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ORIGINAL PAPER

Elucidating the Mechanism Behind Sodium-Coupled Neurotransmitter Transporters by Reconstitution

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Abstract

Sodium-coupled neurotransmitter transporters play a fundamental role in the termination of synaptic neurotransmission, which makes them a major drug target. The reconstitution of these secondary active transporters into liposomes has shed light on their molecular transport mechanisms. From the earliest days of the reconstitution technique up to today's single-molecule studies, insights from live functioning transporters have been indispensable for our understanding of their physiological impact. The two classes of sodium-coupled neurotransmitter transporters, the neurotransmitter: sodium symporters and the excitatory amino acid transporters, have vastly different molecular structures, but complementary proteoliposome studies have sought to unravel their ion-dependence and transport kinetics. Furthermore, reconstitution experiments have been used on both protein classes to investigate the role of e.g. the lipid environment, of posttranslational modifications, and of specific amino acid residues in transport. Techniques that allow the detection of transport at a single-vesicle resolution have been developed, and single-molecule studies have started to reveal single transporter kinetics, which will expand our understanding of how transport across the membrane is facilitated at protein level. Here, we review a selection of the results and applications where the reconstitution of the two classes of neurotransmitter transporters has been instrumental.

Keywords Neurotransmitter transporters · Proteoliposomes · Reconstitution · Membrane transport · Secondary active transporters

Introduction

It is impossible to discuss the reconstitution of neurotransmitter transporters without mentioning the work of Dr. Baruch Kanner—a true pioneer in the field. From the earliest days, he has been central to the development of methods for reconstitution, and he was the first to report the reconstitution of a neurotransmitter: sodium symporter (NSS), namely the GABA transporter, GAT-1. During his career, he has continuously developed the technique, reconstituted several other transporters and characterized their mode of transport and ion dependence in proteoliposomes. Today's scientists are still benefitting from this groundbreaking work that remains of fundamental importance for our continued efforts to better understand the ion-coupled transport of solutes like neurotransmitters across the membrane.

This review will describe functional studies performed on sodium-coupled neurotransmitter transporters reconstituted in liposomes. These are secondary active transporters expressed in the brain and responsible for reuptake of neurotransmitters after release as part of synaptic signaling. Reconstitution is the insertion of purified integral membrane protein, often solubilized in detergent, into a lipid membrane. It enables the study of transport in the simplest possible system. Our motivation for writing this review has been to draw attention to the essential aspects of transport function that have been uncovered in studies of neurotransmitter transporters and their homologues reconstituted into proteoliposome systems. We will first provide a brief introduction to a range of ways by which proteoliposome systems can be tuned depending on the question investigated. We will then provide examples of how proteoliposomes have provided insight to the mechanisms of neurotransmitter transporter function. This should serve as an inspiration to how

Special Issue: In Honor of Baruch Kanner.

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proteoliposomes can be used to directly address key aspects of the molecular mechanisms behind transport function.

The Structure and Function of Sodium-Coupled Neurotransmitter Transporters

Sodium-coupled neurotransmitter transporters are found in the plasma membrane of neurons and glia cells. They are responsible for the removal of neurotransmitters from the extracellular space and thereby terminating the signal [1]. The energy that drives the transport process is harvested from the co-transport of, depending on the transporter, one to three Na^+ ions. Some transport proteins couple their activity to the symport or counter-transport of additional ions, typically the co-transport of Cl^- and/or counter-transport of H^+ or K^+. Because of their important role in maintaining neurotransmission homeostasis, the transporters are targets of pharmaceuticals used to alleviate the symptoms of e.g. ADHD, depression and epilepsy, and by illicit drugs such as cocaine and amphetamine [2].

The neurotransmitter transporters for dopamine, serotonin, norepinephrine, glycine and GABA belong to the NSS family of transporters. The family also includes transporters of amino acids and homologue transporters of bacterial origin [3]. NSSs are functional monomers with 11 or 12 transmembrane helical segments (TMs) [4, 5]. They all share a common structural fold, wherein the first five TMs are symmetrical to the subsequent five through an axis in the plane of the membrane (Fig. 1a). The substrate and ion-binding sites are located centrally in the transporters. One Na^+ binding site, the Na1 site, is situated immediately adjacent to the substrate binding site. In some cases, the Na^+ directly couples to the substrate, contributing to the generation of the substrate binding site [4, 6, 7]. The Na2 site seems to be more important for the translocation mechanism [8–11]. Eukaryotic transporters also bind Cl^-, but its role is less clear [12]. The human serotonin transporter (SERT) shows increased transport when coupled to the counter-transport of

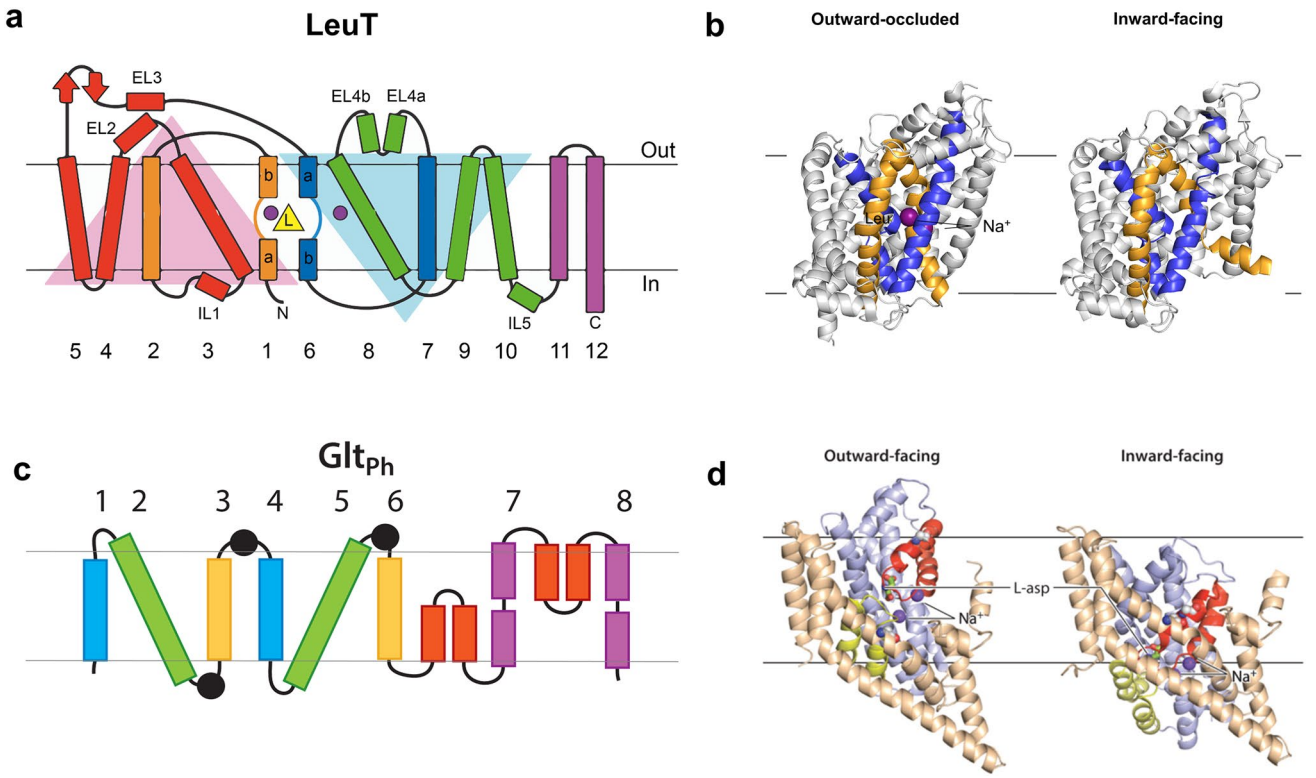

Fig. 1 The molecular structure of the sodium-coupled neurotransmitter transporters. **a** Membrane topology of the prokaryotic neurotransmitter: sodium symporter homologue LeuT. The two Na^+-ions are shown in purple and the substrate is shown in yellow. The triangles indicate the inverted symmkaryotic neurotransmitter: sodium symetry of TM1-5 and TM6-10. Figure modified from [4] with permission. **b** Structure of LeuT in outward-occluded conformation with Na^+ (purple) and substrate bound (PDB 2A65) and the inward-facing conformation after release of Na^+ and substrate (PDB 3TT3). The bundle domain is shown in orange (TM 1+2) and blue (TM 6+7). **c** Membrane topology of Glt_{Ph} monomer. Figure from [20] with permission. **d** Glt_{Ph} in the outward facing conformation and the inward-facing conformation with Na^+ (purple) and substrate bound. The transport domain is shown in blue and the scaffold domain is shown in wheat. Hairpin loop 1 and 2 are shown in green and red, respectively. Figure from [20] with permission

K^+ [13]. Notably, potassium has also been suggested to play a role in the counter-transport mechanism for the intensively studied prokaryotic NSS member, LeuT [9].

The excitatory amino acid transporters (EAATs) mediate the clearance of glutamate in the human brain. There are five EAAT subtypes (EAAT 1–5), which are organized in the membrane as a bowl-shaped homotrimer. Each EAAT monomer is a functional unit with 8 TM regions and two reentrant hairpin loops between TM6-7 (HP1) and TM7-8 (HP2) (Fig. 1c). The hairpin loops, together with residues in TM7 and TM8, form the substrate binding site with at least two Na^+ binding sites in close vicinity [14–16]. The EAATs transport substrate in symport with three Na^+, one H^+ and the counter-transport of one K^+. Furthermore, the transporter conducts a chloride current that is uncoupled from substrate uptake [17]. The prokaryotic aspartate and sodium symporter Glt_{Ph} from *Pyrococcus horikoshii* has the same structural fold as the human glutamate transporters; the crystallization and subsequent determination of the molecular structure of Glt_{Ph} has made the protein a template for studies of the eukaryotic glutamate transporters [18].

The principle behind the mechanisms of transport of neurotransmitter transporters is the alternating access model [19]. The model describes the necessity of internal barriers, which can be formed on either side of the bound substrate. An allosteric coupling must ensure that the substrate is only accessible to one side of the membrane at any given time. The mechanisms behind the model are, however, very different between NSSs and EAATs. Where NSSs use two barriers and switch between the two by rocking a bundle of transmembrane helices, the EAATs use one barrier and 'elevate' the substrate between an extracellular and intracellular exposure. An excellent review describes the transport mechanisms of NSSs and EAATs in great detail [20].

Proteoliposomes—A Practical Approach

The reconstitution method was developed and described by Dr. Racker and colleagues in the beginning of the 1970s. Along with more pioneering work on how to explore the function of membrane proteins, Dr. Racker reconstituted purified Ca^{2+}-ATPase in liposomes formed by phospholipids [21]. Another notable pioneer of the field is Dr. Kaback, whose work on the *E. coli* lac permease has shaped our understanding of membrane transport kinetics for secondary active transporters [22, 23].

There are several methods to achieve reconstitution of an integral membrane protein into liposomes. They all address the challenging procedure of transferring the purified protein from a detergent solution into a lipid bilayer [24]. The earlier methods involved, among others, detergent dilution, dialysis or a sonication procedure. Now, the use of bio-beads has become predominant [25]. The fundamental steps in this reconstitution method include (i) the formation of liposomes with the desired lipid composition in the desired aqueous buffer, (ii) the reconstitution step where liposomes are mixed with protein in the protein's detergent-containing buffer, and (iii) the removal of detergent from the sample with bio-beads. An optional step can be added whereby the intra-vesicular buffer can be changed and/or the finished proteoliposomes can be concentrated (Fig. 2). When the integral membrane protein of interest has been successfully reconstituted this opens the door to a wide range of functional assays.

To obtain the optimal reconstituted sample, important parameters must be optimized. These include the lipid composition, the detergent for liposome destabilization, the protein-to-lipid ratio and the rate of detergent removal. Whether a certain reconstitution procedure proves successful or not highly depends on the nature of the protein of interest. A comprehensive protocol for the general reconstitution of ABC transporters serves as an excellent starting point for the first trials of reconstitution of detergent purified integral membrane transporters [26].

Another option is the reconstitution of integral membrane proteins into nanodiscs. In a nanodisc system the advantage of having the protein in a native lipid environment is preserved; however, planar nanodiscs do not offer the ability to study transport or transporters under the influence of a gradient and the method will not be discussed further here.

Experimental Challenges for Proper Measurement of Transport Kinetics

The experimental read-out in transport assays has often been radiolabeled substrate taken up by or released from the proteoliposome. Alternatively, fluorescence—allowing measurements in real time—can be used. A fluorescence assay can be based on, for example, pH-sensitive or ion-sensitive fluorophores trapped in the lumen of the vesicles, where transport of ions is measured as change in fluorescence signal from the fluorophore. Fluorescence resonance energy transfer (FRET) between fluorophores attached to the transporter may also be an approach, though not for detecting transport per se but rather conformational rearrangements [27]. The ion gradients can be varied across the lipid bilayer, and the electrical potential of the membrane can be affected by applying ionophores with different selectivities to ion permeability. A prerequisite for all uptake experiments is to obtain a significant difference between specific transport and the unspecific contribution from, for example, influx through the membrane or binding.

If the goal is to quantify the uptake kinetics of the transporter, it is important to obtain a method to standardize the uptake between reconstitutions. The number of inserted

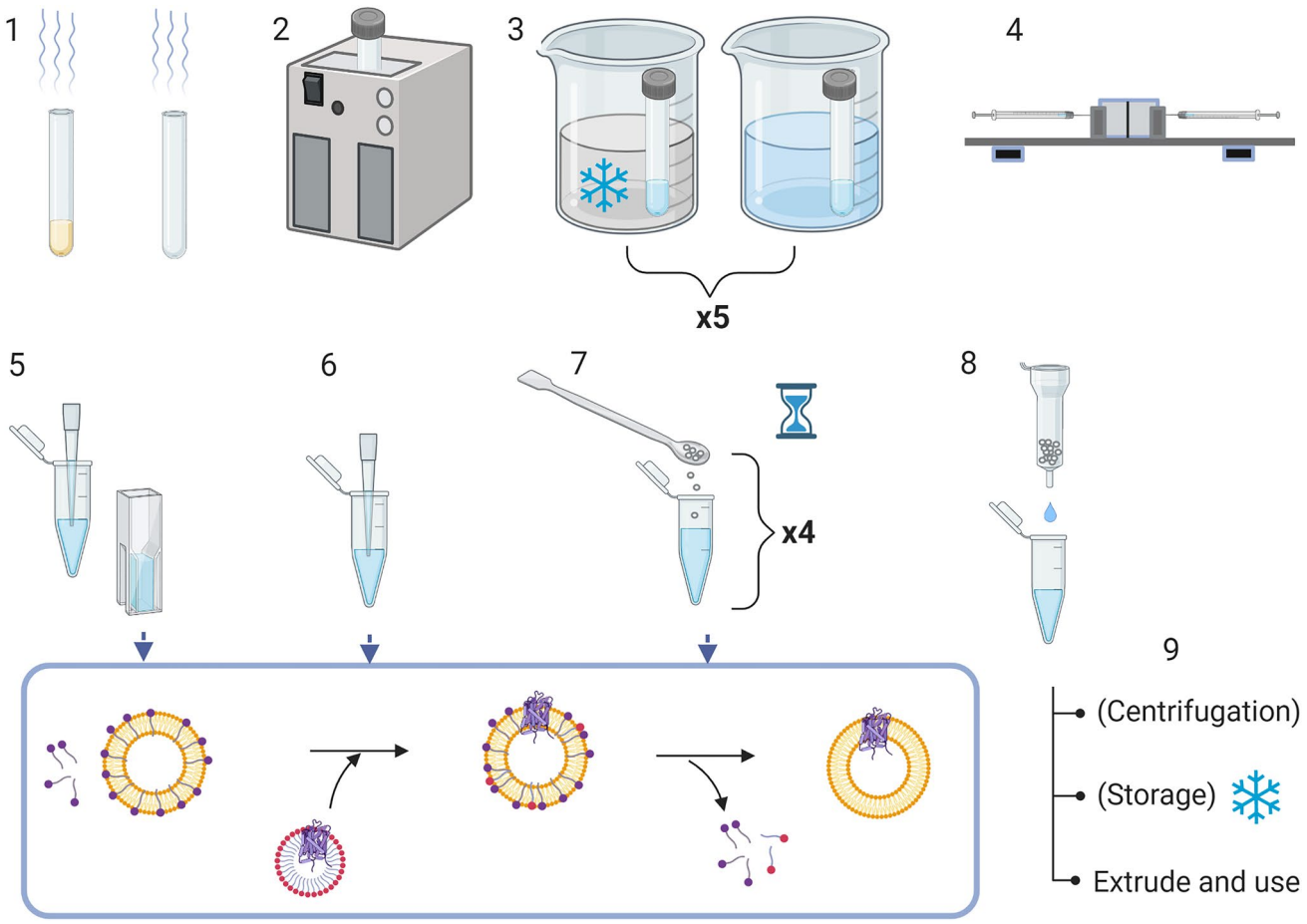

Fig. 2 Overview of the reconstitution process. (1) Evaporation of chloroform from lipid mix under inert gas. (2) Bath sonication of lipid in aqueous buffer to dissolve lipid film. (3) Freeze-thaw cycles in dry ice bath and lukewarm water. (4) Extrusion of liposomes with mini extruder through polycarbonate filter. (5) Addition of detergent (purple) to form mixed lipid-detergent micelles. The process is monitored by measuring the optic density of the sample. (6) Addition of protein (purple transporter) solubilized in detergent micelles (red), which cause insertion of protein into the lipid-detergent micelles. (7) Stepwise addition of SM-2 bio-beads to remove detergent and form tight proteoliposomes. (8) Removal of bio-beads by filtration over poly-prep column. (9) Final steps: Ultra-centrifugation and resuspension to desired concentration (optional), storage at − 80 °C (optional), extrusion and use in uptake assays

proteins per lipid vesicle can be difficult to control and thus might vary between reconstitutions. Quantification of incorporated protein can be assessed by the sensitive amido black staining method [28]. However, some incorporated protein might not be active and to account for this, the fraction of active protein may be determined with a saturation binding assay using a radiolabeled inhibitor.

If the proteoliposomes have remnants of detergent molecules, they can become leaky. The degree of leakiness depends on the purity of the system, the size and integrity of the liposomes and the solvent composition on each side of the membrane. Some level of background leak is common with H^+ ions being the most membrane-permeable ions due to their small size and because they are components of water [29]. The leakiness of the system can be addressed by incorporation of a membrane-impermeable pH-sensitive fluorophore into the lumen of the vesicles, followed by addition of an H^+ pulse to the extra-vesicular buffer. The leak rate is determined by following how the extra-vesicular H^+ leaks in and affects the fluorescence of the fluorophore [30]. Leakiness of other ions could be assessed by measuring the change in fluorescence of other ion sensors, such as fluorescent K^+- and Na^+-sensors, over time when the transporter is blocked with an appropriate inhibitor, and K^+ or Na^+ is present on either side of the membrane.

According to the law of mass action, secondary active transporters should be able to transport their substrate across the lipid bilayer regardless of the orientation of the transporter. However, the orientation will influence the transport kinetics and possibly inhibition by ligands; other features within the protein may exist, which could ensure a rectification of transport [9, 31, 32]. Accordingly, it is highly relevant to know the orientation of the transporters in the membrane. One way to assess the fraction of transporters in the

intracellular side-out orientation is to perform western blots with antibodies binding to a known side of the transporter, such as, to a tag added for the purification procedure (poly-His, strep, etc.). The degree of antibody labeling between samples of intact versus permeabilized vesicles will give an indication of fractional orientation. A similar approach is to cleave off the purification tag and visualize the degree of cleavage [33], or to measure the degree of specific labelling of introducted cysteines on either side of the transporter with a membrane impermeable cysteine-reactive dye [34]. Another option is to selectively obstruct the function of the transporters with a specific orientation. This can be performed by a reaction with membrane-impermeable chemicals, such as certain maleimides or methanethiosulfonates, which inactivate the protein by conjugating to a specific (inserted or endogenous) cysteine residue. If the cysteine is located at a site sensitive to transport on the intracellular side, proteins with an inside-out orientation will be blocked, and vice versa [33, 35–38]. Measurements of transport from only one orientation of reconstituted transporters has also been achieved by the development of synthetic nanobodies that bind to and obstructing transporters in a right-side-out orientation [39].

Reconstituted proteoliposomes can provide a reliable estimate of a transporter's ability to use the provided Na^+ gradient to concentrate the substrate under a given set of experimental conditions. It can also provide kinetic parameters of transport such as K_{cat} and K_m [8, 9, 31, 39, 40] and allow for an estimation of the substrate-to-ion stoichiometry [41, 42]. To obtain an accurate estimate of the substrate concentration inside the vesicles it is necessary to know the size and size distribution of the proteoliposomes. This can be assessed directly by dynamic light scattering [43, 44], or visualized by electron microscopy, but stain artifacts can blur the image and large proteoliposomes have a tendency to collapse on the carbon-coated grids [25]. More reliably is to insert fluorescent dyes into the membrane and assess the size and distribution of proteoliposomes by immobilizing them on a surface for total internal reflection fluorescence microscopy [45, 46] or to perform cryogenic electron microscopy (cryo-EM).

Single-molecule measurements of transporters in liposomes require reconstitution with a very low protein-to-lipid ratio and a detection strategy to identify single-molecule reconstitution or transport kinetics. Typically, the single molecule measurements are done with total internal reflection fluorescence microscopy (TIRFm), which require that the proteoliposomes are tethered to an activated surface via a lipid anchor or via a linker attached to the protein (Fig. 3) [47]. A detailed protocol describing key steps in the process of single-molecule reconstitution and TIRFm measurements is available [48].

Detailed Insights into the Function of Transport Proteins Elucidated with Proteoliposomes

Insights from the Reconstitution of Neurotransmitter: Sodium Symporters

One of the first reconstitutions of a transport protein was performed by Dr. Kanner. He partially purified and reconstituted a GABA transporter (GAT) from rat brain and showed that it was able to transport GABA by imposing an inward directed Na^+ gradient as the energy source [49]. The GABA

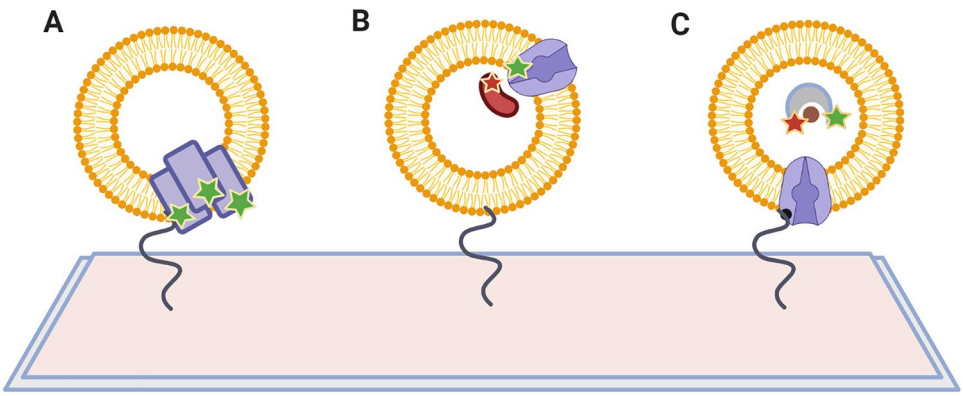

Fig. 3 Different attachment of proteoliposomes with fluorophore probes for TIRF microscopy. **a** Liposome with fluorescently labelled (green stars) transporter subunits (blue) attached to a streptavidin-coated surface via a PEG-biotin linker [73]. **b** Proteoliposome containing biotin-DOPE lipid attached to surface of the TIRF microscopy flow cell coated with streptavidin. FRET was measured between labelled transporter (green star) and labelled interaction partner (red star) in the lumen of vesicle [27]. **c** Proteoliposome attached via maleimide PEG-biotin linker attached to engineered cysteine residue on protein and to streptavidin coated surface. FRET signal from periplasmic binding protein (grey) in the lumen of vesicles detects substrate (brown circle) uptake event [31]

flux and its mutual dependency of Na^+ and Cl^- were later substantiated, also in proteoliposomes, using $^{22}Na^+$, $^{36}Cl^-$, and [^{3}H]GABA [50]. Furthermore, this study established a suggested stoichiometry of 2–3 Na^+ and one Cl^- per transported GABA molecule. The addition of cholesterol to the proteoliposomes potently increased the transport activity due to cholesterol's specific interaction with GAT [51], an interaction that has also been demonstrated for other neurotransmitter transporters [6, 52, 53]. Parallel kinetics measurements on a prokaryotic NSS homologue in proteoliposomes and in *E. coli* cells expressing the transporter showed that with the right lipid composition, proteoliposomes provide an environment that support the same uptake rates as a cell membrane [54]. The rat glycine transporter (GlyT) was also proven to be dependent on Na^+ and Cl^- by an approach similar to the approach to GAT [55]. In GlyT, glycosylation plays an important role in maintaining proper activity [56]: enzymatic deglycosylation of reconstituted glycine transporter 2 (GlyT2) prior to uptake experiments reduced transport by up to 40% [57].

Access to the GABA binding site is thought to be controlled by the opening and closing of external and internal gates. This constitutes a crucial part of the conformational changes within the transport cycle. This hypothesis was supported by mutagenesis studies, which showed that both gates in GAT must be intact to facilitate the concentration of substrate inside proteoliposomes [58]. However, it was still possible to facilitate a substrate exchange situation with only the external gate intact, suggesting that the internal gate is assisting in the conformational rearrangements during the reorientation of the transporter in the return step [58].

The exact role of Cl^- ions in eukaryotic NSS transport had long been unknown, and was investigated in an elegant series of experiments on reconstituted GAT-1 and on a reconstituted bacterial homologue, the tyrosine transporter Tyt1 from *Fusobacterium nucleatum*. In GAT-1, a serine residue is located where the Cl^--independent Tyt1 has an aspartate residue. Substitution of this serine to a residue with a negative charge in GAT-1 made the transporter largely Cl^--independent [59]. Conversely, substituting the negative charge in Tyt1 with a serine residue resulted in [^{3}H] tyrosine transport, which was largely dependent on Cl^-, suggesting that the inserted negative charge could substitute for Cl^- binding. Interestingly, when the charge was removed in Tyt1, transport became largely independent of the pH inside the proteoliposomes. This observation raised the question of whether transport by prokaryotic NSSs required neutralization of the negative charge for the return step to occur. To directly assess if H^+ was counter-transported by Tyt1 the membrane impermeable, pH-sensitive fluorophore pyranine was trapped in the lumen of the proteoliposomes [60]. Indeed, substrate uptake caused an alkalization of the lumen of the proteoliposomes. Valinomycin did not affect the alkalization, excluding the possibility that H^+ escaped from the vesicles to counter-balance the uptake of Na^+ in a non-transporter mediated way. Taken together the reconstitution experiments were able to show that the eukaryotic transporters likely have subsidized the H^+ counter-transport in their bacterial cousins with a Cl^- co-transport.

New aspects of transport kinetics were revealed when NSS proteins were studied at the single-molecule level. Investigation of the bacterial multi-hydrophobic amino acid transporter MhsT, reconstituted in either the extracellular-side-out or extracellular-side-in orientation, showed that transport only took place when MhsT was reconstituted with the extracellular side out [31]. One explanation for this observation is that a secondary allosteric substrate binding site is located on the extracellular side of the protein and acts as an allosteric moderator of transport, ensuring unidirectionality. The single-molecule setup also enabled the distinction between the transport rates of the first and second step in the transport cycle. Data from electrophysiological studies on other NNS proteins show that the return step is the rate-limiting step of the cycle [61, 62]. The single-molecule studies on MhsT showed that the duration of the return step was manipulated by the identity of the substrate, which had just been transported, by a yet unknown mechanism, resulting in the return step being rate-limiting for the transport of some, but not all, substrates [31].

Function of the Glutamate Transporter and Homologue Glt_{Ph}

Glutamate transporters were first partially purified and reconstituted in the 1980s [63, 64]. The experiments revealed the specific Na^+- and K^+-dependence and electrogenicity of glutamate uptake. Reconstituted glutamate transporter was used early on to identify molecular determinants of its ion and substrate selectivity. For EAAT-2 (GLT-1), it was, for example, shown in part based on reconstitution experiments that residue Gln404 is essential for recognizing glutamate and that His326 is possibly involved in the H^+ counter-transport [65, 66]. The elucidation of these functionally important residues was directed by identification and cloning of bacterial homologues [67], eventually leading to the structure of the aspartate transporter (Glt_{ph}) from *Pyrococcus horikoshii* being solved [18]; later followed by the structure of the human glutamate transporter-1 (EAAT-1) [53]. Guided by the crystal structure of Glt_{Ph}, mutations of one of the Na^+-binding sites in EAAT-2 and EAAT-3 revealed an impairment of K^+-stimulated substrate uptake and loss of K^+-stimulated anion conductance. The mutation also made Li^+ unable to substitute for Na^+ in substrate uptake. This suggested that the conserved Asp (Asp455 in EAAT-3 numbering) is involved in cation interactions in the

glutamate transporter in both of the translocation steps in a putative overlapping Na^+ and K^+ binding site [68].

Aspartate uptake by Glt_{Ph} is coupled to symport of Na^+, but unlike EAATs, Glt_{Ph} does not symport H^+ or counter-transport K^+ in the return step [34]. Rather, the stoichiometry of Glt_{Ph} is one aspartate to three Na^+, as determined by uptake studies on proteoliposomes utilizing [^{14}C]aspartate and ^{22}Na [69]. Determination of stoichiometry, however, can be a difficult task. An alternative method relies on the equilibrium between the driving force in the membrane potential and in the chemical gradient, which has been described using the sodium-dependent succinate transporter VcINDY [41].

Glt_{Ph}, like the EAATs, displays a Cl^- conductance uncoupled from the transport of aspartate [70]. Comparing substrate uptake in a reconstituted system with and without valinomycin determined that Glt_{Ph} transport is electrogenic. Without valinomycin, a larger than theoretically expected amount of uptake was still observed in the system, which suggested that the build-up of positive charge from uptake of Na^+ was counter-balanced by another component of the system. Omitting Cl^- from the buffer reduced uptake, which suggested that inflow of Cl^- had to some extent balanced the Na^+ uptake. The Cl^- conductance was then directly detected by incorporation of a chloride-sensitive fluorophore into the proteoliposomes. This showed that influx of Cl^- was highest in symport with Na^+ and substrate, but happened at a markedly higher rate than aspartate uptake, and the Cl^- conductance was observed even in the absence of Na^+. Together, the data suggest that the flux of Cl^- is not stoichiometrically coupled to substrate transport but could be conducted by a channel separate from the substrate translocation mechanism [70]. Interestingly, and supporting these observations, a putative Cl^- channel in Glt_{Ph} has been elegantly identified by the combined use of fluorescence spectroscopy [71], cryo-EM and molecular dynamics simulations [72].

The types of lipid in the bilayer have been shown to affect Glt_{Ph} transport rates. Glt_{Ph} in liposomes consisting primarily of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PE) yielded the highest uptake rate of aspartate, while N-methyl derivatives of PE reduced uptake [33]. The lipid composition also affected the orientation with which Glt_{Ph} inserted itself into the liposomes during reconstitution. The methylated lipid head groups likely increased the percentage of transporters oriented with the intracellular side facing the outside of the liposomes. A tyrosine residue situated in the trimerization domain of the transporter was found to facilitate protein-lipid interaction to the head groups of the membrane lipids, offering a possible explanation for how the lipids can affect rates of conformational change in the transporter. Furthermore, it was found that, whereas the Na^+ dependence of transport is independent of the orientation of Glt_{Ph}, the affinity for aspartate decreases when the transporter is oriented with the intracellular side facing the outside of the liposome. The sided difference in aspartate affinity could serve as a mechanism to minimize counter flow of substrate out of the cell [33].

A direct observation of the "elevator" transport mode in glutamate transporters is difficult, but with a reconstituted system in which only one Glt_{Ph} trimer was inserted per liposome, it was possible to monitor the protein dynamics in the transport domain during substrate translocation [73]. The setup was based on the reporting from single-molecule FRET (smFRET) probes inserted on the three transport domains to probe for protein movement. The rate of transport domain movement in the presence of substrate and ion gradients correlated with the rate of substrate uptake. The rate of dynamics and substrate uptake in Glt_{Ph} was compared to a gain-of-function transporter with two "humanizing" mutations. Reconstituted Glt_{Ph} showed highly interesting single-molecule protein dynamics underlying uptake kinetics. It showed that the transporter can shift between inactive and active periods, and that the binding of substrate increased the length of the inactive periods. In contrast, the humanized transporter showed no evidence of inactive periods, suggesting that the mutated residues interfere with the transporter's ability to remain stabilized in a locked state [73]. In Glt_{Ph}, the transition of the substrate-bound transporter from the outward-facing to the inward-facing state is identified as the rate-limiting step. Using a bioinformatic approach, gain-of-function mutations in HP2 were identified and examined by smFRET in proteoliposomes. Analysis of the results infers that the high-energy transition state of the transporter resembles the inward-facing state, and that the energy barrier can be lowered by the mutations. This information may direct the development of new drugs to modulate the function of glutamate transporters [74]. By keeping the single-molecule setup, but changing the fluorophore to a reporter on the intracellular substrate concentration, it was possible to extract single-molecule transport kinetics on Glt_{Ph} [75]. The data showed that Glt_{Ph} can work in at least three different uptake rates: slow, intermediate and fast, with the rates spanning two orders of magnitude [75]. This suggests that Glt_{Ph} transport is defined by kinetically distinct populations that exhibit long-lasting "molecular memory".

The cryo-EM structure of human EAAT3 has been solved in three different states: the sodium-coupled, the fully loaded sodium, proton and aspartate state and the empty state. None of the inward-facing EAAT3 monomers had asparate bound, whereas the outward-facing had, indicating an increased affinity for asparate in the outward-facing state [76]. The electrogenic uptake activity of the EAAT3 construct used for cryo-EM was measured on purified, reconstituted transporter using solid-supported membrane electrophysiology [76].

Reconstitution into proteoliposomes can also be used to monitor protein dynamics with high-speed atomic force

microscopy (HS-AFM) at the single-molecule level. The spherical proteoliposomes are applied to a mica surface to form a planar lipid bilayer containing the protein of interest [77]. The technique has an impressive temporal resolution, detecting domain dynamics down to 1 millisecond. HS-AFM of Glt_{Ph} has revealed that the transporter can operate much faster than previously anticipated, with state dwell-times in the 50 ms range. It also revealed a novel intermediate transport state between inward and outward facing. Detailed recordings of the transition rates between conformational states showed that the transporter did not always cycle all the way from inward facing to outward facing, but often only made it halfway through the cycle before it turned back to the starting conformation. Additionally, it seemed that the transporter could transition through different intermediate conformations to complete the transport cycle. Even though the transporter is not subjected to a gradient in the HS-AFM setup, the technique adds highly valuable insights into the protein dynamics taking place in the membrane [78].

Future Directions

The recent single-molecule studies on reconstituted transporters have for the first time provided insight into the molecular behaviors of individual transporter molecules that underlie the transport we observe in bulk measurements. Such insights into the transport cycle at the single transporter level might not only be important for our understanding of ion-coupled transmembrane transport, but perhaps also reveal new mechanisms by which we can pharmacologically manipulate this class of membrane proteins.

Yet, of the human dopamine, serotonin and norepinephrine transporters, only SERT has been functionally reconstituted in two proof-of-principle studies [79, 80], despite the great pharmacological interest surrounding these proteins. Finding the right conditions for reconstitution remains an experimentally driven process, but with our more than 40 years of collective experience with the development of robust reconstitution protocols, it should be possible in the near future to reconstitute these transporters. The main obstacle for reconstitution is to obtain sufficient quantities of detergent-solubilized protein. This has been shown possible for the human SERT [6, 81] and partly for the dopamine transporter [82].

Even for the transporters that already have been successfully reconstituted, many questions regarding the nature of their transport are still unanswered. The combination of reconstitution of single transporter molecules into proteoliposomes, with the real-time tracing of change in substrate and ion concentrations inside vesicles is an incredibly powerful tool for zooming in on transporters at work. With our extensive knowledge of the crystal structures of various NNS proteins captured in different conformations as blueprints, knowledge about transport rates of substrate and ions draws a more complete picture of these molecular machines and how they affect the physiology of the systems they are part of. Future researchers will have to find a way to take into account the molecular dynamics observed at single-molecule level to build more true models of the transport mechanisms. Furthermore, cryo-EM offers a way to combine proteoliposomes with studies of the transporter structure. By preparing proteoliposomes and imaging them with cryo-EM, we can in principle determine the conformational ensemble of the transporter under the influence of an ion gradient. We can also visualize potential oligomerization of NSSs in a lipid bilayer; something that cannot be obtained with traditional protein crystallography. Advanced reconstitution systems also offer the possibly of assessing the influence of single variables on transporter function. It could be changes in the lipid composition of the liposome, which has been suggested to have a great impact on transporter function [83–85]. It could also be specific posttranslational modifications of the transporter or co-reconstitution with an interaction partner [86]. Indeed, exciting times lie ahead, not least because we stand on the shoulders of giants like Dr. Baruch Kanner.

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Declarations

Conflict of interest The authors declare no conflicts or competing interests.

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