well), and after 48 hours fixed and labelled with the monoclonal anti-syn 1A antibody (1:1000). Bars, 10 μm . C) T-Rex-SERT cells were induced with 1 $\mu\text{g/ml}$ tetracycline and transfected with pSyn 1A (top panel). HEK cells were cotransfected with an equal amount of pSERT-FLAG and pSyn 1A (bottom panel). Cells were processed for immunofluorescence as described above. Experiments were repeated 3-5 times with similar results.

A



B



C

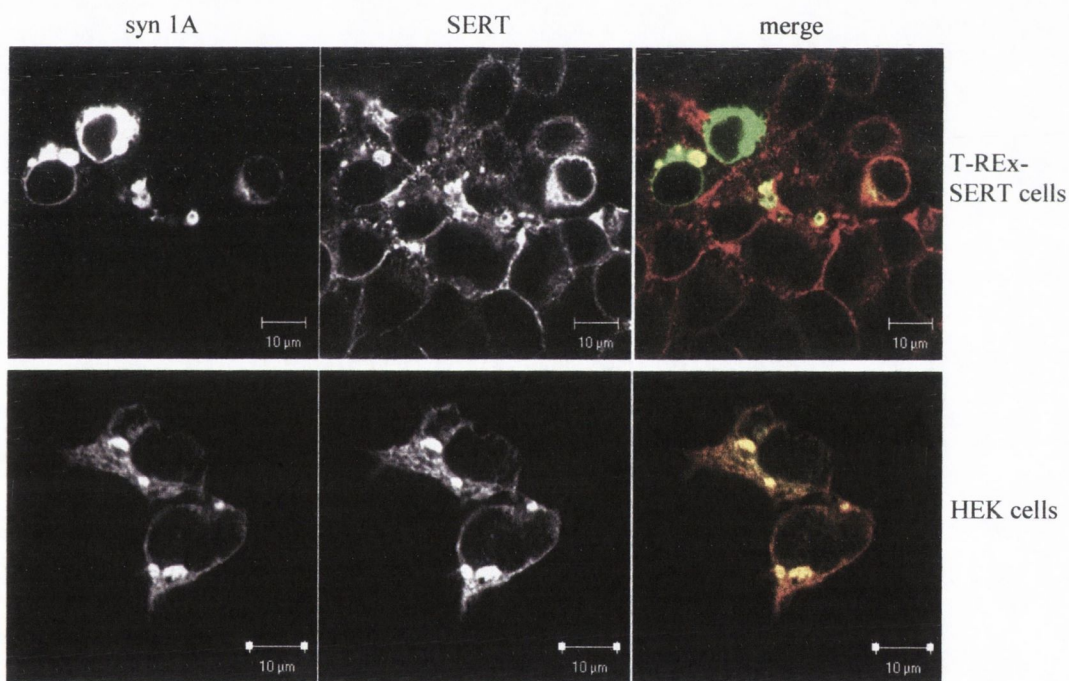
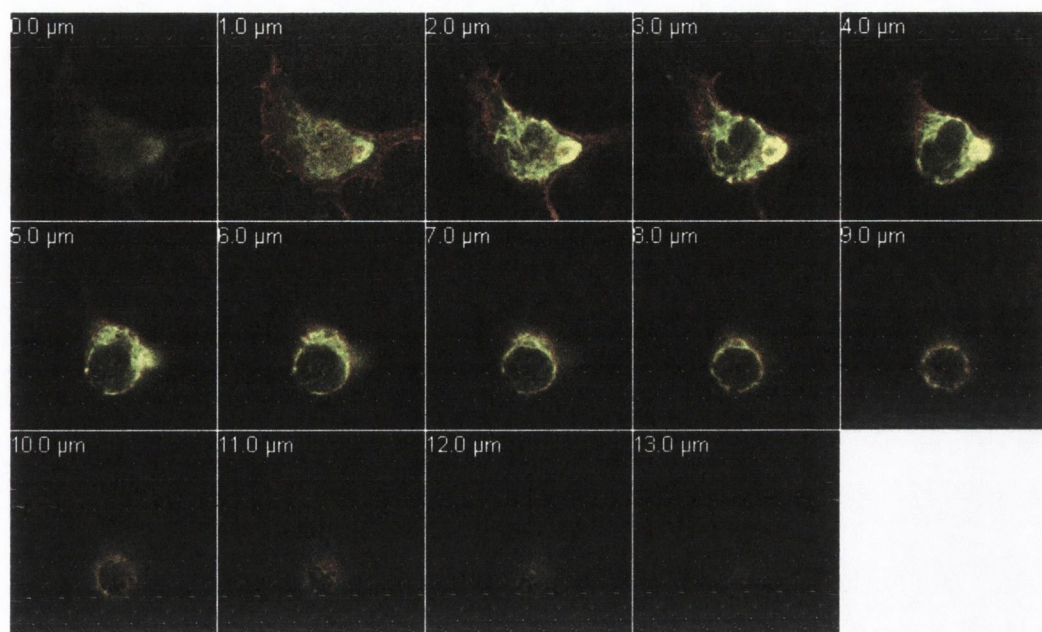


Fig. 4.3

Syn 1A overexpression changes SERT subcellular localization.

HEK cells were cotransfected for syn 1A and SERT and 48 hours later the cells were fixed and stained using the polyclonal anti-NSERT antibody (1:1000) and the monoclonal anti-syn 1A antibody (1:1000). A) The image represents the optical scanning of a representative cell coexpressing SERT (in red) and syn 1A (in green). A stack of 14 images was taken along the z-axis with 1 μm step and used to build a 3D image of the cell with the LaserSharp computer program (B). The 3D image was tilted of a few degrees to the left to show the distribution of the two fluorescent signals within the cell.

A



B

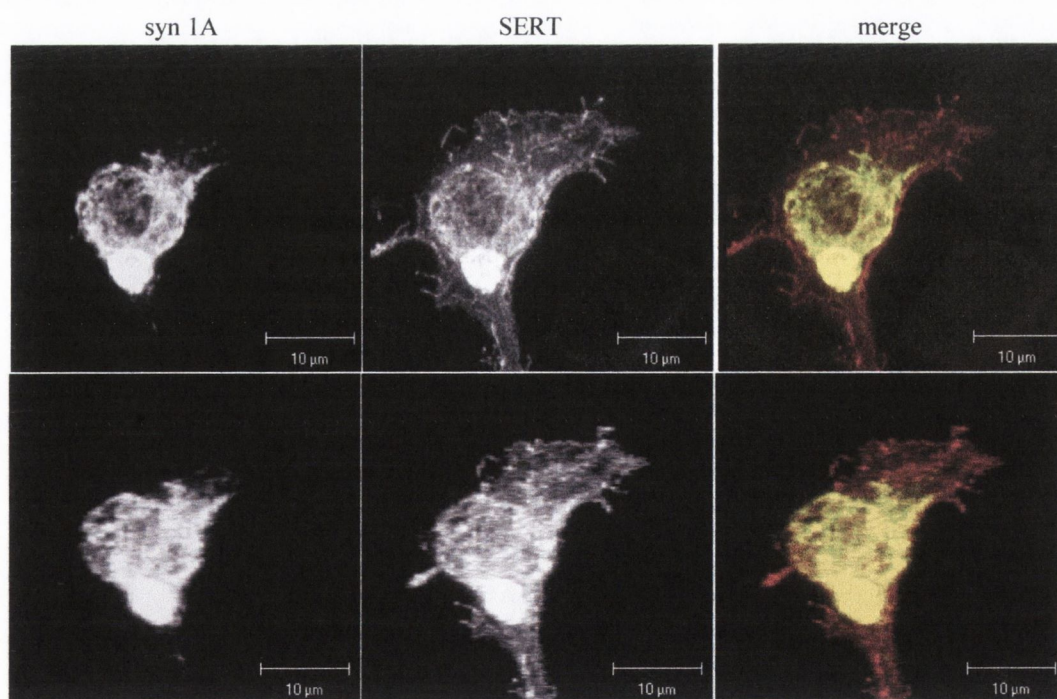
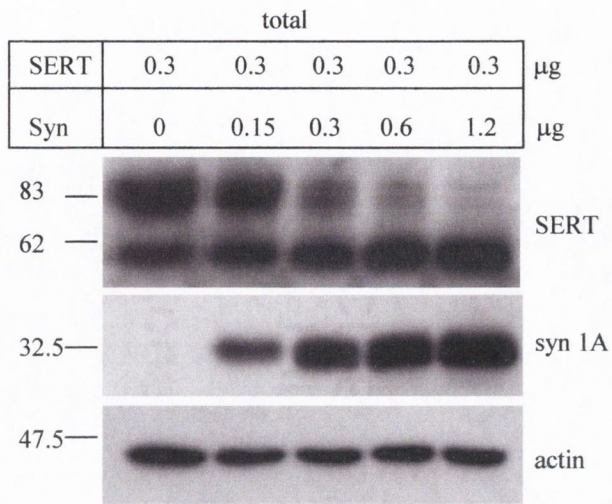


Fig. 4.4

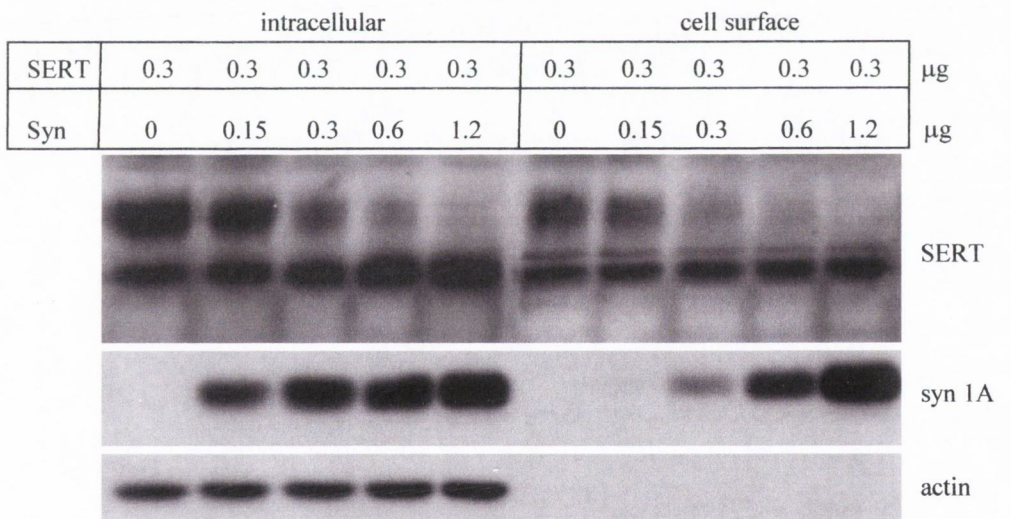
Syn 1A impairs post-translational processing of SERT and reduces its cell surface abundance.

HEK cells were seeded in 6-well plates and after 24 hours were cotransfected with 0.3 μg pSERT-FLAG and increasing amounts of pSyn 1A as indicated. The amount of total transfected DNA was kept constant by adding pcDNA3 to obtain a final 1.5 μg of transfected DNA. 48-72 hours after transfection cell surface biotinylation was carried out as described in Methods. The blot was probed with the polyclonal anti-NSERT antibody (1:6000), and sequentially stripped and reprobed with the monoclonal anti-syn 1A (1:2000) and the monoclonal anti-actin antibody (1:5000). The experiment was repeated three times with similar results.

A



B



These findings indicate that the previously observed decrease in transport capacity of 5HT in the presence of syn 1A (Haase et al., 2001) is likely to be due to a change in the number of fully glycosylated transporters located at the cell surface.

4.2.4 Syn 1A and SERT accumulates in the endoplasmic reticulum

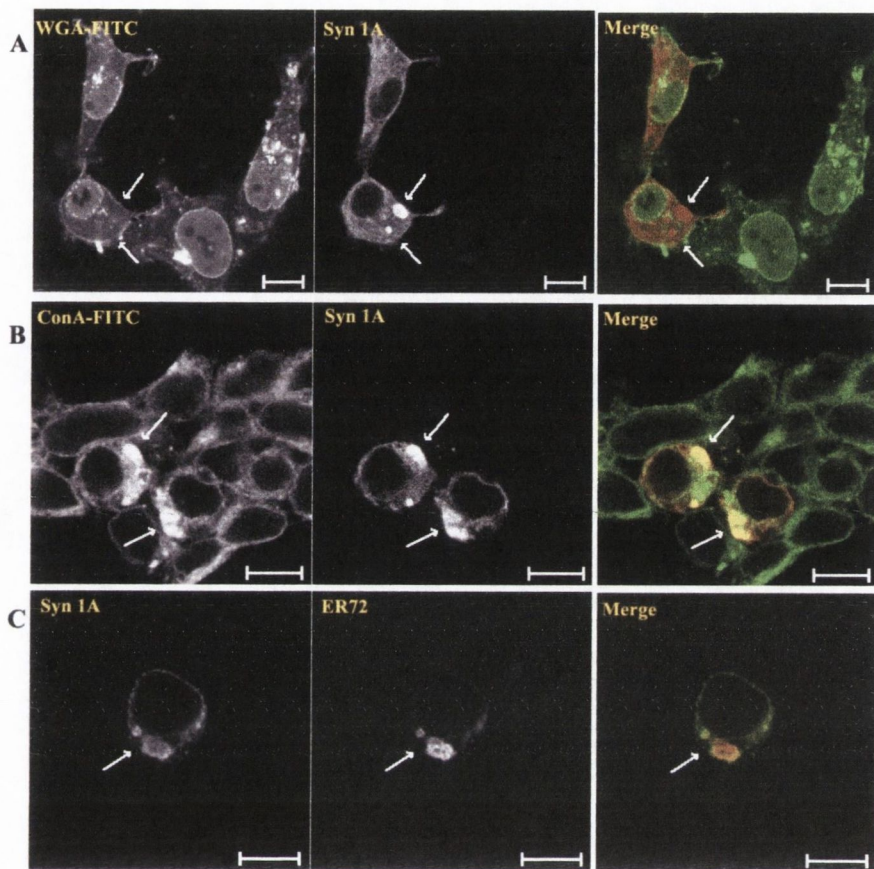
As mentioned above the coexpression of SERT and syn 1A resulted in a marked reduction of fully glycosylated transporter (Fig. 4.4), which suggests that syn 1A may interfere with the maturation of SERT. Because the modification of the protein's glycans is carried out during the passage through the endoplasmic reticulum (ER) to the Golgi, the accumulation of syn 1A (and SERT) was likely to occur in one of these two subcellular organelles. From the morphology of the spheroidal structures that were formed following syn 1A expression it was not obvious in which compartment the accumulation occurred. For this purpose, confocal microscopy was employed to carry out experiments of colocalization using markers for the ER or the Golgi apparatus. The lectin Concanavalin A (ConA) binds to mannose-rich cores in glycoproteins and is used to label the ER, whereas the lectin wheat germ agglutinin (WGA) recognizes complex glycans and thus strongly labels the Golgi apparatus (Virtanen et al., 1980; Parkkinen et al., 1997). As shown in Fig. 4.5, syn 1A was found to accumulate in WGA-negative structures (Fig. 4.5 A), but ConA-positive (Fig. 4.5 B). Additionally, syn 1A colocalized with another ER marker, ER-72 (Fig. 4.5 C), thus confirming that in HEK cells syn 1A accumulates in the ER. In cells that do not express syn 1A, the Golgi apparatus is well preserved and morphologically recognizable in a perinuclear position, whereas in cells expressing syn 1A the morphology of both Golgi and ER seems to be altered.

The reasons why the heterologous expression of syn 1A alters the morphology of ER and Golgi are not clear. The SNARE hypothesis postulates that the assembling of v- and t-SNAREs underlies the specificity of membrane fusion. However, it has been shown that SNAREs interactions are not always

Fig. 4.5

Syn 1A accumulates in the endoplasmic reticulum

HEK cells were transfected with the syn 1A plasmid for 48 hours. Cells were fixed with 4% paraformaldehyde and permeabilized as described in Methods. The cells were then incubated with the monoclonal anti-syn 1A antibody (1:1000; middle panels), followed by the incubation with anti-mouse-TexasRed antibody (1:2000). A) The Golgi apparatus was stained with WGA-FITC (1:200). The ER was labelled using ConA-FITC (1:150; B) or the antibody anti-ER72 (1:200; C). Arrows indicate the intracellular structures where syn 1A accumulates. Bars, 10 μm .



specific and that promiscuous pairing can occur (Grote and Novick, 1999; Yang et al., 1999). Syn 1A together with its cognate SNAREs is implicated in the regulated secretion of neurotransmitter at the synapse, whereas HEK cells are incompetent for regulated secretion. Thus, it is possible that the expression of syn 1A in HEK cells interferes with the coupling of endogenous SNARE proteins, which in this cell line mediate the ER to Golgi vesicular traffic.

4.2.5 Syn 1A affects the trafficking of plasma membrane proteins to the cell surface

In order to investigate whether syn 1A expression specifically affected SERT trafficking or exerted a more general disturbance of subcellular trafficking in HEK cells, cotransfection experiments were carried out with cDNAs encoding for syn 1A and the Toll-like receptor (TLR)-2, a membrane protein non-related to the serotonin transporter. HEK cells were cotransfected with equal amounts of pSyn 1A and pTLR2-FLAG, and 48 hours later processed for immunofluorescence as described in Methods. When expressed alone, TLR2 was homogenously distributed in cells (Fig. 4.6 A). However, when TLR2 was coexpressed with syn 1A it was largely trapped in the syn 1A-immunopositive structures (Fig. 4.6 B). Thus, the heterologous expression of syn 1A in HEK cells appeared to result in the mistargeting of the t-SNARE protein with a consequent impairment of membrane proteins trafficking towards the Golgi apparatus and the cell surface.

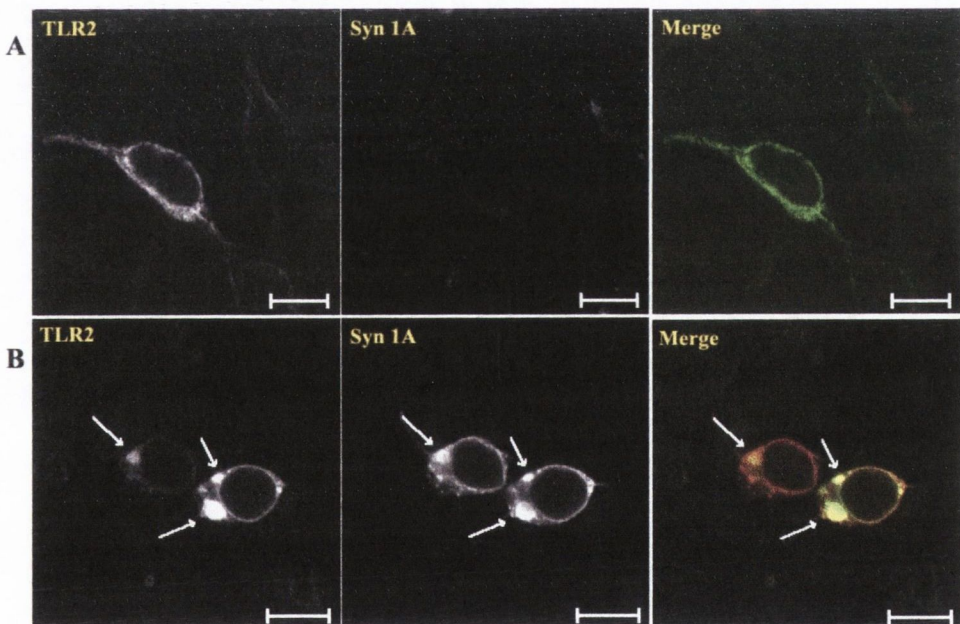
4.2.6 Coexpression of Munc-18-1 with syn 1A does not alter the localization of the syntaxin in HEK cells

Munc 18-1 is a cytosolic protein that binds syn 1A with high-affinity (Hata et al., 1993; Pevsner et al., 1994). Previous studies showed that the interaction of syn 1A with GAT1, GLYT1, GLYT2 and NET is inhibited by the

Fig. 4.6

TLR 2 colocalizes with syn 1A when cotransfected in HEK cells

pTLR2-FLAG was cotransfected in HEK cells with pcDNA3 (A) or pSyn 1A (B). 48 hours after transfection cells were fixed and immunolabelled with the anti-FLAG antibody (1:1000) and the anti-syn 1A polyclonal antibody (1:200). A) TLR2 showed a diffuse localization in the cytoplasm. B) TLR2 is present in the same large cytoplasmic structures containing most of syn 1A (indicated with arrows). Bar, 10 μ m.



coexpression of Munc 18-1 (Beckman et al., 1998; Geerlings et al., 2000; Sung et al., 2003). Thus, the effect of Munc 18-1 on syn 1A subcellular localization in HEK cells was investigated. Munc 18-1 was not detected in mock-transfected HEK cells, whereas in transfected cells appeared as a single immunoreactive band with M_r 83,000 (Fig. 4.7). The immunostaining pattern of Munc 18-1 was widespread in the cytosol (Fig. 4.8A). However, when coexpressed with syn 1A, Munc 18-1 largely colocalized with the syntaxin in large vesicles (Fig. 4.8B).

Fig. 4.7
Expression of Munc 18-1 in HEK cells

HEK cells were seeded in a 6-well plate and 24 hours later transfected with the empty vector pcDNA3 or with pMunc 18-1 (0.6 μ g DNA/well). An equal volume of cell lysate was loaded onto a 10 % polyacrylamide gel. Proteins were transferred onto a PVDF membrane as described in Methods. Following blocking, the membrane was immunoprobed with the monoclonal anti-Munc 18-1 antibody (1:5000). Blots were then reprobed with anti-actin monoclonal antibody (1:5000, bottom panel), showing equal protein loading.

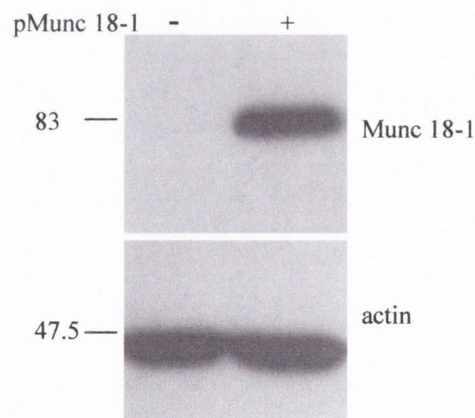
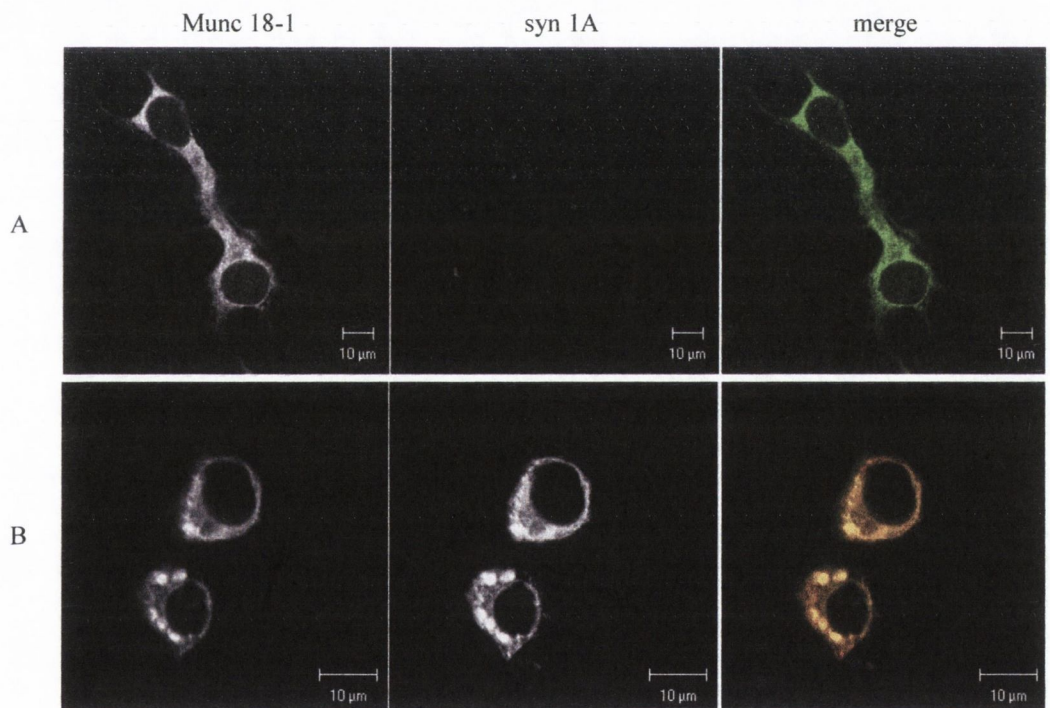


Fig. 4.8

Expression of Munc 18-1 in HEK cells

HEK cells were seeded onto lysine-coated slides in a 6 well-plate and after 24 hours were cotransfected with A) pMunc 18-1 and pcDNA3, or B) pMunc 18-1 and pSyn 1A (0.3 μ g DNA/ well). After 48-72 hours cells were fixed and labelled with the monoclonal anti-Munc 18-1 (1:1500) and anti-syn 1A antibody (1:1000). Bars, 10 μ m. The experiments was repeated three times with similar results.



4.3 Discussion

Several studies recently have brought attention to the physical and functional interaction of transporters for inhibitory amino-acids with the t-SNARE protein syntaxin 1A (Beckman et al., 1998; Deken et al., 2000; Geerlings et al., 2000; Geerlings et al., 2001; Horton and Quick, 2001). These findings suggest that syn 1A may control neurotransmission not only by regulating the fusion of neurotransmitter vesicles with the presynaptic plasma membrane, but also by regulating the traffic to the cell surface of transporters that mediate neurotransmitter re-uptake. During the progress of this project, together with our preliminary report (Haase et al., 2001), others were published reporting that the delivery to the plasma membrane of the serotonin- (Quick, 2002) and norepinephrine-transporter (Sung et al., 2003) is supported by syn 1A. These studies did also show that SERT and NET physically interact with syn 1A *in vivo* (Quick, 2002; Sung et al., 2003). In the present study, heterologous expression systems were used to investigate the functional consequences of the proposed interaction between SERT and syn 1A.

A preliminary study carried out in our laboratory using the GST-pulldown assay showed that SERT appears to form a complex with syn 1A *in vitro* (Haase et al., 2001). The complex was susceptible to increased non-ionic detergent concentrations and was enhanced by high ion concentration, thus suggesting that formation of the SERT-syn 1A complex depends on hydrophobic interactions. However, SERT-syn 1A interaction could not be shown *in vivo* by coimmunoprecipitation of the two proteins either cotransfected in HEK cells or endogenously expressed in brain extracts. In addition, a direct interaction could not be shown in the yeast two-hybrid assay (Jana Haase, personal communication). These difficulties could arise from technical problems, such as difficulties in efficiently solubilizing SERT for the coimmunoprecipitation assay at conditions where the interaction is maintained *in vitro* or misfolding of the transporter in the yeast expression system. Despite the fact that a physical *in vivo*

association between SERT and syn 1A could not be corroborated by us, the coexpression of the two proteins in HEK cells resulted in a marked decrease in the 5HT transport capacity (Haase et al., 2001). This prompted us to evaluate whether the expression of syn 1A could affect the number of transporter molecules at the cell surface.

By confocal microscopy imaging and cell surface biotinylation the transfection of syn 1A into HEK cells was shown to result in a disturbance of SERT trafficking to the plasma membrane. The heterologous expression of syn 1A provoked evident morphological changes of subcellular compartments, with a loss of the reticulate distribution of the ER and the concomitant formation of large vesicles located below the plasma membrane. When coexpressed with syn 1A, the transporter is no longer efficiently delivered to the cell surface: SERT and syn 1A are trapped into intracellular structures that are immunolabelled by ER-markers. Symptomatic of SERT accumulation into ER-positive structures is the inhibition of the transporter post-translational glycosylation: the abundance of SERT fully glycosylated form is reduced, whereas the partially glycosylated transporter appears to be increased. No degradation of the transporter is evident. These findings suggest that the expression of syn 1A in HEK cells affects SERT delivery to the cell surface, which may in turn explain the previously observed $V_{\max}$ reduction of 5HT transport (Haase et al., 2001). Nevertheless, the heterologous expression of syn 1A in HEK cells do not affect specifically the serotonin transporter, but instead appears to generate a similar mistargeting of TLR2, a membrane protein with no homologies with monoamine transporters.

This raises the question why the overexpression of a wild type t-SNARE would cause an inhibition of membrane proteins traffic pathway. Similar effects have been already observed: previous reports showed that syn 1A is not transported to the plasma membrane but rather is retained intracellularly when overexpressed in MDCK, Caco-2 (Rowe et al., 2001) and COS cells (Bittner et al., 1996). Furthermore the overexpression of syn 1A has been shown to inhibit the glucose-stimulated insulin secretion in pancreatic islets cells, whereas the

overexpression of syntaxin 1B (which is not normally expressed in β -cells) had no effect (Nagamatsu et al., 1996).

The transport of membranes and membrane proteins within the cell requires the interaction of v-SNARE proteins on the transport vesicle with their t-SNARE partners on the target membrane, and this binding appears necessary to achieve membrane fusion. The SNARE proteins are generally composed of three protein families: VAMP, syntaxin, and SNAP-25. Together these proteins form a strong coiled-coil complex, which is thought to bridge the membranes to be fused, and in doing so may provide the energy needed for membrane fusion. A large number of different SNARE protein isoforms have been identified that appear to have specific subcellular distributions. This has led to the idea that membrane fusion at each step in the secretory pathway may be mediated by the interactions of a unique set of SNAREs (Sollner et al., 1993; Rothman and Warren, 1994). However, SNARE proteins have been shown to form *in vitro* stable complexes with non-physiological partners (Yang et al., 1999). Even though only certain SNARE complexes are formed *in vivo*, the finding that SNAREs can interact in a non-selective fashion *in vitro* may indicate that a multi-step process regulates membrane fusion specificity. In fact, SNARE proteins are spatially segregated in distinct subcellular compartments, which may restrict their interactions *in vivo*. Furthermore, the traffic of vesicles from a donor compartment to the acceptor one is likely to be regulated by specific sets of coat and motor proteins.

One possible explanation for the observed accumulation of syn 1A in HEK cells is that the stoichiometry of SNARE components may be disturbed, leading to the mistargeting and sequestration of the syntaxin within the ER. It is also possible to speculate that the majority of syn 1A is unable to find a binding partner to form a functional t-SNARE and that the non-functional t-SNAREs cannot mediate vesicle fusion with the Golgi apparatus. As mentioned above, syntaxins can form non-specific complexes with other SNAREs, as it has been shown by using a yeast strain carrying mutant Rab proteins (small GTPases essential for membrane fusion (Grote and Novick, 1999)), and also by analyzing the formation of SNARE complexes *in vitro* (Yang et al., 1999). Whatever the

exact molecular explanation may be, our data indicates that the heterologous expression of syntaxin 1A in HEK cells inhibits the traffic of membrane proteins to the cell surface, causing their intracellular accumulation.

Previous reports have shown that the syn 1A-binding protein Munc 18-1 inhibits the interaction between the t-SNARE and several neurotransmitter transporters (Beckman et al., 1998; Geerlings et al., 2000; Sung et al., 2003). Syn 1A can adopt open and closed conformations. In the closed conformation the N-terminal domain 'Habc', formed by three anti-parallel α -helices, is folded onto the SNARE motif. Munc 18-1 binds with nanomolar affinity syntaxin 1A in the closed conformation, thus preventing it from forming complexes with its SNARE partners (Dulubova et al., 1999). Munc 18-1 has been shown to be able to rescue syn 1A mistargeting in MDCK cells and in neurosecretion incompetent PC12 cells (Rowe et al., 1999; Rowe et al., 2001). These evidences suggest that Munc 18-1 binds syn 1A not only at the plasma membrane, but also in the biosynthetic pathway, thus acting like a chaperone. However, as shown in this study in HEK cells Munc 18-1 appears to not rescue the syn 1A mistargeting, suggesting that the biosynthesis and trafficking of syn 1A require some other component missing in the HEK cell line.

Chapter 5 Results

Partitioning of the serotonin transporter into cholesterol-enriched lipid microdomains modulates transport of serotonin

5.1 Introduction

To carry out clearance of extracellular 5-HT, SERT must be inserted into the plasma membrane in an active form. This may require targeting the transporter to specific plasma membrane regions, but not others. The study of how asymmetry is generated and maintained in polarized cells has changed old concepts of cell membranes. According to the Singer-Nicholson fluid mosaic model the lipid bilayer is a neutral two-dimensional solvent for membrane proteins (Singer and Nicholson, 1972). However, in model lipid bilayers, lipids can exist in different phases: as gel, liquid-ordered (l_o) and liquid-disordered states (as in the Singer-Nicholson model). In the l_o state, saturated hydrocarbonic chains of phospholipids are tightly packed with cholesterol, thus conferring a limited horizontal mobility in the membrane. This model has been confirmed at least partially in cell membranes: the exoplasmic leaflet contains lipid microdomains, enriched in sphingolipids and cholesterol, segregated from glycerolipids. Such lipid microdomains, also known as “rafts”, resist solubilization at 4°C in non-ionic detergents, such as Triton X-100 (Brown and London, 1998; Rietveld and Simons, 1998). The segregation of proteins into lipid microdomains is lost after depletion of cholesterol or sphingolipids. Lipid rafts appear to be involved in several cellular functions, from membrane trafficking to cell signalling (Simons and Ikonen, 1997).

Membrane cholesterol has been shown to be an essential requirement for ligand binding to SERT. Since cholesterol is one of the major components of lipid rafts, in this study the association of SERT with lipid rafts was investigated.

5.2 Results

5.2.1 Methyl- β -cyclodextrin treatment of TREx-SERT cells reduces the transport of 5-HT

The cyclic oligosaccharide methyl- β -cyclodextrin (M β C) has proven extremely useful in manipulating the cholesterol content of cells. Therefore, M β C was used to analyse the effect of cholesterol depletion on SERT function. Firstly, the optimal concentration of M β C was determined, which resulted in sufficient cholesterol depletion without reducing cell viability. TREx-SERT cells were treated with various amounts of M β C and incubated for 30 min at 37 °C. Cell viability was not significantly affected by M β C in the 1.25-20 mg/ml range (Fig. 5.1A). At this concentration range M β C induced a marked depletion of cholesterol in a dose-dependant manner and more than 50% of cholesterol was sequestered by 10-20 mg/ml M β C (Fig. 5.1B). Cholesterol depletion has previously been shown to reduce antidepressant binding activity by SERT expressed in Sf9 insect cells (Baker, 1997) and in HEK cells (Scanlon et al., 2001). In TREx-SERT cells [3 H]-5HT uptake was reduced to less than 50% upon incubation with 10-20 mg/ml M β C (Fig. 5.2A). Saturation kinetic analysis of [3 H]-5HT transport in these cells in order to obtain $V_{\max}$ and K_m values proved difficult. As discussed in Chapter 3, the high level of SERT expression in these cells results in a marked increase of K_m values (Tate et al., 2003). Thus, these experiments were performed in HEK cells, which were transiently transfected with SERT. In accordance with a previous report (Scanlon et al., 2001), in these cells cholesterol depletion by M β C resulted in a decrease of $V_{\max}$ values in a concentration-dependent manner (Fig. 5.2B: control, $V_{\max}$ 8.4 ± 0.2 pmol/min/ 10^6 cells; 10 mg/ml M β C, $V_{\max}$ 4.0 ± 0.6 pmol/min/ 10^6 cells; 20 mg/ml M β C, $V_{\max}$ 3.0 ± 0.6 pmol/min/ 10^6 cells). At the same time the affinity of [3 H]-5HT uptake decreased slightly (control, K_m 446 ± 73 nM; 10 mg/ml M β C, K_m 819 ± 452 nM; 20 mg/ml M β C, K_m 938 ± 717 nM).

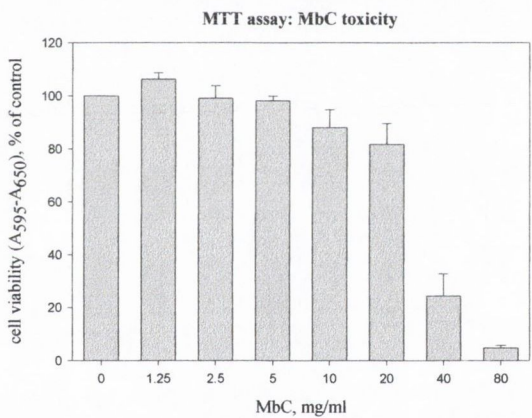
5.2.2 SERT colocalizes with the raft marker GM1 on the plasma membrane

Fig. 5.1

Reduction of cholesterol in cells upon MβC incubation

A) TREx-SERT cells viability upon MβC treatment was determined by MTT assay as described in Methods. Each data point was determined in triplicate and was plotted as mean value ± SEM of three independent experiments. B) Cholesterol quantification following incubation with MβC. TREx-SERT cells were incubated with increasing non-toxic concentrations of MβC for 30 minutes at 37°C. Cells were washed and cholesterol concentration was determined using an enzymatic cholesterol reagent as described in Methods. Each data point was determined in duplicate and was plotted as mean value ± SEM of three independent experiments.

A



B

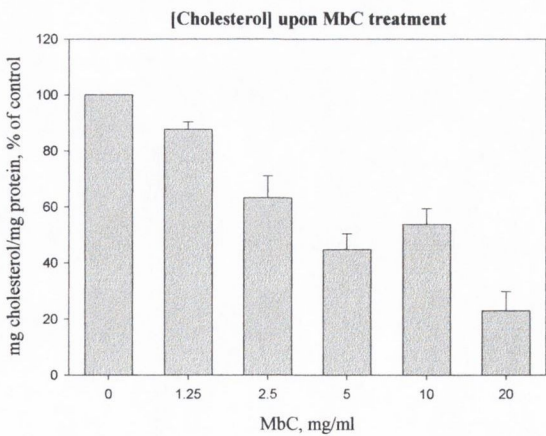
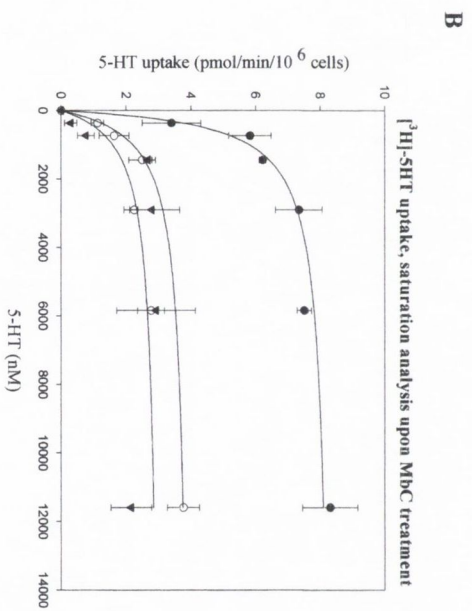
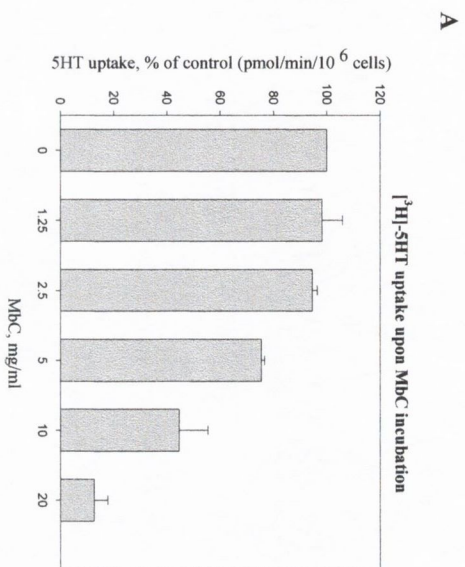


Fig. 5.2

Cholesterol depletion by M β C reduces 5HT-transport

A) Cholesterol depletion reduces [3 H]-5HT uptake in TReX-SERT cells. Following incubation with M β C cells were washed with TB buffer and 5HT-uptake assays were carried out as described in Methods using 500 nM [3 H]-5HT solution. Each data point was determined in triplicate and was plotted as mean value $\pm$ SEM of three independent experiments. B) Saturation analysis of [3 H]-5HT uptake upon cholesterol depletion. HEK cells were plated in 24 well plates for 24 hours and then transfected with 0.3 μ g/well pSERT-FLAG for 48 hours. Cells were incubated with 0 mg/ml ($\bullet$), 10 mg/ml ($\circ$), or 20 mg/ml ($\blacktriangledown$) M β C for 30 minutes at 37°C, washed with TB buffer and incubated with the indicated concentration of [3 H]-5HT for 6 minutes. $V_{\max}$ values for the data are, respectively, 8.4 ± 0.2 , 4.0 ± 0.6 , 3.0 ± 0.6 pmol/min/ 10^6 cells. Each data point was determined in triplicate and was plotted as mean value $\pm$ SEM of three experiments. The saturation curve presented is a representative of two independent experiments, each giving similar results.



Because cholesterol is a component of lipid rafts, it seemed logical to investigate whether the downregulation of SERT upon cholesterol depletion was due to the association of the transporter with lipid rafts. The B-subunit of Cholera toxin (CTxB) is commonly used to specifically label the ganglioside GM1, which is enriched in cholesterol-rich membrane microdomains (Harder et al., 1998). Using confocal microscopy SERT labelling in TREx-SERT cells is concentrated in clusters over the cell surface and CTxB-labelling overlapped extensively with SERT-immunopositive areas (Fig. 5.3). The punctate staining observed did not depend on the fixative used, since it was present when cells were fixed using either 4% paraformaldehyde or cold acetone (see Fig. 3.6). Thus, the similarity in the pattern of SERT cellular localization and GM1 location suggested a possible association of the serotonin transporter with lipid rafts.

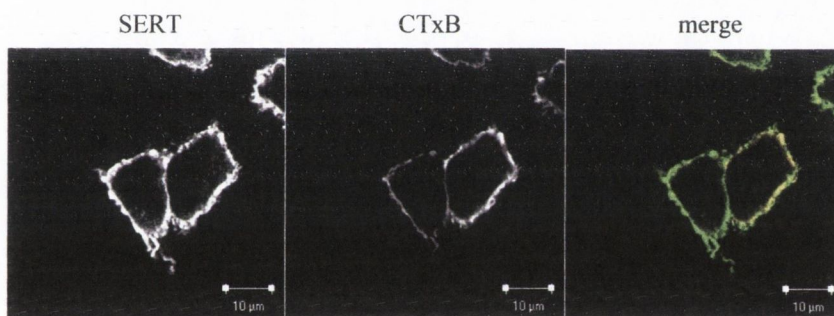
5.2.3 Differential solubilization of SERT in various detergents

A feature of lipid microdomains is their resistance to extraction by non-ionic detergents at 4°C, such as Triton X-100 (Simons and Ikonen, 1997; Brown and London, 1998). This property has been widely used to isolate rafts. Due to the high lipid content, detergent-resistant complexes manifest high buoyancy upon centrifugation in a density gradient, and thus float in the upper layers of a density gradient. SERT solubilization was firstly tested in three non-ionic detergents, Triton X-100, Nonidet P-40 and Brij 58, in a concentration range from 0.1% to 1.5 %. Cells were lysed at 4°C in the appropriate detergent diluted in TNE buffer, homogenized and subjected to centrifugation at 830 g for 10 minutes in order to clear the lysates from nuclei and unbroken cells. Cell lysates were placed at the bottom of a discontinuous sucrose gradient (5%-30%-40%) and centrifuged at 134,400 g for 17 hours at 4°C. Fully solubilized proteins remain in the bottom layers, whilst insoluble proteins inserted in lipid microdomains float in the upper layers at the 5-30% interface. Triton X-100 appeared to fully solubilize the transporter. Only when the blots were overexposed the presence of SERT immunoreactive bands could be detected in fractions 1 and 2, containing the interface 5-30% of sucrose (Fig. 5.4, left column). Similarly, SERT was

Fig. 5.3

SERT colocalizes with GM1.

TREx-SERT cells were incubated with 3 $\mu\text{g/ml}$ CTxB to label GM1, fixed with 4% w/v paraformaldehyde and incubated with antibodies as described in Methods. The left panel shows SERT distribution and the middle panel shows CTxB distribution. SERT (green)- and CTxB (red)-immunofluorescences are merged in the right panel. Colocalization of the two signals is indicated in yellow.



solubilized in Nonidet P-40 at all the tested concentrations (Fig. 5.4, middle column). A different flotation profile was given when cells were solubilized in Brij 58: SERT fractionated at the top fractions of the gradient and its distribution along the density gradient was similar when Brij 58 was used in a 0.1-3% range (Fig. 5.4, right column), suggesting that the variation of the detergent/protein mass ratio does not affect SERT solubility in Brij 58. About 40-50% of SERT was recovered at the interface 5-30% of sucrose. The differential solubilization pattern seen here for SERT when cells are treated with different non-ionic detergents, may be explained by the finding that lipid and protein composition of lipid rafts has been in fact shown to differ when different detergents are used (Taylor et al., 2002; Schuck et al., 2003). For this reason, a more systematic approach was used and the solubilization of SERT was assayed in four non-ionic detergents having both different structure and hydrophilic-lipophilic balance (HLB) values (Table 5.1): Triton X-100, Tween 80, Brij 58, and Tween 20. Critical micellar concentration (CMC) values should be taken into account when assessing the efficacy of different detergent in solubilizing a given protein. Thus, the four detergents were initially compared at concentrations corresponding to 10-fold of their respective CMC. A positive control, GM1, was used to follow the distribution of lipid rafts in the density gradient. Blots were also re-probed for the cytoplasmic protein actin, in order to rule out the possibility that SERT insolubility may arise from the association with the cytoskeleton. When cells were lysed in Tween 80 and Tween 20, a consistent amount of SERT and CTxB immunoreactivity was recovered in the pellet (P_1) probably resulting from a partial cell lysis. In the attempt to overcome this, the experiment was repeated at 100-fold and 300-fold the CMC (shown in Fig. 5.5). However, similar flotation profiles for SERT, GM1 and actin were obtained independent of whether cells were solubilized in 10-, 100- or 300-fold the CMC of each detergent. SERT was fully solubilized in Triton X-100, whereas GM1 was recovered in the upper fractions. However, SERT was partially insoluble in Brij 58 (approximately 40%), in Tween 80 (10%) and Tween 20 (20%). The vast majority of actin was retrieved at the bottom of the gradient (Fig. 5.5, right column), supporting the conclusion that the insolubility of SERT is due to the association with low-buoyancy lipid rafts and not with the cytoskeleton. The enrichment of GM1 in

Fig. 5.4

Effect of various non-ionic detergents on SERT solubilization

TREx-SERT cells were lysed with Triton X-100 (left), Nonidet P-40 (middle) or Brij 58 (right) at the indicated concentrations. Lysates were cleared by centrifugation for 10 minutes at 830 g. 500 μ l of each supernatant was mixed with an equal volume of 80% sucrose in TNE buffer, placed at the bottom of an ultracentrifuge tube, and overlaid with 1 ml of 30% sucrose in TNE buffer and then with 500 μ l of 5% sucrose in TNE buffer. Density gradients were centrifuged for 17 hours at 134,400 g, 4°C. Eight fractions were collected from the top to the bottom of the gradient and analysed by SDS-PAGE followed by immunoblotting for SERT (anti-NSERT antibody, 1:6000). The numbers in brackets refer to the fold CMC value corresponding to the indicated concentration of each detergent.

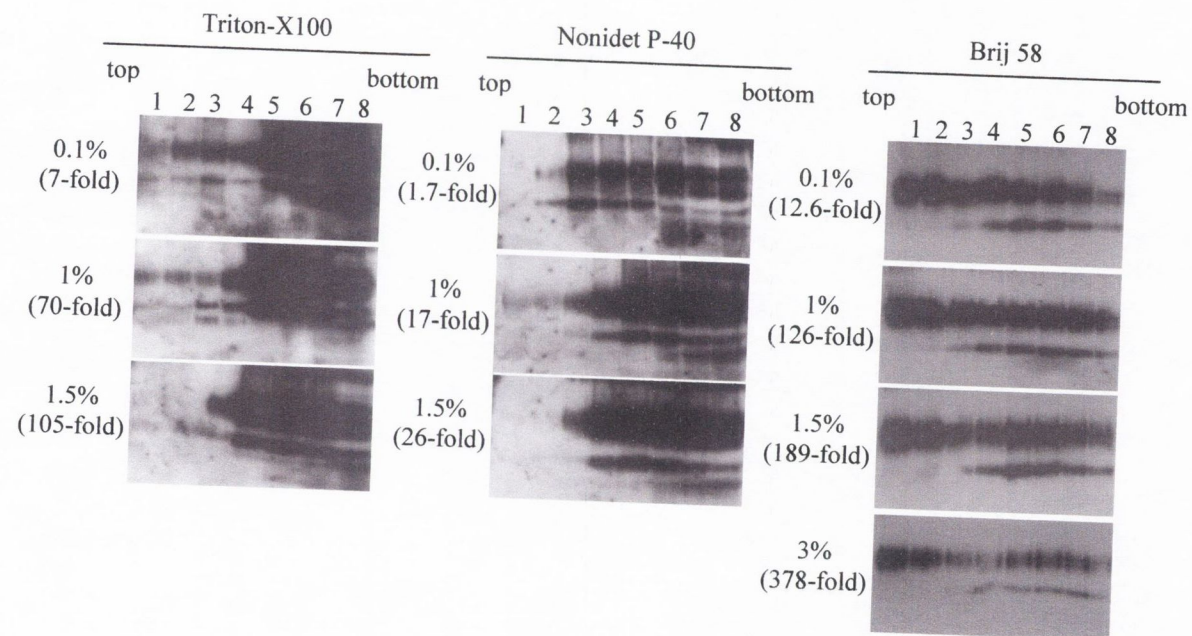


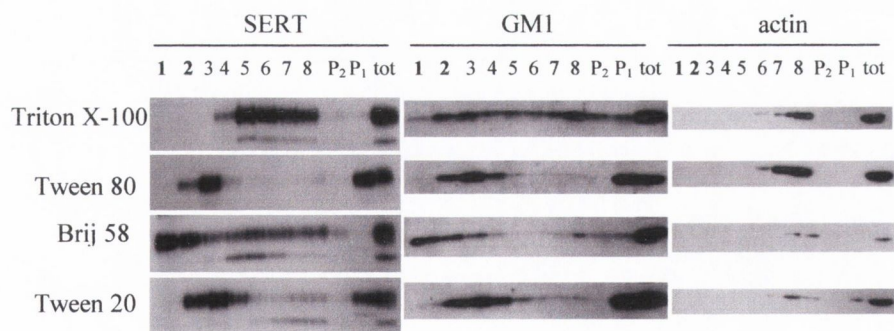
Table 5.1 HLB value and CMC for some non-ionic detergents (modified from (Helenius and Simons, 1975)).

Commercial name	Detergent	HLB value	CMC (mM)	mw (average)	Percentage at 10 x CMC (w/v)	Percentage at 100 x CMC (w/v)	Percentage at 300 x CMC (w/v)
Nonidet P-40	PEG(9) p-t-octylphenol	13.1	0.29	2000	0.58	5.8	17.4
Triton X-100	PEG(9-10) p-t-octylphenol	13.5	0.24	625	0.15	1.5	4.5
Tween 80	PEG(20) sorbitol monooleate	15	0.01	1308	0.013	0.13	0.39
Brij 58	PEG(20) cetyl alcohol	15.7	0.077	1123	0.086	0.86	2.58
Tween 20	PEG(20) sorbitan monolaureate	16.7	0.049	1225	0.06	0.6	1.8

Fig. 5.5

**Comparison of floatation gradient-profiles obtained with various detergents
at 300-fold their CMC value**

TREx-SERT cells were incubated with 3 μ g/ml CTxB for 10 minutes at RT. Lysates were obtained using the indicated detergent at a concentration corresponding 300-fold CMC (see Table 1). Lysates were cleared by centrifugation for 10 minutes at 830 g. Pellets (P₁) were resuspended and 500 μ l of each supernatant was centrifuged for 17 hours at 134,400 g, 4°C. Eight fractions were collected from the top to the bottom of the gradient and the pellet (P₂) was resuspended. The eight fractions, the pellets and an aliquot of the precleared lysate (designated 'tot') were analysed by SDS-PAGE followed by immunoblotting for SERT (anti-NSERT antibody, 1:6000; left panel), GM1 (anti-CTxB, 1:1000, middle panel), and actin (anti-actin, 1:5000, right panel). Fraction 1 and 2 (indicated in bold) contain detergent-insoluble complexes (rafts).



lipid microdomains varied as well as SERT distribution along the density gradient depending on the detergent used. Most (>50%) of CTxB was recovered in the upper fractions of the gradient when cells were lysed in Brij 58 (Fig. 5.5, middle column). No differences were noticed whether the detergent was included in the gradient or not: even if the samples were diluted in four volumes of sucrose gradient, the concentration of detergent is likely to be still above its CMC along the gradient.

To confirm that SERT was associated with lipid microdomains, a negative control was also introduced. The solubilization profile of the Toll-like receptor (TLR)-4, a membrane protein non-related to the neurotransmitter transporter-family, was examined. As seen before, SERT was fully solubilized by Triton X-100, whereas about 40% of SERT was insoluble in Brij 58 and therefore recovered in the upper layers of the sucrose gradient (Fig. 5.6). In contrast, TLR-4 was fully solubilized by both detergents, indicating that SERT insolubility in Brij 58 is not due to an incomplete solubilization of bulk membrane.

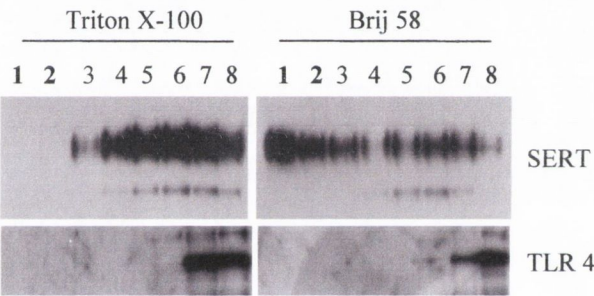
5.2.4 Effect of cholesterol depletion on solubilization of SERT in Brij 58-resistant complexes

If SERT insolubility in Brij 58 reflects its association with lipid rafts, the question arises whether cholesterol is needed to maintain the structure of these low-buoyancy complexes. M β C selectively depletes cholesterol from membranes, therefore disrupting lipid microdomains whose integrity depends on cholesterol. For this purpose, TREx-SERT cells were incubated with 10 or 20 mg/ml M β C for 30 min at 37°C at which concentration more than 50% of cholesterol was depleted from cells (see above, Fig. 5.1 B). Cells were subsequently lysed in 1% w/v Brij 58 at 4°C and precleared lysates were loaded at the bottom of a discontinuous sucrose gradient. Flotation profiles for SERT on density gradient centrifugation revealed that M β C treatment, and thus cholesterol depletion, caused SERT to shift to the higher buoyant density (Fig. 5.7 A). Similarly, when cholesterol was sequestered from cells by a combination of 0.5% w/v Brij 58 and 0.2% w/v saponin, which is known to form insoluble complexes with cholesterol, SERT

Fig. 5.6

The membrane protein TLR4 is solubilized by both Triton X-100 and Brij 58

TREx-SERT cells were transfected with pTLR4-FLAG (4 μ g/10 cm Petri dish) and 48 hours later cells were lysed with 1% w/v Triton X-100 (left panel) or 1% w/v Brij 58 (right panel). Flootation experiment was carried out as described in Methods and blots were probed for SERT (anti-NSERT, 1:6000) and TLR 4 (anti-FLAG, 1:1000). Fraction 1 and 2 (indicated in bold) contain detergent-insoluble complexes (rafts).



was effectively solubilized and no longer recovered in low buoyancy fractions of sucrose gradient (Fig. 5.7 B).

5.2.5 Disruption of lipid rafts causes a decrease in SERT turnover number

When cholesterol is removed from cell membranes, the saturation analysis of 5HT transport reveals a decrease in the maximum transport velocity ($V_{\max}$) and changes in affinity (K_m) (Fig. 5.2 B; (Scanlon et al., 2001)). The observed change of $V_{\max}$ could be produced either by reducing the number of SERT molecules on the plasma membrane or by decreasing the turnover number of transported 5-HT.

In order to discriminate between these two possibilities, cells were subjected to cholesterol depletion followed by cell surface biotinylation and lipid rafts isolation. To avoid handling a high number of samples and overcomplications due to the presence of different sucrose concentrations along the density gradient, which may interfere with the assay, Brij 58-resistant complexes were isolated not by flotation experiments but by differential centrifugation. In order to assess the condition at which lipid microdomains sediment, cells were lysed in Brij 58, and cleared lysate was centrifuged at 15,000 g for 20 min or 101,300 g for 1 hour. To monitor the sedimentation of lipid rafts, pellets ('rafts') were resuspended in lysis buffer and supernatants were subjected to flotation experiment. Brij 58-resistant domains containing insoluble SERT and GM1 were only partially pelleted at 15,000 g for 20 min, whereas they sedimented after centrifugation at 101,300 g for 1 hour (Fig. 5.8). This indicates that SERT associates with Brij 58-resistant complexes of different sizes: a smaller type that sediments at 101,300 g (for 1 hour) and a larger type that sediments at 15,000 g (for 20 min).

To establish whether the localization of SERT on the plasma membrane is affected by cholesterol depletion, TREx-SERT cells were incubated with 10 mg/ml M β C for 30 min at 37°C to deplete membranes of cholesterol. Cells were then placed on ice and incubated with 1.5 mg/ml impermeant NHS-sulfo-biotin for 30 min at 4°C to label proteins at the cell surface, followed by quenching of

Fig. 5.7

Insolubility of SERT in Brij 58 reflects its association with rafts.

A) Cholesterol was depleted in TREx-SERT cells with the indicated concentrations of M β C for 30 minutes at 37°C. Cells were then lysed in 1% w/v Brij 58, and subjected to floatation centrifugation on a discontinuous sucrose gradient. B) TREx-SERT cells were lysed in 1% w/v Brij 58, or in 0.5% w/v Brij 58 / 0.2% w/v saponin, to sequester cholesterol. Precleared lysates were then subjected to floatation experiment as described in Methods. The gradient fractions were analysed by immunoblotting for SERT. Fraction 1 and 2 (indicated in bold) contain detergent-insoluble complexes (rafts).

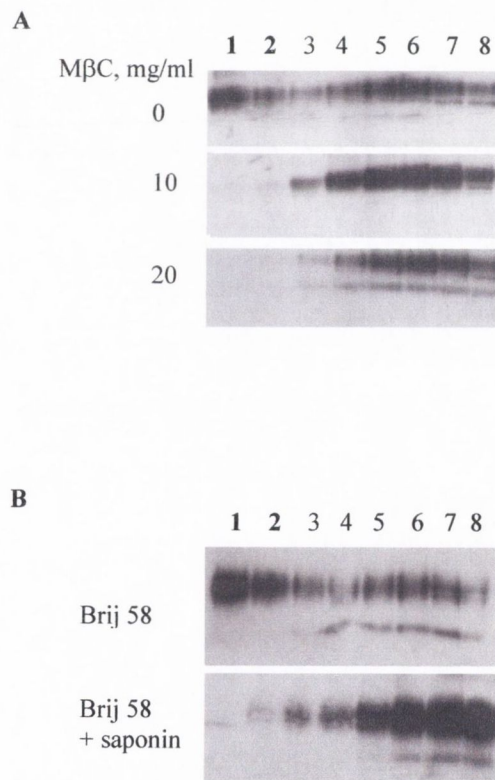
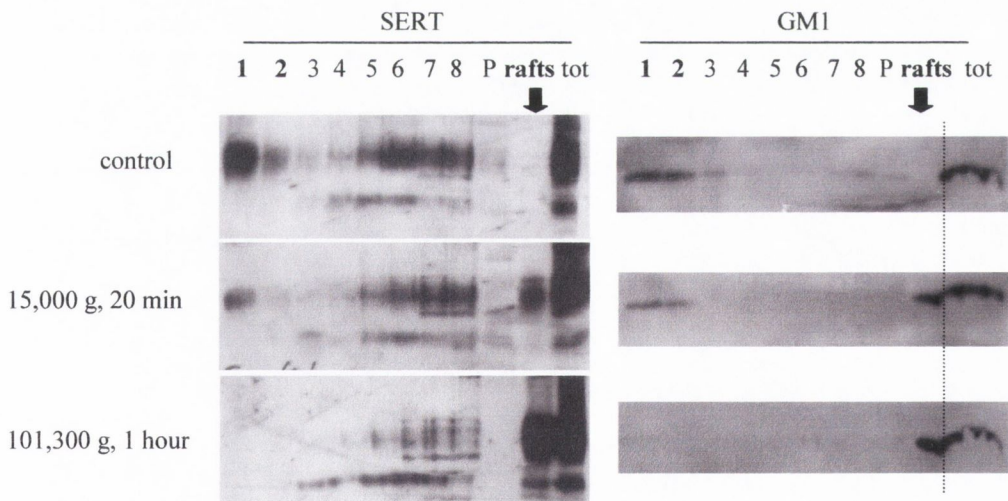


Fig. 5.8

Isolation of detergent-insoluble complexes by differential centrifugation.

TREx-SERT cells were incubated with 3 $\mu\text{g/ml}$ CTxB for 10 minutes at RT. Cells were lysed in 1% w/v Brij 58, and the cleared lysate was divided into three aliquots. One aliquot was centrifuged at 15,000 g for 20 min and a second one at 101,300 g for 1 hour. Pellets ('rafts') were resuspended in lysis buffer and stored at -20°C . Supernatants were subjected to floatation sucrose gradient centrifugation, in parallel with a third aliquot of precleared lysate ('control'). Eight fractions were collected from the top to the bottom of the gradient and the pellet (P) was resuspended into solubilization buffer. Gradient fractions, pellet P, rafts, and an aliquot of precleared lysate ('tot') were analyzed by immunoblotting for SERT and GM1.



unbound biotin. Cells were subsequently lysed in ice-cold 1% Brij 58 as described in Methods and lipid rafts were isolated by centrifugation at 101,300 g for 1 hour. Using this method, the majority of SERT was found in the raft fractions. Fig. 5.9 A shows that cholesterol depletion led to a partial solubilization of SERT, due to desegregation of lipid microdomains, as seen before. Raft-associated SERT was found in both the intracellular and the cell-surface fractions. Cholesterol depletion did not affect the amount of SERT localized on the plasma membrane (Fig. 5.9 B). Interestingly, by lowering cellular cholesterol levels although SERT was still present on the plasma membrane, it was shifted into detergent-soluble membrane domains (Fig. 5.9 C). Therefore, the $V_{\max}$ reduction observed in [^3H]-5HT transport following cholesterol depletion appears not to be caused by a decrease of SERT on the plasma membrane, but by a change in the turnover number.

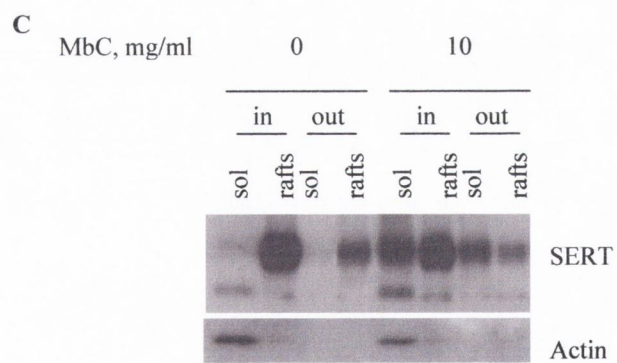
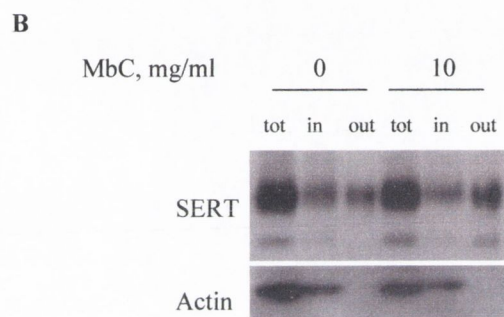
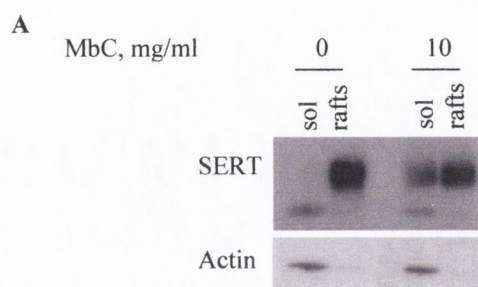
5.2.6 Loss of rafts' integrity results in an even distribution of SERT along the plasma membrane

The lipid raft hypothesis proposes that phase separation causes cholesterol, sphingolipids, and proteins to aggregate forming distinct microdomains within the plane of the membrane determining their insolubility in non-ionic detergents (Schroeder et al., 1998). Indeed the segregation of raft markers in distinct clusters on the plasma membrane is lost upon cholesterol depletion (Harder et al., 1998). As described above, SERT colocalised with the lipid raft marker GM1 and showed a punctate staining pattern, which is likely to arise from phase segregation within the membrane bilayer. If this is the case, it should be possible to observe a redistribution of SERT and GM1 by confocal microscopy, when lipid rafts are desegregated in intact cells. Although there is no general accord on the size of lipid microdomains, membrane rafts appear to be below the resolution of light microscopy. Thus, crosslinking with antibodies is used to stabilize lipid microdomains on the cell surface by clustering together raft components in larger patches of μm -size, thus allowing their observation by light microscopy (Harder et al., 1998; Harder and Simons, 1999). For this purpose, GM1 was clustered with CTxB and anti-CTxB antibody, cells were fixed and immunofluorescent staining

Fig. 5.9

Cholesterol depletion does not alter the number of SERT molecules on the plasma membrane.

TREx-SERT cells were incubated with the indicated amount of M β C for 30 minutes at 37°C, washed and then processed for cell surface biotinylation as described in Methods. Cells were lysed with 1% Brij 58 and lipid rafts were separated from soluble material by differential centrifugation for 1 hour at 101,300 g. Soluble material ('sol') and lipid raft fractions were analysed by immunoblotting for SERT and actin (A). Actin was not detected in raft fractions, indicating that no soluble material was sedimented following differential centrifugation. B) An aliquot of each cleared lysate (designated 'tot') was then incubated with NeutrAvidin beads in order to separate cell surface proteins ('out') from intracellular proteins ('in'). C) Similarly, soluble and raft fractions obtained by differential centrifugation were incubated with NeutrAvidin beads to monitor changes in SERT distribution upon cholesterol depletion. Actin was not detected in cell surface ('out') fractions indicating that only cell surface proteins were labelled by NHS-sulfo-biotin.



of SERT and GM1 was visualized by confocal microscopy. In control cells, SERT labelling localized preferentially to clusters in the areas of cross-linked GM1 patches (Fig. 5.10, top row). In cells treated with 10 mg/ml M β C, SERT and GM1 immunoreactivity seemed essentially diffusely distributed (Fig. 5.10, middle row). When cholesterol was further reduced with 20 mg/ml M β C, SERT and GM1 staining appeared uniformly localized along the plasma membrane (Fig. 5.10, bottom row). At this M β C concentration, cells showed morphological changes, such as rounding-up and a loss of branches as well as a tendency of detaching more easily from the support. This is likely to be caused by loss of interaction between adhesion molecules associated with lipid microdomains and the extracellular matrix (Berditchevski, 2001; Leitinger and Hogg, 2002).

5.2.7 SERT is associated with lipid rafts in brain

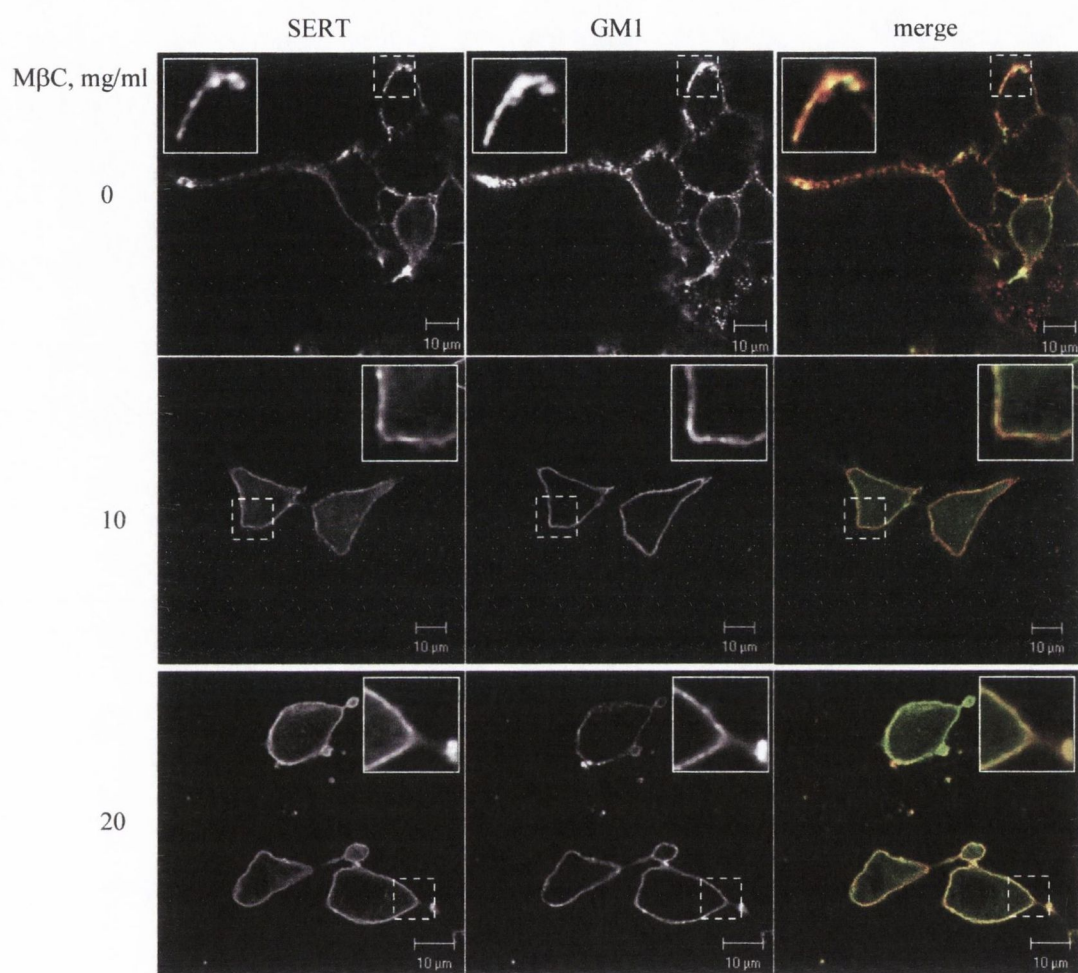
To determine whether the serotonin transporter is associated with lipid microdomains in native tissues, the solubility and flotation behaviour of SERT in rat brain were examined. Midbrain and forebrain regions, rich in serotonergic neurons, were homogenized and lysed with either Triton X-100, Tween 80, Brij 58, and Tween 20 at concentrations corresponding to 300-fold CMC (see Table 5.1). After detergent-extraction, soluble and insoluble material was separated by density gradient centrifugation. Samples were then analyzed by immunoblotting. SERT was identified as a single band of approximately 76 kDa and was only weakly associated with Triton X-100-resistant complexes (Fig. 5.11). SERT was recovered in the raft fractions (1 and 2) upon lysis with Tween 80, Tween 20, or Brij 58. Thus, SERT from rat brain tissue showed a similar pattern of association with lipid rafts compared to heterologously expressed SERT in cultured cells.

The SNARE protein syntaxin 1A has been shown to associate with lipid microdomains in PC12 cells, whereas its binding partner Munc 18-1 (nSec1) is not associated with rafts (Chamberlain et al., 2001; Lang et al., 2001). Similarly, in rat brain Munc 18-1 was fully solubilized by either 1% w/v Triton X-100 or 1% w/v Brij 58, whereas syn 1A exhibited low buoyancy and was recovered in the

Fig. 5.10

The punctate staining of SERT on the plasma membrane depends on rafts' integrity.

CTxB and anti-CTxB antibody were used to patch and stain GM1 in TREx-SERT cells as described in Methods. Cells were depleted of cholesterol using M β C. In control cells (first row), SERT (green) colocalizes extensively with GM1 (red) in cell surface clusters. SERT and CTxB punctate staining is lost following cholesterol depletion (second and third row).



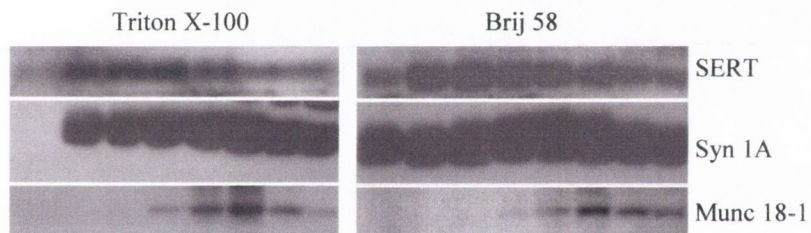
SERT is associated with lipid rafts in brain

	SERT							
	1	2	3	4	5	6	7	8
Triton X-100								
Tween 80								
Brij 58								
Tween 20								

Fig. 5.12

SERT and syntaxin 1A, but not Munc 18-1, are associated with lipid rafts in rat brain

Midbrain and forebrain regions of female Wistar rats were homogenated and then lysed with 1% w/v Triton X-100 or Brij 58. Lysates were subjected to floatation sucrose gradient as described in Methods and eight fractions were collected from the top to the bottom of the gradient. Fractions were analyzed by immunoblotting with the polyclonal anti-NSERT antibody (1:6000), the monoclonal anti-syn 1A (1:6000), and the monoclonal anti-Munc 18 (1:2000).



upper fractions of the density gradient (Fig. 5.12), thus providing an internal negative and positive controls for the assay. Moreover, considering the similar biochemical behaviour, it is likely that in brain SERT and syn 1A might be present in the same type of lipid rafts, whereas Munc 18-1 appears to be spatially segregated in a different membrane environment.

5.3 Discussion

The lateral organization of membranes in 'rafts' proposed by Simons and Ikonen (Simons and Ikonen, 1997) is of particular interest for membrane protein function since these lipid microdomains have been shown to selectively incorporate or exclude proteins. The data presented here provide biochemical and morphological evidence that SERT is associated with cholesterol-enriched microdomains and that this association is required for optimal 5HT-transport activity.

By means of flotation experiments, SERT resistance to solubilization in different non-ionic detergents was examined. SERT is solubilized in Triton X-100, and it is partially insoluble in Brij 58, Tween 20, and Tween 80. From these data it seems that SERT detergent-insolubility is associated with an HLB value ≥ 15 , similarly to the microvillar protein prominin (Roper et al., 2000). However, the structure of detergents may also affect SERT solubilization (i.e., presence of a phenol ring between the hydrophobic tail and the hydrophilic head of Triton X-100 and Nonidet P-40). The flotation profiles of both SERT and GM1 change markedly when different detergents are used. This may arise from a differential ability of detergents to insert into the tight acyl chain packing of lipids in rafts, thus resulting in the isolation of various subpopulations of microdomains, each characterized by a specific lipid composition (Schuck et al., 2003). Interestingly, the data presented here resemble quite closely the findings of Talvenheimo and Rudnick (Talvenheimo and Rudnick, 1980). The authors tested the ability of several detergents in solubilizing the imipramine binding sites of SERT-expressing platelets. They found that solubilization in Triton X-100 inactivates SERT, whereas in other detergents, such as Brij 58, Tween 20 and Tween 80, SERT is not solubilized and imipramine binding activity is recovered in particulate material that sediments at 226,000 g for 30 minutes. The conditions used by these authors are quite similar to the conditions found to be necessary to sediment lipid rafts (101,300 g for 1 hour, Fig.5.8). The similarity between the data presented in this study data and the data of Talvenheimo and Rudnick (Talvenheimo and Rudnick, 1980) led us to hypothesize that the population of

SERT molecules found to be associated with lipid microdomains were in an active form. This hypothesis has been corroborated by the finding that when cholesterol is depleted from cells, lipid rafts are desegregated, leading to the full solubilization of SERT (Fig. 5.7) and to the concomitant impairing of 5HT-transport (Fig. 5.2 A).

Specifically, the disruption of rafts by cholesterol depletion affects the $V_{\max}$ of 5HT-transport (Fig. 5.2 B). This could result either from a reduction of transporters located on the plasma membrane or from a decrease in the number of transported 5HT molecules per unit of time ('turnover number'). Upon desegregation of lipid rafts, the relative amount of SERT located on the cell surface is shown to be unchanged. Therefore, the $V_{\max}$ reduction caused by cholesterol depletion most likely reflects a change in the turnover number of 5HT-transport. Thus, SERT appears to require the association with lipid rafts in order to catalyse the transport of 5HT: the loss of interaction with lipid microdomains is detrimental to SERT function, possibly due to conformational changes that mainly affect the translocation of substrate across the plasma membrane. The increase in K_m for 5HT, although only slight, may provide an additional indication for a structural change of the transporter. The alteration of rafts' physical properties (e.g., bilayer thickness and membrane curvature) may cause SERT to assume an inactive conformation, similarly to what was observed for some ion-channels (Martinac and Hamill, 2002; Tillman and Cascio, 2003). 5HT can still bind reasonably well to SERT, but can no longer be efficiently transported. Lipid raft association might therefore be important for conformational changes associated with transport steps subsequent to 5HT binding. Such changes could affect, for example, ion binding and could be caused either by loss of direct interaction with cholesterol or by loss of interaction between SERT and other raft-proteins.

The reduction of cholesterol levels was also shown to affect the binding of SERT inhibitors in membrane preparations of Sf9 and HEK cells expressing SERT (Baker, 1997; Scanlon et al., 2001). Thus, further analysis of the

involvement of rafts in SERT function may shed some light on the mechanism of action of antidepressants and on their therapeutic efficacy.

This study also provides morphological evidence that in intact cells SERT is spatially segregated in lipid rafts and is redistributed evenly along the plasma membrane upon cholesterol depletion. In untreated cells, SERT colocalizes with the ganglioside GM1 in discrete clusters along the plasma membrane irrespective of the mode of fixation and whether rafts had been stabilized by co-patching using CTxB (compare Fig. 5.3 and Fig. 5.10, control). This may indicate that in HEK cells lipid rafts have a size in the μ -range, thus above the resolution of light microscopy. When lipid microdomains are desegregated by reduction of cholesterol levels, the segregation of SERT and GM1 into discrete patches is lost and they appear diffusely distributed along the plasma membrane (Fig. 5.10). It is conceivable that the spatial distribution of SERT in heterologous expression systems might indicate a raft-dependent targeted localisation of the transporter to discrete domains also in native tissue.

In neurons, SERT functions in the reuptake of 5HT following its release at the presynaptic membrane. It has been shown that SERT is present all along the axolemma and in axon terminals, but is present at very low levels at the plasma membrane of cell soma and dendrites (Zhou et al., 1998; Tao-Cheng and Zhou, 1999). This suggests that SERT function is tightly linked to its position in specific regions of the plasma membrane. Thus, a regulated mechanism would be required to target SERT to the plasma membrane of neurons in a polarized manner. In the past few years a body of data has become available on the role of lipid rafts both in neuronal trafficking and in cell signalling (Ledesma et al., 1998; Ledesma et al., 1999; Tsui-Pierchala et al., 2002). In this study SERT is shown to associate with lipid rafts also in rat brain. Similarly to TREx-SERT cells, neuronal SERT is partially recovered at the high-buoyant raft-fractions (1 and 2) when solubilization is carried out in Brij 58, Tween 20, Tween 80, and to a lesser extent in Triton X-100 (Fig. 5.11).

An interesting hypothesis is that the localization of SERT in neuronal lipid rafts may physiologically regulate its function within the axolemma. Plasma membrane bound SERT might be found in equilibrium between an active raft-associated state and an inactive state residing outside lipid rafts. The relative distribution between these two states might depend on the physiological situation; for example, the raft-associated transport-competent SERT conformation might be favoured following neurotransmitter release. The question is what triggers the association with lipid microdomains. Interestingly, cholesterol appears to regulate different steps of neurotransmitter release. In the central nervous system, cholesterol is released by glial cells through apoE-containing lipoproteins and is taken up by neurons, where it contributes to an increase in the number of synapses (Mauch et al., 2001). Cholesterol also positively regulates the formation of synaptic vesicles (Thiele et al., 2000) and enhances the assembling of SNARE proteins in defined synaptic vesicles-docking and fusion sites (Lang et al., 2001). Thus, it would be interesting to investigate whether, by regulating the levels of cholesterol in neurons, SERT activity could be modulated at the synapse by a dynamic association with lipid rafts.

In cultured cells the population of SERT-containing rafts was shown not to be homogenous in terms of size. In fact, a fraction of lipid rafts is sedimented by centrifugation at 101,300 g for 1 hour ('small' rafts), but not at 15,000 g for 20 minutes ('large' rafts). Similar raft-subsets have been identified for the protein prominin and are involved in the transport of prominin from the trans-Golgi/TGN to the apical plasma membrane (Roper et al., 2000). The finding presented here that SERT-rafts are found in intracellular membranes (Fig. 5.9 B) may suggest that lipid microdomains play a role in trafficking of SERT to the plasma membrane and is consistent with a possible involvement of lipid rafts in the apical delivery of SERT to the axolemma and axon terminals. In order to corroborate such a hypothesis, further studies on the involvement of lipid microdomains in SERT trafficking and targeting are necessary.

Chapter 6 Results

Is N-glycosylation necessary for SERT association with lipid rafts?

6.1 Introduction

6.1.1 Protein N-glycosylation

Glycans can be attached to proteins by a covalent bond between a preformed glycan to the amide group of asparagine at the specific sequence Asn-X-Ser/Thr- (X can be any amino-acid, except proline). The presence of this motif in the amino-acid sequence of a protein is necessary but not sufficient for glycosylation to occur. Other factors, such as secondary structure of the peptide in the vicinity of the consensus sequence, may influence the process.

A given glycoprotein has to be considered as a population of molecules, characterized by the same polypeptide but with different glycosylation states ('glycoforms'). Even though the population of glycoforms shows a conserved and reproducible pattern in a given cell line, it can be altered by pathological conditions and is strictly cell-type specific.

The precursor glycan is synthesized in the endoplasmic reticulum (ER) by glycosyltransferases that build the oligosaccharide [(glucose)₃(mannose)₉(N-acetylglucosamine)₂] on a dolichol lipid carrier, a process called "the dolichol-cycle". This basic core is transferred from the dolichol carrier to the asparagine of a specific -Asn-X-Ser/Thr- sequence, while the protein is being translated. The precursor glycan is then sequentially elongated and trimmed in the ER and Golgi apparatus. The processing that N-glycans undergo in the ER confers limited diversity to the glycoprotein, and these alterations are shared by all glycoproteins. The pattern of glycoforms is rather uniform until the glycoproteins are transferred to the medial Golgi, where structural diversification is progressively introduced. In the ER, glycans promote mainly protein folding and quality control. Misfolded proteins fail to pass the quality control and are selectively degraded by unfolded protein response (UPR) or ER-associated degradation (ERAD), a stress response characterized by the upregulation of several chaperones in the ER (McMillan et al., 1994; Brewer et al., 1999). Glycans have several functions in the cell, including enhancing protein stability, mediating ligand-receptor interactions and

controlling the quality of proteins destined for secretion (Alberts et al., 1994). Several of the enzymes of the N-glycosylation process can be inhibited, arresting the maturation of a protein at a particular stage. These inhibitions are valuable tools to investigate the role of N-glycans in the function of glycoproteins.

6.1.2 SERT is co-translationally modified by addition of N-glycans

A feature common to all members of the $\text{Na}^+\text{-Cl}^-$ dependent transporter family is a large extracellular loop between TMD 3 and 4, which contains 1 to 4 potential N-linked glycosylation sites. The position of the first glycosylation consensus sequence, the most N-terminal one, is conserved amongst the members of the transporter family, whilst the position of the other consensus sequences varies (Tate and Blakely, 1994).

The primary sequence of SERT contains two putative N-glycosylation sites, located at Asn²⁰⁸ and Asn²¹⁷, which have been both demonstrated to be used for the glycosylation of the transporter in Sf9 insect cells (Tate and Blakely, 1994). SERT produced in HEK cells (and in other cell lines) exhibits structural heterogeneity that is evidenced by aberrant migration in an SDS-PAGE. Different N-glycosylation states of SERT can typically be shown by Western blotting: a 55 kDa unglycosylated form, a 70 kDa partially glycosylated form, and a third, broader band at ~ 85 kDa (fully glycosylated form) (Tate et al., 2003). The presence of glycans increases the molecular mass of the polypeptide and alters the binding capacity of SDS to the protein, ultimately affecting the transporter electrophoretic mobility.

6.1.3 N-glycosylation as an apical sorting signal and targeting signal to rafts

It has been proposed that N-glycans function as apical targeting signals for proteins in polarized cells. For example, the addition of N-glycosylation consensus sequences promotes the apical release of the growth hormone, a protein normally non-glycosylated and secreted in a non-polarized fashion (Scheiffele et al., 1995). Similarly, the recombinant addition of N-glycans to membrane proteins has been shown to promote apical surface delivery (Gut et al., 1998). N-glycans

are proposed to be recognized by intracellular lectins such as VIP36 at the trans-Golgi network (Fiedler et al., 1994), which may mediate apical trafficking. However, the role of N-glycans as an apical sorting signal is not unequivocal. Several proteins have been reported to traffic in a non-polarized manner, even though they are N-glycosylated (for a review see (Rodriguez-Boulán and Gonzalez, 1999)). Therefore, N-glycans do not appear to be a universal signal for apical sorting of membrane proteins or secretory proteins and their role in protein function needs to be investigated on a case-by-case basis.

Mechanisms involved in apical sorting include association with lipid rafts. N-glycosylation can promote raft association and/or be required in addition to raft association for apical destination (Alfalah et al., 2002; Kohno et al., 2002). Whereas in non-polarized cells, such as fibroblasts, lipid microdomains are distributed along the whole cell surface, in polarized cells, such as neurons, rafts preferentially accumulate in the apical (axonal) plasma membrane (Simons and Ikonen, 1997).

In neurons, the serotonin transporter has been mainly found to be located in the axolemma and in the presynaptic membrane, whereas it is scarcely present on the plasma membrane of soma and dendrites (Zhou et al., 1998; Tao-Cheng and Zhou, 1999). Therefore, it seems likely that SERT may be targeted to the apical membrane of neurons (the axolemma) by a specific mechanism. The finding that SERT is associated with lipid rafts (see Chapter 5) suggests that cholesterol-enriched microdomains may play a role in the apical targeting of the transporter. As discussed in the previous chapter, cholesterol appears to be a specific requirement for SERT function, perhaps by interacting directly with the transporter or, alternatively, because cholesterol is required to form lipid rafts within the plasma membrane of cells. If cholesterol does not interact directly with SERT, thus mediating the association with lipid microdomains (a hypothesis that, however, needs to be investigated: see Chapter 7), the question of how the transporter is inserted in lipid rafts is left open. It is possible that the association of the transporter with rafts might be modulated via SERT interacting proteins,

associated themselves with rafts (e.g. syntaxin 1A). However, the fact that the serotonin transporter is a glycosylated protein opens a third possibility, that its N-glycans might mediate the association with lipid rafts, as has been found for other two glycoproteins, the intestinal dipeptidyl peptidase IV and the sphingosine 1-phosphate receptor Edg-1 (Alfalah et al., 2002; Kohno et al., 2002). Thus, the aim of the studies presented in this chapter was to investigate whether N-glycosylation was a necessary requirement for SERT association with lipid rafts.

6.2 Results

6.2.1 Tunicamycin treatment of TREx-SERT cells

In order to investigate whether N-glycans play a role in associating SERT with lipid rafts the antibiotic tunicamycin was used. This compound inhibits the enzyme dolichol pyrophosphate N-acetylglucosamine transferase, which is one of the initial transferases of the dolichol-cycle. Thus, tunicamycin prevents the formation of dolichol-linked oligosaccharides and results in the blockage of N-glycosylation of proteins. TREx-SERT cells were grown in presence of 2 µg/ml tunicamycin diluted in DMSO for 0, 24 or 48 hours. In parallel, control cells were incubated with an equivalent volume of DMSO for the indicated time. Cells were then lysed in RIPA buffer and cleared lysates were analyzed by SDS-PAGE. In control cells the transporter was detected by Western blotting as a mixture of polypeptides with different glycosylation status. SERT-immunoreactive bands were detected at about 85 kDa (fully glycosylated form) and at 70 kDa (partially glycosylated form). The mobility of SERT changed markedly upon tunicamycin treatment. Following 24 or 48 hours incubation with tunicamycin, a single SERT-immunoreactive band was detected with M_r of about 55,000 (Fig. 6.1A).

The typical fibroblastoid morphology of HEK cells (stretched and branched) changed dramatically upon 24-48 hours incubation with tunicamycin to a rounded one (not shown). It is possible that tunicamycin interfered with adhesion of cells to the extracellular matrix by inhibiting N-glycosylation of cellular adhesion molecules (CAM).

In the attempt to overcome this 'toxic' effect, cells were incubated for a shorter time with 2 µg/ml tunicamycin. The mobility of the SERT-immunoreactive bands was affected after 2 hours incubation with the antibiotic (Fig. 6.1B). By increasing the length of incubation with tunicamycin, the partially and fully glycosylated forms of SERT were progressively less detectable. The maximal tunicamycin effect was observed after 16 hours, when the 85- and 70-kDa bands were no longer detected and SERT immunopositivity was reduced to less than 1/10 of control. The partially glycosylated transporter appeared to be affected by the tunicamycin treatment already after two hours incubation, perhaps

due to a lower stability of this glycoform compared to the fully glycosylated transporter (Rudd et al., 1994; Rudd et al., 1995). The blot was reprobed for actin, showing that equal amounts of protein samples were loaded onto the polyacrylamide gel (Fig. 6.1B). Cell morphology was only mildly affected after 16 hours incubation with tunicamycin (not shown).

6.2.2 Inhibition of N-glycosylation affects SERT association with lipid rafts

TREx-SERT cells were incubated with 2 μ g/ml tunicamycin in DMSO for 16 hours or with an equivalent volume of DMSO (control cells). Cells were then lysed in 1% w/v Brij 58 in TNE buffer and processed for floatation gradient centrifugation as described in Methods. Protein concentration of cleared lysates was determined and samples were brought to the same protein concentration by addition of lysis buffer. Equal volumes of lysates were then loaded at the bottom of a discontinuous sucrose gradients and lipid rafts were isolated by flotation centrifugation. Eight fractions were collected, and an aliquot of each fraction, pellets and total sample was analyzed by Western blotting. In tunicamycin-treated cells, SERT-immunoreactive bands were no longer detected in raft fractions (number 1 and 2, Fig. 6.2). A comparison of SERT-bands optical density between control and treated cells revealed that tunicamycin incubation of TREx cells resulted in a loss of SERT, as seen in section 6.2.1, Fig. 6.1B. Usually a decrease in the half-life of a protein synthesized in the presence of tunicamycin reflects endoplasmic reticulum-mediated response to unfolded protein (Brewer et al., 1997; Sidrauski et al., 1998). Therefore, unglycosylated SERT may not be correctly folded and therefore susceptible to degradation. The accumulation of misfolded transporter in the ER could also account for the apparent loss of association with lipid rafts.

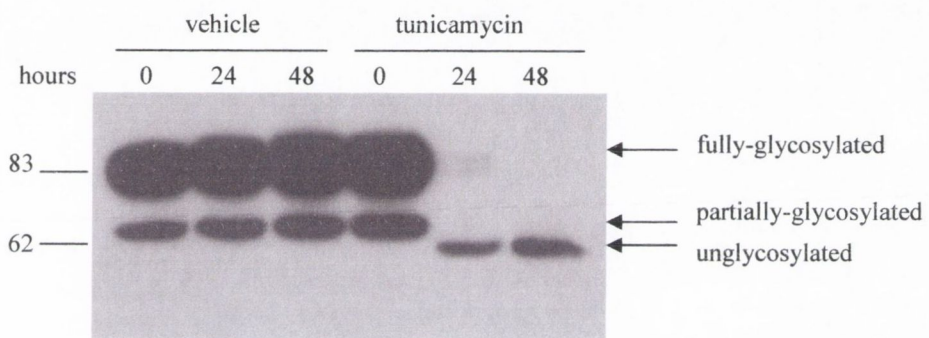
6.2.3 Removal of N-glycosylation signals by site-directed mutagenesis

Fig. 6.1

Effect of tunicamycin on SERT electrophoretic mobility

A) After treatment with 2 $\mu\text{g/ml}$ tunicamycin or DMSO (vehicle) for the indicated time, TReX-SERT cells were lysed, subjected to 10% SDS-PAGE, blotted and probed with anti-NSERT antibody (1:6000). B) Cells were treated with 2 $\mu\text{g/ml}$ tunicamycin or DMSO as above for the indicated time. Protein concentration of each sample was determined by Markwell assay as described in Methods. The blot was probed with anti-NSERT antibody, stripped and reprobed with anti-actin antibody (1:5000). Blots are representative of 2-3 independent experiments.

A



B

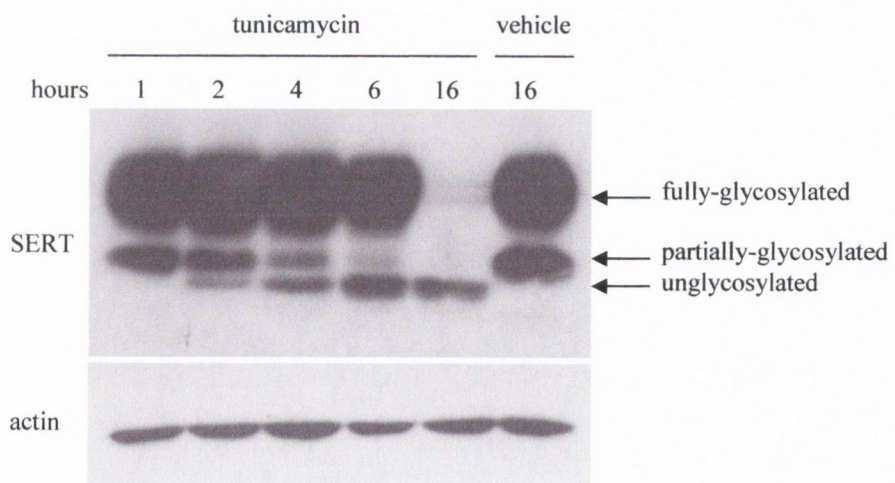
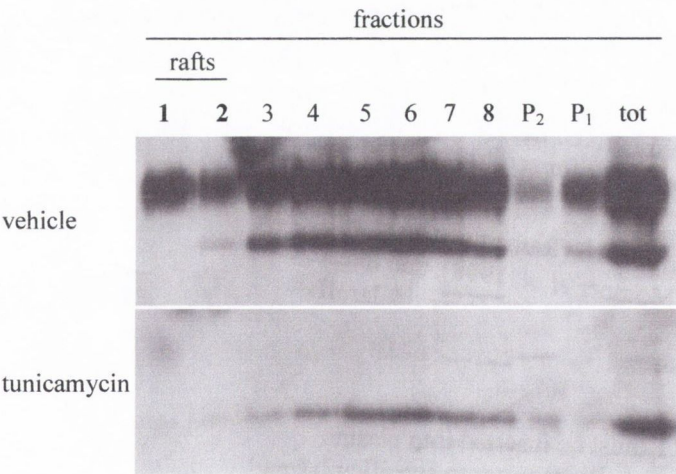


Fig. 6.2
Distribution of SERT in sucrose density gradient upon tunicamycin treatment.

TREx-SERT cells were incubated with tunicamycin (2 µg/ml, 16 hours; lower panel) or an equivalent amount of DMSO (upper panel), lysed in 1% Brij 58, and fractionated by discontinuous sucrose gradient centrifugation (see details in Methods). Fraction 1 represents the lower density sucrose layer and fraction 8 represents the higher density sucrose fraction. Fractions 1 and 2 (in bold) contain the detergent-insoluble material ('rafts'). Each fraction was analyzed by Western blotting using the anti-NSERT antibody (1:6000). 'P₂' is the pellet recovered from the bottom of the density gradient, 'P₁' represents the pellet recovered from the initial centrifugation at 830 g for 10 minutes, and 'tot' represents the precleared lysed loaded at the bottom of the sucrose gradient. Three independent experiments gave similar results.



In order to establish whether N-glycans are directly correlated with the association of SERT with rafts, site-directed mutagenesis was employed to selectively remove one or both asparagine-based glycosylation signals.

The two N-linked glycosylation consensus-sites (Asn²⁰⁸ and Asn²¹⁷) were changed to glutamine. Glutamine is the amino-acid that bears the most similar chemical structure to asparagine (side chains (R): R_{Asn} = -CH₂(CO)NH₂; R_{Gln} = -CH₂CH₂(CO)NH₂). Thus, the mutation of Asn with Gln is a conservative change, less likely to alter protein folding/structure *per se*.

The QuickChange Site-directed mutagenesis kit (Stratagene) was used to generate such mutations directly in SERT cloned into p5b-FLAG vector (Sigma) containing SERT (pSERT-FLAG; Magnani, Killian, et al., manuscript in preparation). Two sets of oligonucleotide primers were synthesized containing the desired mutation from the Asn codon (AAC) to the Gln one (CAA) (Fig. 6.3), and were used to generate the single mutants pSERT^{N208Q} and pSERT^{N217Q}. Pfu-Turbo DNA-polymerase replicated both plasmid strands with high fidelity by extending the mutagenic primers and generating a mutated plasmid containing nicked ends. Following PCR, the samples were then treated with the methylation-sensitive Dpn I endonuclease to digest the methylated DNA template. The PCR products were subsequently transformed into the E.coli strain DH5 α . Mutated plasmids were isolated by a small-scale plasmid preparation, analyzed by restriction analysis and sequencing to confirm the Asn to Gln mutations with no additional change of SERT sequence.

pSERT^{wt} and glycosylation-deficient mutants were transfected in HEK cells in order to verify whether the mutant SERT constructs were expressed and to assess the glycosylation status of resulting proteins. Cell membranes were prepared from transfected cells and 200 μ g of membranes were incubated with 1 unit of endoglycosidase PNGaseF at 37°C overnight as described in Methods. HEK cells expressing the single mutants SERT^{N208Q} and SERT^{N217Q} produced a pattern of polypeptides ranging from 55 kDa to about 70 kDa, corresponding to a different glycosylation status of the mutated transporter (Fig. 6.4). The SERT immunoreactive band of M_r 83,000 present in the lysates of cells expressing the

Fig. 6.3

Site direct mutagenesis of N-glycosylation sites in N²⁰⁸ and N²¹⁷

A) Sequence of primers used to remove from the rSERT-coding region the potential N-glycosylation sites. Oligonucleotides listed in (1) converted Asn-208 to Gln and oligonucleotides listed in (2) converted Asn-217 to Gln, generating the single glycosylation mutants pSERT^{N208Q} and pSERT^{N217Q} respectively. The double mutant pSERT^{N208,217Q} was generated by site direct mutagenesis using pSERT^{N217Q} as a template and the set of primers listed in (1). B) Schematic representation of the location of the N-glycosylation consensus sequences (in bold) in the second extracellular loop in rSERT. Asn208 and Asn217 are indicated in red. Below, results of sequencing of the rSERT glycosylation mutants: the figure compares the nucleotidic sequences of pSERT^{N208Q}, pSERT^{N217Q}, and pSERT^{N208,217Q} to pSERT^{wt} (only the alignment of sequences between base-851 and -900 is shown). The conservative mutation of Asn- to Gln-codon is highlighted in red.

(1) N208Q-primers

reverse 5' CGAAGTAGTTGGTGCATTGGCCAGTGTTCC 3'

(2) N217Q-primers

reverse 5' GCGTCCAGGTGATTGGTCCTGGGCGAAG 3'

TMD3 PWTSC TNSWNTGNCTNYFAQDNITWTLHSTSPAE . . . **TMD4**

↓ ↓

-GCAACTGCACCAACTACTTCGCCCAGGACAACATCACCTGGACGCTGCAT- (SERT^{WT})

-GCCAATGCACCAACTACTTCGCCCAGGACAACATCACCTGGACGCTGCAT- (SERT^{N208Q})

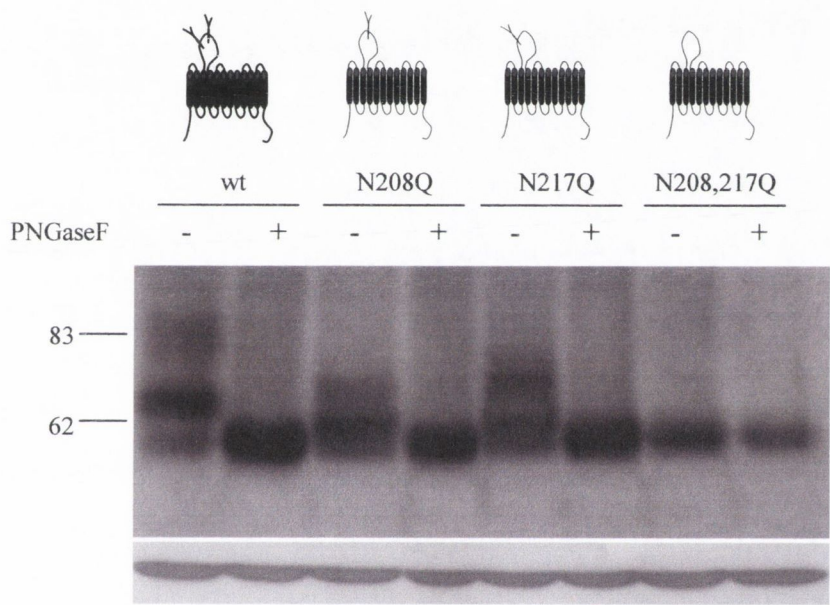
-GCAACTGCACCAACTACTTCGCCCAGGACCAATCACCTGGACGCTGCAT- (SERT^{N217Q})

-GCCAATGCACCAACTACTTCGCCCAGGACCAATCACCTGGACGCTGCAT- (SERT^{N208, 217Q})

Fig. 6.4

Western blotting analysis of membranes prepared from HEK cells expressing wild type SERT and glycosylation-deficient transporters

200 μ g of membranes were incubated with 1 unit of PNGaseF (+) or with a corresponding volume of reaction buffer (-) at 37°C overnight, as described in Methods. Blots probed with anti-NSERT antibody (1:6000), and subsequently stripped and reprobed with anti-actin antibody (1:5000) to confirm equal loading of samples.



wild type transporter was not detected in cell extracts of HEK cells expressing the single glycosylation-deficient mutants, due to the use of only one glycosylation site instead of the two present in the wild type. The electrophoretic mobility of SERT^{N208,217Q} was unchanged after PNGaseF treatment (apparent Mr ~ 55,000), indicating that the removal of the two putative N-glycosylation consensus sequences resulted in the expression of unglycosylated SERT.

6.2.4 Glycosylation-status of SERT affects the velocity of 5HT transport

The consequences of N-glycosylation site disruption on the kinetic of serotonin transport were investigated.

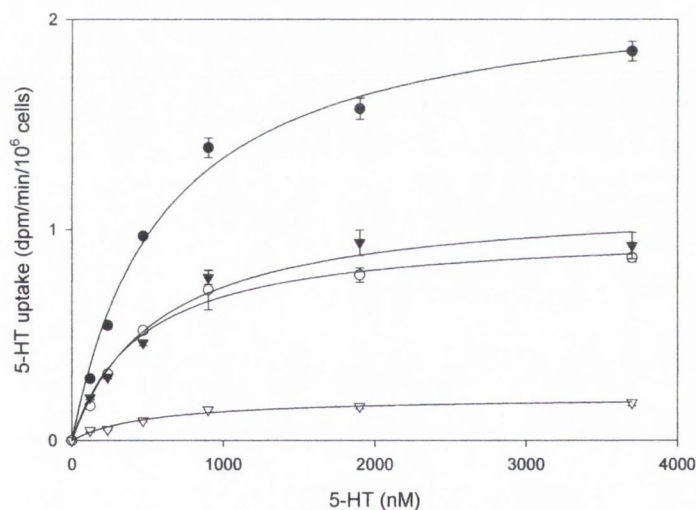
SERT^{wt} and its glycosylation-deficient mutants were transfected in parallel into HEK cells. After 48 hours, [³H]-5HT uptake was measured as described in Methods by incubating cells with various concentration of [³H]-5HT solution. The four transporters showed a consistent $V_{\max}$ ratio of 10:5:5:1 for wt:N208Q:N217Q:N208,217Q in three independent experiments. Compared with the SERT^{wt}, the two single mutants showed a significant 2-fold reduction in transport (Fig. 6.5: SERT^{wt}, $V_{\max}$ 2.16 ± 0.09 pmol/min/ 10^6 cells; SERT^{N208Q}, $V_{\max}$ 0.99 ± 0.04 pmol/min/ 10^6 cells; SERT^{N217Q}, $V_{\max}$ 1.14 ± 0.08 pmol/min/ 10^6 cells), with no significant change in the affinity for 5HT (SERT^{wt}, K_m 607 ± 78 mM; SERT^{N208Q}, K_m 457 ± 62 mM; SERT^{N217Q}, K_m 570 ± 120 mM). The unglycosylated transporter showed a 10-fold decrease in 5HT-transport velocity compared with SERT^{wt} with no significant reduction in its affinity for serotonin (Fig. 6.5: SERT^{N208,217Q}, $V_{\max}$ 0.20 ± 0.01 pmol/min/ 10^6 cells; K_m 521 ± 98 mM).

6.2.5 Glycosylation-deficient SERTs are not efficiently targeted on cell surface

The reduction in $V_{\max}$ observed for SERT glycosylation-deficient mutants could result from a decrease in turnover number, cell surface expression, or synthesis of the transporter. A reduction of the transporter's synthesis was already apparent for the glycosylation mutants by Western blotting analysis (Fig. 6.4), and this may account for the change of $V_{\max}$ observed. However, SERT needs to be

Fig. 6.5
Uptake of [3 H]-5HT into HEK cells expressing glycosylation-deficient mutants and the control wild type-SERT.

HEK cells were seeded onto poly-L-lysine coated 24-well plates and 24 hours later transiently transfected with 0.3 μ g/well of either pSERT^{wt} (●), pSERT^{N208Q} (○), pSERT^{N217Q} (▼), or pSERT^{N208,217Q} (▽) for 48 hours. [3 H]-5HT was added to the indicated final concentrations and incubated for 6 minutes. The assay was carried out as described in Methods. Each data point was determined in triplicate as was plotted as mean value $\pm$ SE. The saturation curve shown is representative of three independent experiments each giving similar results.



located on the cell surface to catalyze the transport of 5HT. Therefore, it is important to investigate whether the observed decrease in 5HT transport was due a specific reduction in cell surface expression of glycosylation-deficient mutants. It should be noted that transient transfection resulted in lower levels of fully glycosylated SERT (Fig. 6.4) compared to the TREx-SERT stable expression system (Fig. 6.1; see paragraph 6.2.6 for further comments).

For this purpose, HEK cells were transfected in parallel with constructs for wild type- and glycosylation mutant-SERTs. After 48 hours, cell surface biotinylation was carried out as described in Methods. As revealed by biotinylation, cells expressing SERT^{wt} showed two polypeptides of about 85- and 70-kDa at the cell surface (Fig. 6.6, top panel). Cells transfected with either the mutant SERT^{N208Q} or SERT^{N217Q} exhibited a SERT-immunopositive band of about 70 kDa at the cell surface. The combined intensity of SERT^{wt} 85- and 70-kDa species appeared to be similar to the intensity of the 70 kDa species of the single mutant SERT^{N208Q}. The immunopositivity of the SERT^{N217Q} mutant appear to be slightly reduced compared to the SERT^{N208Q} mutant. The double mutant was detected at the cell surface as a faint 55 kDa SERT-immunopositive band. Although rather weak, the unglycosylated transporter was detected at the cell surface for all the four constructs. The absence of actin- immunoreactive bands from the cell surface fractions indicated that no intracellular proteins were non-specifically biotinylated (Fig. 6.7, middle panel). The blot was stripped and reprobed with streptavidin-HRP to detect biotinylated proteins: no biotin was detected in unbound protein fractions, indicating that biotinylated proteins were efficiently recovered in the bound fractions (Fig. 6.6, bottom panel).

This result suggests that the observed reduction in 5HT transport for the double mutant may be explained by a reduction of SERT molecules at the cell surface, as a consequence of N-glycans' removal. However, this does not seem to be the case for the two single mutants: whereas the $V_{\max}$ for the two single mutants is reduced to about 50% the $V_{\max}$ measured for the wild type transporter, their cell surface expression is not halved. This suggests that the observed decrease of $V_{\max}$ is to some extent attributable to a change of transporter conformation due to the removal of N-glycans.

Fig. 6.6
Cell surface labelling of SERT^{wt}, SERT^{N208Q}, SERT^{N217Q}, and SERT^{N208,217Q}
in transiently transfected HEK cells.

Cell surface biotinylation was performed 48 hours after transfection as described in Methods. Blots were first probed with anti-NSERT antibody (1:6000) and then stripped and reprobed with anti-actin antibody (1:5000). Blots were stripped again and reprobed with streptavidin-HRP (1:500).

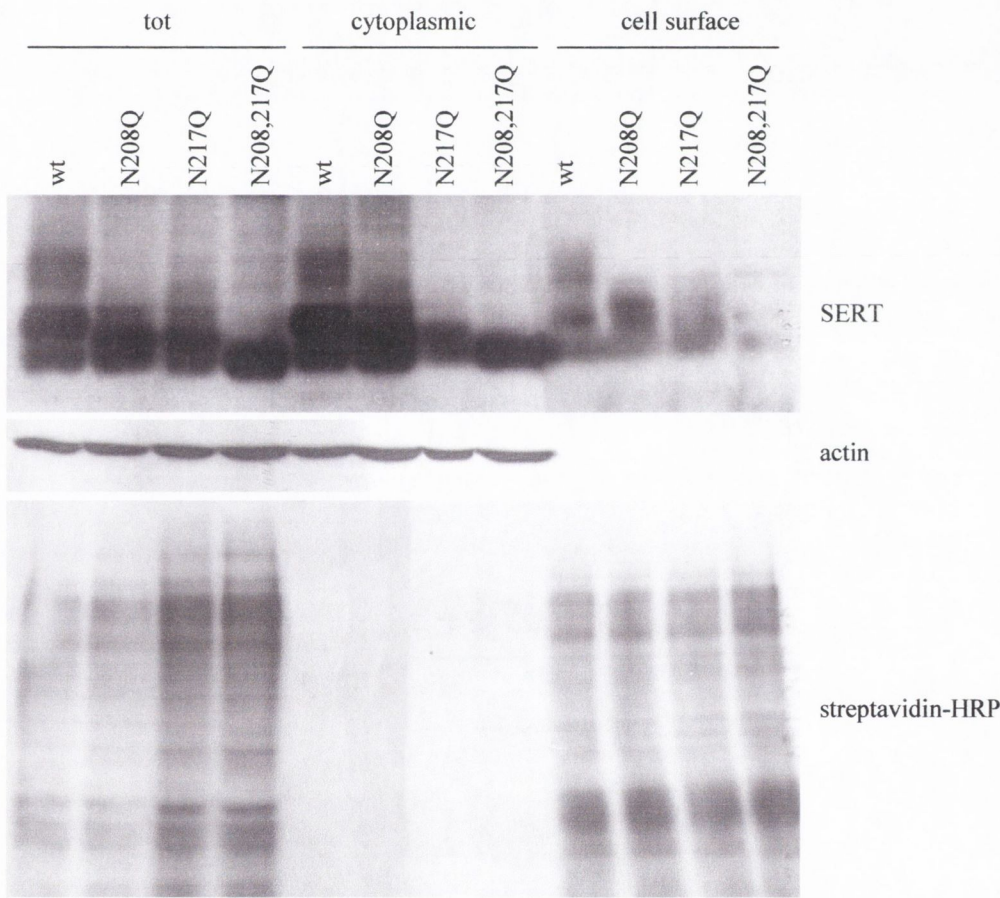
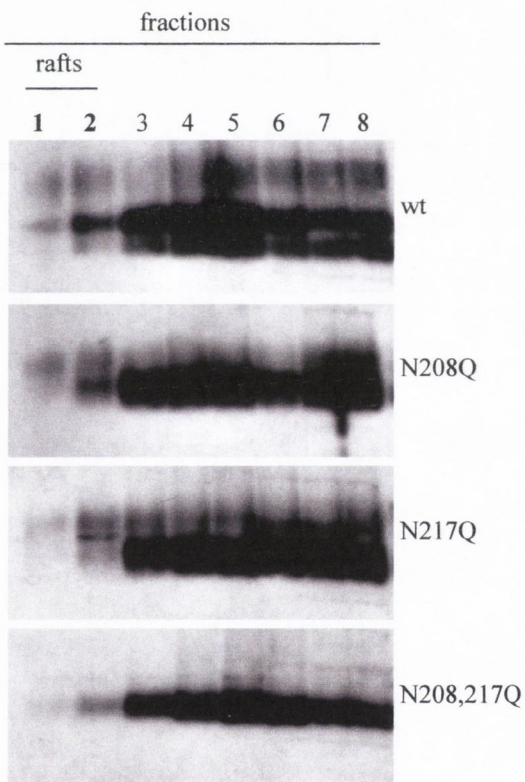


Fig. 6.7

Distribution of wild type and glycosylation-deficient SERT in sucrose density gradient.

HEK cells were transfected for SERT^{wt} and its glycosylation-mutants (4 µg DNA/ 10 cm Petri dish). After 48 hours, cells were lysed with 1% w/v Brij 58 and processed for flotation density centrifugation as described in Methods. Eight fractions were collected from the top (fraction number 1) to the bottom (fraction number 8) of the gradient, and analyzed by Western blotting. Blots were probed with anti-NSERT antibody (1:6000). Fractions 1 and 2, corresponding to the interface 5-30% sucrose, contain the detergent-insoluble material ('rafts'). Blots shown are representative of three separate experiments that gave similar results.



6.2.6 The association of SERT with rafts is not mediated by its N-glycans

The localization of glycoproteins in lipid rafts has been shown in some cases to depend on their glycans-moiety (Alfalah et al., 2002; Kohno et al., 2002). Therefore, the association of glycosylation-deficient SERTs with lipid microdomains was investigated.

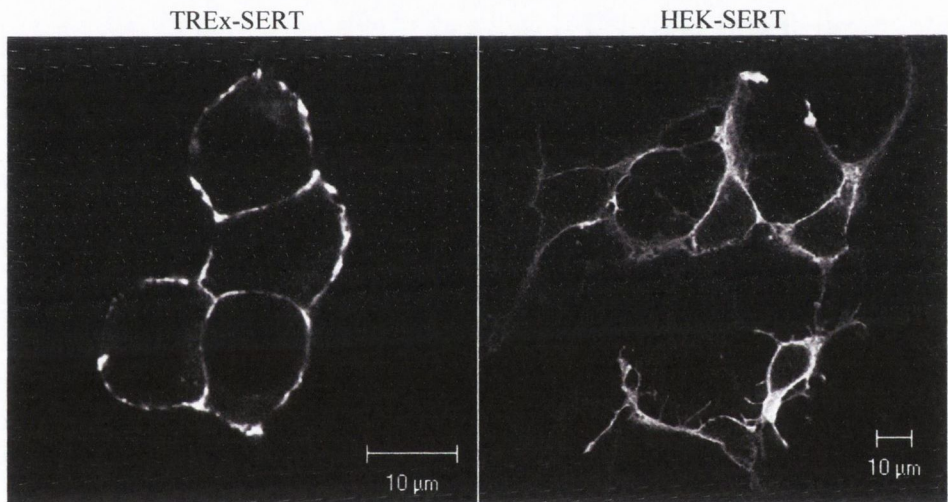
HEK cells were transfected in parallel with the constructs for SERT^{wt}, SERT^{N208Q}, SERT^{N217Q}, and SERT^{N208,217Q}, and 48 hours later cells were lysed in 1% w/v Brij 58. Cleared lysates were subjected to sucrose gradient centrifugation as described in Methods to isolate lipid rafts. The association of glycosylation-deficient transporters with lipid rafts appears to be reduced (Fig. 6.7). However, such a reduction seems to correlate to the decreased expression levels observed for the glycosylation-deficient transporters (Fig. 6.5). Taken together, these results suggest that N-glycans do not seem to mediate SERT association with lipid raft microdomains.

Notably, the localization of SERT^{wt} in rafts was lower when the transporter was transiently expressed in HEK cells compared to SERT expressed in the inducible cell line TREx-SERT (see Chapter 3). Despite the fact that HEK is similar to the parental cell line used to produce the TREx-SERT cell line (Tate et al., 2003), it is evident that SERT is processed differently in the two expression systems. Transient transfection of SERT in HEK cells produced three polypeptides of 85-, 70-, and 55-kDa (see Fig. 6.5), whilst in the TREx-SERT cells the 55 kDa-unglycosylated transporter was barely detectable (see Fig. 6.1). HEK cells showed a diffuse SERT-immunofluorescence throughout the cell cytoplasm and plasma membrane (Fig. 6.8, right panel). However, TREx-SERT cells showed a strong labelling along the plasma membrane (Fig. 6.8, left panel). For transient expression, the plasmid, once it enters a cell, is episomally maintained and these cells would have higher expression levels since the copy number of the plasmid is higher. In general, for stable transfectants only one or two copies are integrated. Therefore, stably transfected cells will usually have lower expression levels than transiently transfected cells. It is thus likely that high expression levels of SERT cause an overload of the glycosylation machinery

Fig. 6.8

Subcellular distribution of SERT in stably transfected TREx-SERT cells and transiently transfected HEK cells.

HEK cells (right panel) were transfected with pSERT^{wt} (4 μ g DNA/ 10 cm Petri dish) and after 48 hours processed for immunofluorescence as described in Methods. TREx-SERT cells (left panel) were induced with 1 μ g/ml tetracycline and 24 hours later fixed and stained as above. SERT was probed with anti-NSERT antibody (1:500).



capacity in the ER of transiently transfected HEK; consequently, transporter maturation and its trafficking to the Golgi apparatus and plasma membrane may be slowed down. The high ratio fully glycosylated/ partially glycosylated SERT in the induced TReX-SERT cells may account for the elevated velocity of 5HT transport measured (73.6 pmol/min/ 10^6 cells, Fig. 3.14) compared to the values obtained for transiently transfected HEK cells (2.16 pmol/min/ 10^6 cells, Fig. 6.5). Therefore, the higher number of SERT molecules per cell expressed by transient transfection compared to the inducible expression system may generate the observed differences in SERT association with rafts.

6.3 Discussion

The results presented in this chapter suggest that N-glycans are not necessary for SERT association with lipid rafts. Although a reduction in the association with rafts is evident, this appears to be a secondary effect of glycosylation-deficient transporters' reduced expression. The inefficient folding of glycosylation-mutants, which, in turn, would prompt ER-mediated proteolysis, may cause such a reduction in expression.

One of the proposed functions of asparagine-linked oligosaccharides is to protect the polypeptide from proteolysis (Varki, 1993). By comparing glycosylated proteins with their unglycosylated counterparts, glycosylation has been shown to increase thermal stability, solubility, and resistance to protease digestion (Rudd et al., 1994; Rudd et al., 1995). Additionally, for some proteins glycosylation functions as a signal for apical targeting in polarized cells (Scheiffele et al., 1995; Gut et al., 1998). More recently, N-glycosylation has been also shown to promote the association of the intestinal dipeptidyl peptidase IV and sphingosine 1-phosphate receptor Edg-1 with lipid rafts (Alfalah et al., 2002; Kohno et al., 2002).

Experiments designed to analyze the importance of glycosylation in protecting endogenous proteins from proteolysis in cells have used approaches that prevent the initial glycosylation of the nascent protein. Most of these studies have used tunicamycin, an inhibitor of UDP-GlcNAc:dolichyl-phosphate GlcNAc-

1-phosphate transferase, to produce unglycosylated proteins *in vivo*. Following tunicamycin treatment of TREx-SERT cells, SERT immunoreactivity was evidently reduced, probably due to proteolysis of the unglycosylated transporter (Fig. 6.1B). An overall effect of the compound on cell morphology was also noticed. The morphology of TREx-SERT cells was altered upon tunicamycin incubation from an elongated and branched fibroblastoid-like shape to a rounded one. This was possibly caused by reduced cellular adhesion to the extracellular matrix, due to a loss of CAMs within the cell surface glycocalyx. It is known that the inhibition of initial glycosylation by tunicamycin disrupts protein folding to such an extent that it induces the 'unfolded protein response', a complex stress response characterized by the up-regulation of several chaperones in the ER (Brewer et al., 1997). Consequently, many of the misfolded proteins are rapidly degraded in the ER (Sidrauski et al., 1998).

Even though cholesterol is synthesized in the ER, the head-group modification of gangliosides and sphingomyelin is completed in the Golgi apparatus. This implies that cholesterol-sphingolipids enriched-rafts are first formed in the Golgi. We cannot however rule out the possibility that lipid microdomains could form in the ER in presence of high local levels of cholesterol and ceramide, as shown to be the case in artificial membranes (Xu et al., 2001). Nevertheless, at the present the most likely hypothesis is that the observed loss of association between the unglycosylated transporter and lipid microdomains (Fig. 6.2) may in fact result from degradation of misfolded glycosylation-deficient transporters.

The use of site-directed mutagenesis to remove Asn-linked glycosylation signals has provided a means for exploring the role of individual glycosylation sites on the behaviour of a target protein. Three glycosylation-deficient mutants of SERT were generated: two single mutants, SERT^{N208Q} and SERT^{N217Q}, and a double mutant, SERT^{N208,217Q}, where both N-glycosylation consensus sequences were depleted. Immunoblot analysis of SERT glycosylation-deficient mutants showed an altered electrophoretic mobility with respect to the wild type. This indicates that in TREx-SERT cells both N-glycosylation sites were used (Fig.

6.5). Similarly to the tunicamycin treatment, the recombinant depletion of N-glycans caused a significant decrease in the amount of expressed transporter. Although the affinity of 5HT-transport was not significantly affected, the $V_{\max}$ was halved for the two single mutants, whereas for the double mutant the $V_{\max}$ was reduced to one tenth of the control (Fig. 6.6). These results agree with a previous report where SERT glycosylation-deficient mutants were expressed in Sf9 insect cells (Tate and Blakely, 1994). Similar observations have been also made for other neurotransmitter transporters (Olivares et al., 1995; Melikian et al., 1996; Nguyen and Amara, 1996), suggesting that N-glycans are not essential for substrate or inhibitor recognition. As pointed out in Chapter 5, changes in $V_{\max}$ could reflect alterations in either biosynthesis of the transporter, turnover number, or cell surface expression of the carrier. By using the impermeant reagent sulfo-NHS-biotin to label proteins at the cell surface, the cell surface expression of the double mutant SERT^{N208,217Q} was significantly reduced. However, the cell surface expression of the SERT^{N208Q}- and SERT^{N217Q}-transporters were only slightly reduced (Fig. 6.6). Therefore, it is possible that N-glycans support not only the folding of SERT, but also the efficient transport of 5HT, perhaps affecting the conformation or stability of the transporter at the cell surface.

The recombinant removal of N-glycosylation sites circumvented some of the problems that arose with tunicamycin incubation of cells, particularly the detrimental effect on their viability arising from inhibition of cellular N-glycosylation. However, Fig. 6.5 shows that problems with proper protein folding of glycosylation-deficient transporters may remain. In fact, the expression levels of the two single mutants, and even more so of the double mutant, were reduced with respect to SERT^{wt}. Glycosylation-deficient transporters may have misfolded in the ER and been recognized as an abnormal protein by the quality control system (Barriocanal et al., 1986). Although reduced, all the transporter glycoforms produced by the glycosylation-deficient mutants reached the cell surface (Fig. 6.6) and were able to transport 5HT with a substrate affinity similar to the wild type (Fig. 6.5). It is conceivable that N-glycans are required not just for proper folding of SERT, but may also affect its efficient traffic to the plasma membrane or its stability, similarly to what has been shown for the

norepinephrine transporter (Nguyen and Amara, 1996). Unfortunately, the expression of SERT^{wt} in transiently transfected HEK cells produced a lower amount of fully glycosylated protein, than in the stable TREx-SERT cell line. In fact, the majority of SERT produced in transiently expressed HEK cells was partially glycosylated or unglycosylated. This does not appear to be cell type-dependent, because similar observations have been made in Sf9 insect cells transiently transfected for SERT and in HeLa cells transfected for NET (Tate and Blakely, 1994; Nguyen and Amara, 1996). Moreover, the expression of SERT by stable transfectants generally results in a high level of fully glycosylated SERT, as seen for TREx-SERT cells and IMI270 cells, both generated from HEK cells (Chapter 3; (Tate et al., 2003)). The less efficient processing of SERT in transient transfectants is likely to result from the high plasmid copy number per cell introduced; very high levels of transporter are thus synthesized, which may overload the glycosylation machinery of ER and Golgi. Similar differences between transient expression- and stable expression-systems are in fact not unique to monoamine transporters, but appears to be shared by other glycoproteins, such as the cytokine Leukemia Inhibitory Factor (Geisse et al., 1996).

Chapter 7

Concluding remarks

This study was concerned with the investigation of regulation and trafficking of the serotonin transporter. In the past years, due to the presence of putative phosphorylation sites in the amino acid sequence of the transporter, the study of SERT regulation has been mainly focused on the role of protein kinases. More recently, the investigation has been directed to the search of interacting proteins, which would regulate the activity of the transporter. One of such proteins is syntaxin 1A. Syn 1A is a SNARE protein that functions in the exocytosis of neurotransmitters and has been shown to physically interact with a number of neurotransmitter transporters (GAT1, GLYT2, NET and SERT; (Beckman et al., 1998; Geerlings et al., 2001; Haase et al., 2001; Horton and Quick, 2001; Quick, 2002, 2003; Sung et al., 2003)) and voltage gated-ion channels (calcium and potassium; (Bezprozvanny et al., 1995; Fili et al., 2001)) at the presynaptic membrane of neurons. Members of the syntaxin gene family are components of protein complexes that regulate vesicle docking and/or fusion during transport of cargo through the constitutive and regulated secretory pathways of eukaryotic cells. Two of the syntaxins, 1A and 1B, have been suggested to be the docking receptors for synaptic vesicles with the presynaptic membrane. In this study, the heterologous expression of syntaxin 1A caused a specific inhibition of the biosynthetic transport from the ER to the TGN of two non-related membrane proteins, SERT and TLR2. The heterologous expression of syn 1A creates a disturbance of the ER to Golgi trafficking as indicated both by the inhibition of SERT glycosylation and by colocalization studies. Syn 1A and other cargo proteins, such as SERT and TLR 4, are found trapped in intracellular structures, identified as ER. The reason for such an inhibition is not clear, and more information regarding the molecular mechanism by which SNAREs and other molecules catalyze membrane fusion are needed. However, it is clear that membrane fusion requires the sequential assembly and disassembly of several protein complexes involving SNAREs. It is likely that in 293HEK cells one or more syntaxin 1A cognate proteins are absent, thus inhibiting the fusion of ER vesicles with the *cis*-Golgi stack and causing a disturbance of subcellular

trafficking. During the course of this study, two reports were published showing that in neurons syn 1A interacts with the N-terminal of SERT, regulating both the cell surface expression and the activity of the transporter (Quick, 2002, 2003). In HEK cells expressing SERT we observed a decrease in the velocity of 5HT transport in presence of syn 1A. However, this appeared to be the effect of the aforementioned traffic disturbance caused by the heterologous expression of syn 1A in this cell line. Taken together, these findings indicate that the HEK cell line, which does not have a regulatory pathway of exocytosis, does not constitute a suitable system for the study of the interplay between SERT and syn 1A. Therefore, further studies should be carried out in a polarized cell line, such as the RN46A-B14 cell line, derived from immortalized rat serotonergic neurons (Eaton and Whittemore, 1996).

Interestingly, in rat brain SERT and syn 1A were both found associated with lipid rafts. SERT and syn 1A respond similarly to the solubilization in different non-ionic detergents, as seen in their floatation gradient profiles. On the other hand, Munc 18-1, which acts as a chaperone and inhibits the interaction of syn 1A with other interacting proteins, in particular SNARE proteins, does not associate with rafts. These data corroborate similar findings for syn 1A and Munc 18-1 in PC12 cells and in synaptosomes (Chamberlain et al., 2001; Lang et al., 2001; Taverna et al., 2004), thus indicating that at least a fraction of SERT and syn 1A is spatially segregated from Munc 18-1. This may have important functional implications in the regulation of serotonergic neurotransmission, by allowing the efficient coupling of 5HT release with the reuptake of the neurotransmitter by SERT. The sequence of events occurring between SERT and syn 1A following the arrival of the presynaptic action potential needs to be further characterized. Specifically, it needs to be confirmed whether SERT and syn 1A segregate in the same subpopulation of rafts at the presynaptic active zones (where the neurotransmitter is released) and how lipid rafts dynamically regulate the interplay between SERT, syn 1A, and Munc 18-1.

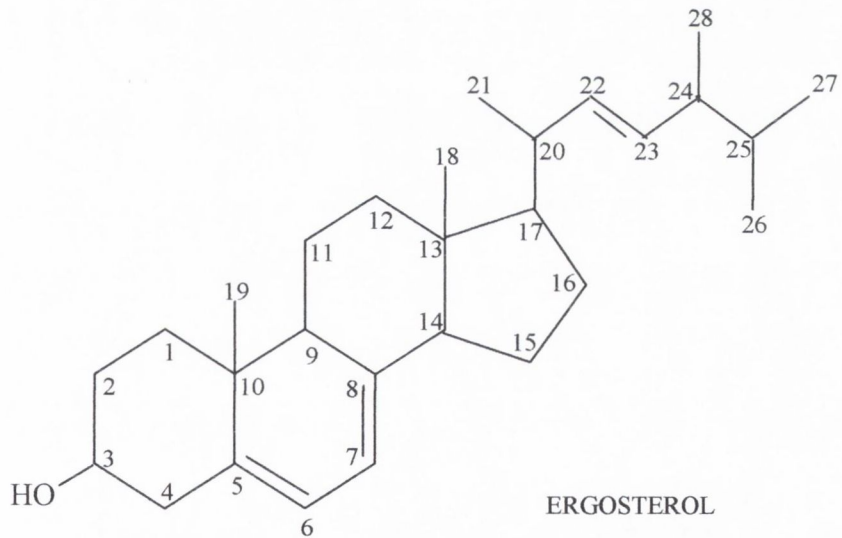
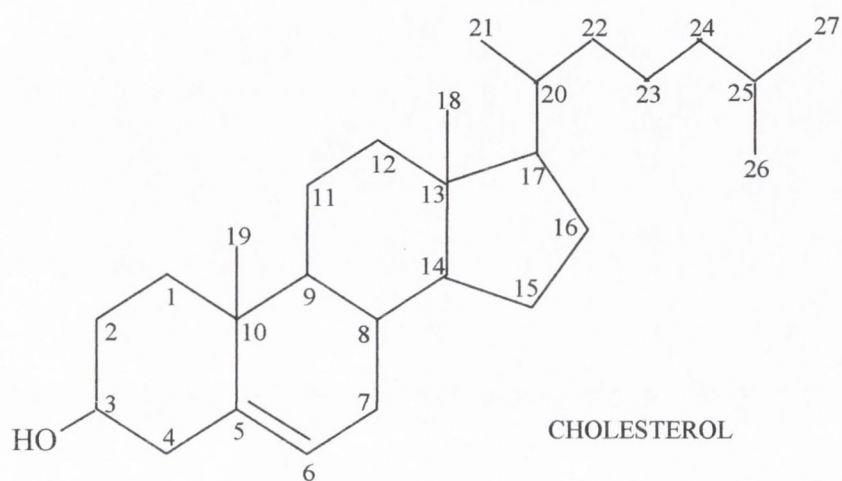
In this study, the disassembly of rafts by sequestration of cholesterol from cell membranes was shown to affect the serotonin transporter turnover number.

However, it is not clear whether it is the integrity of lipid rafts or the presence of cholesterol itself to be required for SERT activity.

Whether a direct interaction exists between SERT and cholesterol could be investigated by using a photoactivatable [^3H]-cholesterol molecule (Thiele et al., 2000). Cells would be incubated with the photoactivatable [^3H]-cholesterol and then irradiated with UV to generate a short-lived and extremely reactive intermediate that reacts with nearby molecules to form covalent and stable complexes. Then, SERT could be immunoprecipitated and the incorporation of radioactivity in the immunoprecipitated SERT would indicate the direct interaction with cholesterol. Previous reports have shown that cholesterol is required for the efficient binding of antidepressants to SERT, and that ergosterol cannot efficiently substitute cholesterol (Baker, 1997; Scanlon et al., 2001), thus suggesting that cholesterol may directly interact with SERT. However, the authors did not show whether ergosterol had been efficiently incorporated into the membranes, and there are in fact indications that ergosterol does not form stable complexes with M β C (Eroglu et al., 2003). Therefore, it is also possible that cholesterol does not directly interact with the serotonin transporter, and its presence may be required to promote the formation of lipid rafts in cell membranes. Thus, the role of specific sterols in modulating SERT activity needs to be further investigated.

Sterols are essential components of all eukaryotic membranes. Ergosterol is the major sterol in fungal cells and has functions similar to cholesterol in higher eukaryotic cells. The structure of ergosterol is identical to cholesterol, except for two additional conjugated double bonds in the B-ring and a methyl residue at C24 (Fig. 7.1). Ergosterol has been shown to promote the formation of liquid ordered domains in artificial membranes more strongly than cholesterol (Xu et al., 2001), and it is also a constituent of lipid microdomains in yeast (Bagnat et al., 2000; Bagnat and Simons, 2002) and *Drosophila melanogaster* (Rietveld et al., 1999). An effective way to determine the role of sterols in modulating the activity of the transporter would be to reconstitute SERT into liposomes of a defined lipid composition, and to monitor 5HT transport and ligand binding. Unfortunately, the reconstitution of rSERT has not been achieved so far, due to difficulties in

Fig. 7.1
Structure of cholesterol and ergosterol



purifying the transporter in a functional state. Nonetheless, other means of investigations are conceivable. Whereas mammals synthesize cholesterol, some invertebrates, such as arthropods (e.g. *D. melanogaster*), molluscs, platyhelminthes, nematodes (e.g. *C. elegans*), terrestrial annelids, are auxotrophic for sterols and require a dietary source. Therefore, their cell membranes vary in lipid composition depending on the diet. For instance, when *Drosophila* is fed on yeast, ergosterol constitutes the majority of membrane sterols, accounting for 68% of total sterols in embryos (Rietveld et al., 1999; Eroglu et al., 2003). Therefore, contrary to mammalian cells, insect cells appear to have an intrinsic capacity to adapt to different sterols. The Sf (*Spodoptera frugiperda*)-9 insect cell line may have retained this adaptive capacity. The baculovirus expression of proteins in the Sf9 cell line is widely used, because these cells are easy to grow and the culture can be scaled-up in bioreactors, allowing the large-scale purification of recombinant proteins. The expression of rSERT has already been achieved in Sf9 cells. However, even though large amounts of SERT could be expressed in these cells, only a fraction of it was active, probably due to the poor glycosylation of the transporter (Tate and Blakely, 1994; Tate, 1998). In fact, contrary to mammals, insects lack glycosyltransferases for the elongation of N-glycans, thus making difficult to express mammalian glycoproteins. However, recently the Sf9 cell line has been engineered with several glycosyltransferase and sialyltransferase genes for the addition of complex glycans to glycoproteins (Aumiller et al., 2003; Tomiya et al., 2003). The resulting SFSWT-3 cell line can grow in absence of serum, allowing the low cost production of high levels of recombinant glycoproteins. By using this expression system, it might be possible to obtain high levels of active transporter. Because the SFSWT-3 cell line can grow in absence of serum, it would be relatively easy to manipulate the lipid composition of cell membranes. Specifically, the addition to the growth medium of either cholesterol (complexed with M β C or LDL) or ergosterol (in liposomes) should clarify the function of sterols, and, more in general, of lipid rafts, in regulating SERT.

There are indications, presented in this study, that SERT may associate with lipid rafts during its maturation. This hypothesis is supported by cell surface biotinylation experiments and also by the finding that SERT is associated with lipid microdomains of various sizes, as defined by differential centrifugation: a small type and a large type. Similar lipid raft populations have been described for the microvillar protein prominin. The small type is assembled in the *trans* Golgi/TGN, whereas the large type is associated with the apical plasma membrane of epithelial cells (Roper et al., 2000). The combined use of metabolic labelling, subcellular fractionation, and cholesterol depletion should clarify whether lipid rafts are involved in the apical delivery of SERT in neurons (RN46A cells).

A similar approach can be also used to assess the function of SERT's N-glycans in the apical targeting of the transporter in RN46A cells. The experiments carried out in this study in non-polarized HEK cells indicated that N-glycans do not mediate the interaction of SERT with lipid rafts. However, it has to be investigated whether N-glycans and/or rafts are involved in the apical targeting of the transporter in neurons. The single glycosylation deficient mutants of SERT generated for this study could be used for this purpose, by transiently expressing them in RN46A cells. However, as discussed in chapter 6, the recombinant removal of both N-glycosylation consensus sequences caused a severe reduction in the expression of SERT, possibly due to protein misfolding. An alternative approach to investigate whether glycans promote the stability of SERT and/or its traffic to the cell surface would be to excise the N-linked oligosaccharides from mature SERT in intact RN46A cells. The advantage of this approach would be to allow the transporter to be properly folded and to traffic to the Golgi and the plasma membrane, thus gaining more insights into the role of N-glycans in the trafficking of SERT in polarized cells. This method would be based on the efficient removal of high mannose oligosaccharides from mature proteins by endoglycosidase H (endoH) (Varki, 1993). EndoH is unable to cleave high mannose oligosaccharides from complex-type glycans, whose [(glucose)₃(mannose)₉(N-acetylglucosamine)₂]-core has been modified by the addition of galactose, fucose, and/or sialic acid. Thus, only the biosynthetic intermediates prior to the action of mannosidase II in the Golgi apparatus are

sensible to endoH. Therefore, the partially glycosylated transporter would be susceptible to endoH, whilst the fully glycosylated form, modified in the Golgi, would no longer be sensible to the action of endoH, as it is been shown in TREx-SERT cells (Tate et al., 2003). A possible way to retain SERT sensibility to endoH would require the incubation of cells with 1-deoxymannojirimycin (DMJ), which reversibly inhibits mannosidase I in the Golgi apparatus, causing the accumulation of high-mannose glycoproteins (Overton, 1988). Even though most of the cellular functions are not likely to be affected by treatments with DMJ and endoH, the N-glycans of every glycoproteins will be removed. Nonetheless, the expected phenotype is likely to be less severely affected than following tunicamycin treatment, because it avoids problems arising from protein misfolding.

The finding that SERT associates with lipid microdomains may also help to elucidate the mechanism of action of some antidepressants. Imipramine freely diffuses through the brain-blood barrier and distributes unevenly within the rat brain, particularly concentrating in areas rich in serotonergic terminals, such as the frontal cortex, hippocampus and thalamus (Duncan et al., 1992). It is well established that imipramine binds to SERT and NET. However, Duncan and coworkers demonstrated that, both in rat and in *postmortem* human brains, a majority of imipramine is bound to other sites in addition to serotonergic and noradrenergic terminals (Duncan et al., 1992). These are low affinity binding sites but of pharmacological relevance, and, even though they have not been yet identified, are protease-insensitive and thus presumably of lipid nature (Marcusson et al., 1985). Additionally, some responses of the nematode *C. elegans* to the SSRI paroxetine appear to be mediated not by the serotonin transporter, MOD-5, but through other unknown target(s) (Ranganathan et al., 2001).

Another piece of evidence that suggests imipramine may bind to lipids comes from a freeze-fracture study (Bal and Bird, 1991). The authors of this study showed that chronic treatment (two weeks) with either imipramine (tricyclic antidepressant) or mianserin (tetracyclic) reduced the number of filipin-

cholesterol complexes within the rat cingulate cortex. Surprisingly, the acute treatment with these antidepressants (24 hours) generated the opposite effect, increasing the number of filipin-cholesterol complexes. Interestingly, imipramine and other amphiphiles, such as U18666A, progesterone and chlorpromazine, are used to mimic the Niemann-Pick type C disease, an inherited recessive lipid storage disorder that causes a precocious neurodegeneration. These compounds inhibit intracellular cholesterol transport pathways and cause an abnormal accumulation of cholesterol and gangliosides within lysosomes, probably by inhibiting cholesterol export from lysosomes to the ER and the plasma membrane (Lange et al., 1998; Sato et al., 1998). However, the basis for the cholesterol accumulation in the Niemann-Pick type C disease or in cells treated with amphiphilic drugs remains uncertain.

In the light of these findings and considering that the association of SERT with cholesterol-enriched lipid microdomains is required for 5HT transport, it seems logical to investigate whether antidepressants, such as imipramine, may inhibit the transporter by affecting also its interaction with lipid rafts. This investigation might help to understand why antidepressants require more than two weeks to commence their therapeutic effects, and whether a functional link exists between hypercholesterolemia and the non-responsiveness to antidepressants, as indicated by clinical studies (Sonawalla et al., 2002; Papakostas et al., 2003a; Papakostas et al., 2003b)

Cholesterol is known to be necessary for the reconstitution of the GABA transporter (Shouffani and Kanner, 1990). More recently, the norepinephrine transporter has been shown to associate with lipid rafts and that this association appears to mediate NET internalization upon PKC activation (Jayanthy et al., 2004). Taken together with the study presented here, these findings suggest that all neurotransmitter transporters may associate with cholesterol-enriched lipid microdomains, and that this association may be essential for their function and for their regulation at the cell surface. As shown for the GABA transporter, the requirement of cholesterol may be pivotal to succeed in the reconstitution of SERT. The main obstacles to SERT reconstitution were the lack of a convenient source of material and its purification in an active form. The baculovirus

expression of proteins in the Sf9 cell line is the more convenient for the large-scale production of membrane proteins for purification purposes. However, as aforementioned, Sf9 insect cells lack of glycosylation enzymes responsible for the formation of complex N-glycans, which resulted in the production of low levels of active SERT (Tate, 1998). However today, a novel system for the expression of N-glycosylated proteins, the SFSWT-3 insect cell line, has been produced (Aumiller et al., 2003). Moreover, a new class of non-denaturing detergents is now commercially available. These detergents share structural similarities to various lipids and are thus more suitable for the purification of membrane proteins. The current availability of these new tools may allow the purification of neurotransmitter transporters in an active form, hopefully leading to its crystallization and determination of its structure with good resolution.

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