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The reverse operation of Na^+/Cl^--coupled neurotransmitter transporters–why amphetamines take two to tango

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Abstract

Sodium-chloride coupled neurotransmitter transporters achieve reuptake of their physiological substrate by exploiting the pre-existing sodium-gradient across the cellular membrane. This terminates the action of previously released substrate in the synaptic cleft. However, a change of the transmembrane ionic gradients or specific binding of some psychostimulant drugs to these proteins, like amphetamine and its derivatives, induce reverse operation of neurotransmitter:sodium symporters. This effect eventually leads to an increase in the synaptic concentration of non-exocytotically released neurotransmitters [and – in the case of the norepinephrine transporters, underlies the well-known indirect sympathomimetic activity]. While this action has long been appreciated, the underlying mechanistic details have been surprisingly difficult to understand. Some aspects can be resolved by incorporating insights into the oligomeric nature of transporters, into the nature of the accompanying ion fluxes, and changes in protein kinase activities.

Keywords

carrier-mediated release; clinical therapeutics; drug targets; neurotransmitter transporters; reverse operation; transporter-mediated release

Synaptic transmission is terminated by diffusion of neurotransmitter out of the synaptic cleft, by enzymatic degradation or by the action of neurotransmitter transporters (Iversen 1971). The latter re-accumulate the neurotransmitter into the presynaptic specialization by using the sodium gradient as the driving force (Rudnick and Clark 1993). This secondary-active transport process constitutes the most economical – and also, fastest means to retrieve released neurotransmitter from the neuronal vicinity. Among the transporters using electrochemical gradients to drive the accumulation of substrates, the neurotransmitter:sodium symporters (NSS; Saier *et al*. 2006) comprise the largest family (Nelson 1998). They include transporters for monoamines (i.e. dopamine, DAT, norepinephrine, NET, and serotonin, SERT, also abbreviated as 5-HTT), amino acids [i.e. GABA (GABA transporter), glycine, proline and taurine], as well as osmolytes (i.e. betaine and creatine) and the so-called ‘orphan’ transporters with currently unknown substrate

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specificity (Masson *et al*. 1999; Saier *et al*. 2006). The NSS family is of obvious medical relevance since a number of different disorders can be efficiently treated by inhibiting transporters: here, it is primarily mental disease like depression and inhibition of SERT and NET and epilepsy and inhibition of GABA-transporter-1 (GAT1) (Iversen 2000).

There is a wealth of data on ionic requirements, stoichiometry, regulation, physiological and pharmacological properties of the re-uptake process mediated by NSS (for review see Torres *et al*. 2003b). Moreover, there is general consensus that these transport proteins share a common structural motif of 12 hydrophobic transmembrane helices as indicated by hydropathy plot analysis (Amara and Kuhar 1993), studies employing antibodies (Bruss *et al*. 1995), electron microscopic immunocytochemistry (Nirenberg *et al*. 1997) and functional assays using thiol-reactive reagents (Chen and Reith 2000; Sen *et al*. 2005).

Currently, the interest in the transport process has been rekindled because several snapshots have become available that allow to re-examine previous educated guesses: the x-ray crystal structures of the bacterial transporter $LeuT_{Aa}$ from *Aquifex aeolicus* has been solved in several conformations. $LeuT_{Aa}$ is homologous to NSS transport proteins (Yamashita *et al*. 2005; Singh 2008; Singh *et al*. 2008). In spite of the low overall homology (some 20–25% residues in $LeuT_{Aa}$ and mammalian NSS are identical), there is remarkable conservation around the binding site (i.e., more than 50% identity): this information clearly allows for making more educated guesses than those relying solely on site-directed mutagenesis. It is also gratifying to see that the crystallographic snapshots delineate a sequence of events (Singh 2008) that is consistent with the venerable alternating access model: some 40 years ago, Jardetzky (1966) conceptualized the conformational steps required for substrate translocation by inferring a minimum of two states during the transport cycle that differed by substrate accessibility: ‘open-to-out/closed-to-in’ (i.e. ‘outward-facing conformation’) or ‘closed-to-out/open-to-in’ (i.e. ‘inward-facing conformation’. Importantly, only one of the gates (extracellular or cytoplasmic) should be open at any given time point (otherwise the protein functions as a channel rather than a transporter). Furthermore, the hypothesis predicted a transition state in which the protein changes from outward- to inward-facing conformations via an occluded state. Here, both putative gates are closed. The various crystal structures (Singh 2008) support this scheme and thus have proven very fruitful in guiding attempts to elucidate the molecular mechanism of transport (Forrest *et al*. 2008; Shi *et al*. 2008).

It is currently well established that NSS are constitutive oligomers (Sitte and Freissmuth 2003; Sitte *et al*. 2004); this has been shown by numerous research groups by using a plethora of different biochemical methods (Jess *et al*. 1996; Kilic and Rudnick 2000; Hastrup *et al*. 2001) and microscopic techniques in living cells (Schmid *et al*. 2001; Sorkina *et al*. 2003; Just *et al*. 2004; Egana *et al*. 2009) including neurons expressing transporters in their natural environment (Egana *et al*. 2009; Fjorback *et al*. 2009). It is not clear, why transporters should adopt a quaternary structure: in fact, the transport process is apparently not affected in mutants that have been rendered monomeric (Scholze *et al*. 2002a). From a teleological perspective, it can be argued that oligomer formation has evolved to support efficient export of transporters from the endoplasmic reticulum (Farhan *et al*. 2006). Here, we will argue that the oligomeric nature of NSS does have an impact on the transport

process because it is also essential for the action of amphetamine and its congeners on DAT, SERT and NET.

Plasmalemmal neurotransmitter transporters are necessary to support reverse transport

The discovery of reverse transport is intricately linked to the analysis of the pharmacological action of sympathomimetic amines: the original classification of sympathomimetic amines did not differentiate between a direct action of catechol compounds (that is now known to arise from a stimulation of the pertinent receptors) and the indirect action of non-catechol compounds (that is now appreciated to be contingent on reverse transport; Barger and Dale 1910). However, it became evident that cocaine potentiated the action of adrenaline (Fröhlich and Loewi 1910) but abolished the action of tyramine (Tainter and Chang 1927). Subsequent experiments documented that the effect of tyramine was contingent on the presence of endogenous noradrenaline because it was absent in denervated organs (Fleckenstein and Burn 1953) and in reserpine-treated animals (Burn and Rand 1958). These and related experiments, where sympathetic nerve endings in organ preparations were challenged with amphetamine or its congeners (Axelrod *et al*. 1961; Furchgott *et al*. 1963; Glowinski and Axelrod 1965; Ross and Renyi 1966), paved the way for a concept, in which release of noradrenaline (or dopamine) by reverse transport was the crucial step to understand their mode of action. This concept was also extensively verified in brain slices or synaptosomes (Gobbi *et al*. 1997). However, these paradigms share the common feature that they contain presynaptic nerve terminals with the complete machinery for synaptic vesicle exocytosis. The same is true for platelets and membranes prepared thereof, which contain SERT and the exocytotic machinery of platelets (Fishkes and Rudnick 1982; Rudnick and Wall 1992). Because of the simultaneous presence of vesicular and membrane transporters – and hence of different pools of substrates – it is difficult to dissect the contribution of each substrate pool to reverse transport under these conditions.

With the availability of the cDNA's encoding individual NSS's, it was possible to heterologously express a given monoamine transporter and to perform uptake and release experiments in cells that only contain the transporter of interest. This approach by definition eliminates the confounding effects of vesicular storage mechanisms and of the exocytotic release machinery: under these conditions – i.e., in cells heterologously expressing a given monoamine transporter – amphetamine and its derivatives induce reverse transport (Eshleman *et al*. 1994; Pifl *et al*. 1995, 1999; Wall *et al*. 1995; Pifl and Singer 1999). These observations and observations in DAT-knockout mice (Jones *et al*. 1998) provided a formal proof for the interpretation that monoamine transporters are the principle site of action of amphetamine and its congeners. While this statement may appear like a trivial truism, it is worth pointing out that, *in vivo*, the action of amphetamines and other indirectly acting sympathomimetic amines were abrogated in reserpine-treated animals (Burn and Rand 1958). Reserpine, however, targets the vesicular monoamine transporters-1 and -2 (VMAT1 and 2; Chaudhry *et al*. 2008; Schuldiner *et al*. 1993, 1995). However, it may be worth mentioning that genetic deletion of VMAT2 decreased the amount of amphetamine-induced release by 65% (Fon *et al*. 1997).

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A unifying concept of counter-transport versus several modes of reverse transport

Importantly, not only amphetamines are capable of inducing reverse transport. In fact, all compounds recognized as substrates which are inwardly transported trigger reverse transport in NSS expressing cells (Eshleman *et al*. 1994; Pifl *et al*. 1995; Cinquanta *et al*. 1997; Sitte *et al*. 1998, 2001; Scholze *et al*. 2000; Gobbi *et al*. 2002, 2008; Willeit *et al*. 2008). We argue, though, that this is an experimental artefact: physiological substrates do not act as releasers under physiological conditions: in fact, this would lead to futile cycling – rather than efficient removal of neurotransmitter from the synaptic cleft – and there isn't any evidence that this occurs *in vivo* (see below). In contrast, the biological actions of amphetamines and other naturally occurring amphetamine-like releasers (like 'khat', 'ephedrine', and 'tyramine'; see also Sulzer *et al*. 2005) are contingent on transport reversal. In addition, if transmembrane ion gradients are changed, reverse transport is triggered: this is true for both, lowering the extracellular sodium concentration (Pifl *et al*. 1995, 1997; Pifl and Singer 1999) or raising the extracellular potassium concentration (Scholze *et al*. 2000, 2002a). Thus, it may be conceptually important to consider at least two different types of reverse transport: the first one is triggered upon binding of releasers to the transporter protein and the second one upon changes of the ion composition of the extra- or intracellular fluid. The latter situation differs from the former by the fact that releasers need not be present on the extracellular side. Obviously, in either case, a releasable pool of substrate must be accessible on the cytoplasmic side.

Several models have been put forth to account for and explain reverse transport; these were initially based on the 'alternating access hypothesis' that explained the inward transport of substrates from the extracellular side by a substrate-induced conformational change in a sodium- and chloride-dependent manner. Hence, based on this hypothesis, reverse transport was conceptualized as a revolving door, where outward transport of a given substrate located within the cell was driven by and coupled to the inward transport of another substrate residing on the extracellular side. The fact that both, forward and reverse transport is accomplished by the very same transporter molecule was inferred from the substrate selectivity and the blockage by specific inhibitors; e.g., cocaine blocked both substrate uptake and amphetamine-induced substrate efflux. This revolving door model was termed 'facilitated exchange diffusion model' (Widdas 1952; Stein 1967; Fischer and Cho 1979; Trendelenburg *et al*. 1987; Bönisch and Trendelenburg 1989).

In brief, the releaser (R) binds to the outward-facing conformation (T*o*) of the transporter together with the co-substrates sodium and chloride to produce R-T*o*. A change in conformation transports and releases the bound substrates instantaneously across the plasma membrane to the intracellular side. The unloading of the transported substrate on the intracellular side may be caused by the concentration gradient for the co-substrate(s): there is a steep concentration gradient for sodium (≥ 30-fold) and an additional electrical driving force between the extracellular and intracellular side. Given the low intracellular Na^+ concentration, Na^+ must be instantaneously released on the intracellular side resulting in a drop in the affinity for the substrate and rapid dissociation (*i.e.*, cytoplasmic release) of the

substrate-transporter complex. Subsequently, the inward-facing conformation of the transporter protein (T*i*) is available for binding of intracellular substrate (S) (together with co-transported substrates to produce S-T*i*). Reverse transport to the extracellular side of this substrate requires a reorientation step of the transporter protein. Here, the exchanged substrate is released by the transporter protein, which is then available for the next releaser-molecule to be taken up to the cytoplasmic side.

While the individual steps in this reaction cycle are readily delineated in the schematic rendering of Fig. 1, it does not allow to understand what keeps the transporter from futile cycling. Obviously, inward transport is the favored mode and outward transport of substrate is *per se* a rare event (because $[Na^+]_i$ is low). If, however, the transporter supports influx of large amounts of substrate, reverse transport is more likely to occur (e.g., because $[Na^+]_i$ builds up). Accordingly, EC_{50} for substrate-induced substrate release is higher than the K_M for inward transport (Scholze *et al*. 2000). Similarly, V_{max} for outward transport only reaches < 20% of V_{max} for inward transport (Sitte *et al*. 2001, 2002).

The scheme in Fig. 1 also postulates that the switch from outward-facing to inward-facing conformation enhances the availability of inward-facing transporters by any given substrate. This, in turn, enhances the probability of the binding and subsequent translocation of cytoplasmic substrates to the cell exterior. Fischer and Cho (1979) noted that a tight coupling between substrate uptake and reverse transport is to be expected: *If uptake of the releasing amine is the first step in the chemical release process, then it must follow that the initial rate of release of [^{3}H]dopamine induced by an IAS amine must be related to the initial rate of uptake of the releasing agent*. This ‘facilitated exchange diffusion model’ was supported by numerous publications on reverse transport examined in different NSS family members (Hilgemann and Lu 1999; Lu and Hilgemann 1999a,b; Roux and Supplisson 2000; Wang *et al*. 2003).

As mentioned, appreciable transporter-mediated reverse transport is not expected to occur under basal conditions. In neurons (and platelets), the free cytosolic concentration of substrate is low because neurotransmitters are sequestered into synaptic vesicles by the action of vesicular transporters. This presumably limited the selective pressure, because there was no need to further optimize the system and preclude reverse transport completely. $[dopamine]_i$ has been estimated to about 2 μM (Ewing *et al*. 1992). This concentration, however, is too small to initiate a detectable reverse transport, because the K_M for outward transport exceeds that for inward transport by three orders of magnitude (Sitte *et al*. 2001). Jones *et al*. (1999) measured reverse transport of dopamine by cyclic voltammetry in mouse striatal slices: a $[dopamine]_{in}$ elevation by VMAT inhibition, however, failed to induce DAT-mediated reverse transport. The detection limit for cyclic voltammetry is 25 nM; thus, the concentration did not apparently raise enough to allow for measurement of $[dopamine]_{out}$. In this context, it may be noted that, once redistributed from the vesicle to the cytosol, DA is quickly metabolized by monoamine-oxidase (MAO), often accompanied by an increase in its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC). However, reverse transport can be initiated by raising the intracellular dopamine concentration (in the absence of any further manipulation other than the co-injected Cl^-): Sulzer *et al*. (1995) observed a clear increase in $[dopamine]_{out}$ after injecting 4 pL of 0.5 mM dopamine (i.e., 2 fmol) into

the giant DA neuron of *Planorbis corneus*. It is difficult to interpret the phenomenon, because the dilution of dopamine cannot be estimated; however, under these conditions, reverse transport was specifically triggered because dopamine efflux was blocked by nomifensine (a transport inhibitor) (Sulzer *et al*. 1995). It is evident that this efflux did not require the presence of substrate on the extracellular side; it is also quite clear that the amount of co-injected Cl^- did not suffice to change the ionic composition; apparently, no additional ions were co-injected. To the best of our knowledge, this is the only instance where raising dopamine alone – in the absence of an increase on intracellular Na^+ – sufficed to support efflux. Mosharov *et al*. (2003) further characterized the mechanisms, by which amphetamine affects cytosolic dopamine levels by using intracellular patch electrochemistry in chromaffin cells: extracellular amphetamine (50 μM) was found to increase cytosolic dopamine levels 6-fold during the first 10–15 min, i.e., amphetamine can increase cytosolic substrate concentration also in the presence of vesicles. A follow-up study in neuronal cell bodies (Mosharov *et al*. 2009) showed decreased cytosolic dopamine levels on amphetamine exposure, presumably because there was no vesicular dopamine in neuronal cell bodies (in contrast to chromaffin cells); this decrease was blocked by cocaine or nomifensine, indicating that extracellular amphetamine caused release of cytosolic dopamine via reverse transport: the authors concluded that reverse transport was not induced by the elevated cytosolic dopamine alone, but required additional amphetamine-mediated effects on DAT.

As mentioned above, we proposed two conceptually separate triggers for favoring the efflux mode in the transport cycle outlined in Fig. 1: (i) Inward facing conformations are favored, if Na^+ accumulates on the intracellular side. Similarly, a reduction in external Na^+ eliminates the inward driving force and precludes the accumulation of outward facing conformations. The latter statement is supported by the following circumstantial evidence: inhibitors of NET, DAT and SERT (e.g., cocaine analogues, tricyclic anti-depressants) bind preferentially to the outward facing conformations (Singh 2008; Singh *et al*. 2008); high-affinity binding is strictly dependent on Na^+ (Humphreys *et al*. 1994) and proceeds in an ordered fashion with Na^+ binding prior to the inhibitor (Korkhov *et al*. 2006). Last but not least, currents associated with amphetamine translocation are also consistent with the interpretation that high Na^+ drives the transporter into the outward conformation (Erreger *et al*. 2008). Manipulations of the Na^+-gradient result in the predicted change in influx and efflux rates: Ouabain blocks the Na^+/K^+-ATPase and thereby enhances the $[Na^+]_{in}$; this has long been known to reduce substrate uptake and facilitate efflux (Bönisch 1986; Trendelenburg *et al*. 1987; Wolfel and Graefe 1992; Sitte *et al*. 1998; Jones *et al*. 1999) and this can readily be recapitulated upon heterologous expression, i.e. in the absence of vesicular transporters (Scholze *et al*. 2000; Sitte *et al*. 2000, 2002). Khoshbouei *et al*. (2003) directly correlated an increase in the cytoplasmic $[Na^+]$ and DAT-mediated dopamine efflux. Taken together, these results indicate that substrate efflux can be triggered by an increase in the intracellular $[Na^+]$ (see also below and Fig. 2). (ii) Amphetamines and other releasers must elicit a conformational change that drives reverse transport.

Transport associated currents as a prerequisite for release

The second trigger that allows for efflux is the presence of a releasing substrate, the prototype being amphetamine and its congeners. However, it will be evident that in all

instances amphetamine triggers a substantial movement of excess positive charges, i.e. Na^+ influx. Na^+ is a co-transported substrate in all NSS family members. In the alternating access model the concomitant translocation of substrates and co-transported ions is strictly stoichiometric. While the majority of the transporters of the NSS-family work in an electrogenic manner, SERT is thought to be an exception because it works in an electroneutral manner because of the counter-transport of K^+ (Rudnick 1999): in theory, charge-flow must not necessarily be translated in measureable steady-state current; for SERT, the prediction would be that no net current can be measured. Theoretically, this movement of ions may be assessed in electrophysiological experiments by an increased noise upon transporter activity. Similar to the unexpected observations of SERT-mediated currents (Bruns *et al*. 1993; Mager *et al*. 1994), there was surprising excessive current measured in DAT-expressing cells (Sonders *et al*. 1997; Sitte *et al*. 1998). Hence, electrophysiological investigations have shown that plasma membrane neurotransmitter transporters can function in a way similar to ion channels (Sonders and Amara 1996). Still, the charge fluxes carried by NSS-members are an enigmatic property of the transporters, but they are central to understanding amphetamine-induced efflux.

Currents in transporters and their role in amphetamine-induced efflux

Excess ion flux is observed during substrate influx in many members of the NSS family; in addition, there is leak current, which can be detected under basal conditions and blocked by inhibitors (Mager *et al*. 1994, 1996; Galli *et al*. 1995, 1997, 1998). While the substrate-induced ion flow through neurotransmitter transporters is reminiscent of ligand-gated ion channels, it is worth noting that the currents of the transporters are smaller by orders magnitudes than those of ion channels and their conductance is presumably also much smaller.

It is a challenge to reconcile the view of the transporter as a ‘pump with a channel’ with the alternating access model, because the transport mechanism may be more complicated than predicted from the scheme in Fig. 1. The issue is succinctly summarized by Accardi and Miller (2004) who concluded their description of secondary active transport mediated by a prokaryotic homologue of ClC Cl^- channels in that ‘transporters and channels may be separated by an exceedingly fine line’. Segments in the structure of $LeuT_{Aa}$ proteins can also be conceptually treated as gates that may open simultaneously and thus convert the transporter into a channel. However, there isn’t any evidence so far that $LeuT_{Aa}$ can also generate a transport-associated current (see also below).

Small structural changes suffice to cross the ominous ‘line’ between a transporter and a channel, and this line is crossed in nature: a good example for such subtleties is the sodium-glucose transporter SGLT-3: a mere glucose transporter in pigs, but a glucose-activated Na^+-channel in man and functional as a glucose sensor (Diez-Sampedro *et al*. 2003). Conversely, both bacterial (Accardi and Miller 2004) and mammalian chloride channels are actually H^+/Cl^--antiporters (Scheel *et al*. 2005). Similarly, the mammalian SERT has significantly lower conducting properties than its homolog from *Drosophila melanogaster*: in flies, the currents carry 5- to 10-times more charged moieties/transport cycle (Galli *et al*. 1997; Petersen and DeFelice 1999). In theory, a channel like-mode can be envisaged by assuming

that the transporter moves excess Na^+ (and Cl^-) because the upper (external) gate opens faster than the substrate is released at the internal vestibule. In this instance, the transporter would still be considered to operate in a transport mode albeit a leaky one. However, a true channel-like mode was observed for the substrate translocation in SERT of *Drosophila melanogaster*: at high concentrations, substrate and ions move through the translocation pathway in random order (Petersen and DeFelice 1999) consistent with a 'single file model' (Lester *et al*. 1996; Su *et al*. 1996) that is indicative of diffusion through a pore. Ramsey and DeFelice (2002) suggested that the formation of a pore might rely on oligomerization of the SERT at the plasma membrane; however, further evidence in support of this notion is still lacking.

Uncoupled conductance can only be explained by the formation of a pore within the transporter molecule. The difficulty in interpreting the data at the current stage also arises from the fact that – in spite of the presence of candidate gates at the external and internal vestibule – the crystal structure of $LeuT_{Aa}$ does not reveal a cavity consistent with the random movement of charge through the molecule (Singh 2008).

Regardless of these conceptual difficulties, currents are important to understand the actions of amphetamines: binding of amphetamine to the transporter appears to favour a channel mode, i.e., a conducting pore may be stabilized and support efflux: brief spike-like elevations of extracellular dopamine can be observed by amperometry in DAT-expressing cells. These spikes were detected only after application of amphetamine but not of dopamine (Kahlig *et al*. 2005). The spikes are consistent with fast, burst-like release events of neurotransmitter through a channel. Amperometry is limited in its sensitivity (and the amount of released detectable substrate), thus, it is difficult to discern the relative proportion of dopamine that is released by bursts and by counter-transport. Kahlig *et al*. (2005) estimated a ratio of 9 : 1 in favour of continuous efflux; this estimate suggests that amphetamine does allow the transporter to cycle efficiently through outward and inward facing conformations and that the channel-like mode is a comparably rare event.

Originally, reverse transport was defined as a carrier-mediated, Ca^{2+}-independent non-exocytotic release of neurotransmitter (Levi and Raiteri 1993). This led to the implicit assumption that the membrane potential was irrelevant for amphetamine-induced efflux. However, monoamine substrates are transported as charged species (Berfield *et al*. 1999). Hence, a negative membrane potential provides an additional driving force for influx. Similarly reverse transport also depends on the membrane potential (Khoshbouei *et al*. 2003) and depolarization adds some additional driving force for counter-transport. It is worth noting that the action of amphetamine on DAT *per se* depolarizes the cell (Carvelli *et al*. 2004; Meinild *et al*. 2004) and that Na^+ is the principal charge carrier involved in this process (Sitte *et al*. 1998). Similarly, experiments in SERT-expressing Xenopus laevis oocytes also support Na^+ as the charge carrier (Quick *et al*. 2004). The uncoupled conductance is postulated to supply enough Na^+ to trigger reverse transport: based on the scheme in Fig. 1, the local accumulation of intracellular Na^+ is predicted to favour the accumulation of transporters in the inward facing conformation and thus render the transporter more prone to support substrate efflux; binding of substrate to the inward-facing

conformation of the transporter becomes a more likely event as $[Na^+]_{in}$ rises. The transporter will return to the outward-facing conformation in a substrate-loaded state.

The physiological role of the uncoupled currents in neurotransmitter transporters remains enigmatic. Moreover, the channel within the DAT is also permeable for anions (usually chloride; Ingram *et al*. 2002; Meinild *et al*. 2004) which can be decisive in shifting the membrane potential: depending on the actual membrane potential and the reversal potential for Cl^-, the net effect can either result in hyperpolarisation or depolarization of the cell membrane, (Ingram *et al*. 2002; Sulzer and Galli 2003; Meinild *et al*. 2004). Thus, it is conceivable that neurotransmitter transport modulates the excitability of neurons (Falkenburger *et al*. 2001; Ingram *et al*. 2002) and, in addition, that reverse transport takes place if cells are overloaded with Na^+, e.g. by depolarization via glutamate receptors (Falkenburger *et al*. 2001) or by hypoxia/anoxia (Schömig *et al*. 1987).

Taken together, the observations show that all transporter substrates induce a transport-associated current. This current, however, only rarely suffices to trigger substrate efflux. However, the pharmacological actions of amphetamines are contingent on reverse transport. Reverse transport by monoamine transporters has also been discovered as a target during evolution: long before man has elaborated the synthesis of amphetamine, plants have acquired the ability to produce related alkaloids such as ephedrine and cathinon to fend off grazers and insects.

What are the differences between physiological substrates and amphetamines that finally lead to their dissimilar properties? The apparent affinity in substrate and amphetamine uptake is very similar in the low micromolar range. But the number of transported substrate molecules differs. Admittedly, it is difficult to measure uptake of amphetamines precisely owing to their notorious lipophilic nature (which causes rapid back-diffusion; Bonisch 1984; Zaczek *et al*. 1991). Thus, there are only a few systematic investigations in this area: HEK293 cells stably expressing the human DAT differed in substrate uptake rates (dopamine, tyramine and the two isomers of amphetamine) by more than a factor of 20 (Sitte *et al*. 1998). In contrast, superfusion experiments performed in parallel sister cultures revealed that D-amphetamine induced RT with the highest potency and efficacy. It is worth pointing out that the released substrate was MPP^+, which is metabolically stable (Langston *et al*. 1984; Javitch *et al*. 1985); this obviates any confounding effects because of the action of amphetamines on MAO-A or MAO-B. Furthermore, patch-clamp experiments carried out in parallel showed that the rank order of potency for the induction of inwardly directed current was comparable to that obtained in superfusion experiments (Sitte *et al*. 1998). Importantly, when currents were consecutively assessed in the same DAT-expressing cells (Sitte *et al*. 1998), amphetamine induced a 25% higher inwardly-directed uncoupled conductance compared to dopamine. Hence, the propensity of amphetamines to induce larger currents suggests that they are more effective in triggering the channel mode in neurotransmitter transporters. This may account for the differences between amphetamines and physiological substrates.

There is only one observation that is inconsistent with the conjecture that transport-associated currents are essential to drive the transporter into an efflux mode: this is the

action of syntaxin 1a on DAT mediated efflux. Syntaxin 1a is a SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) that supports fusion of neurotransmitter vesicles with the target membrane (i.e. the presynaptic membrane). Syntaxin 1A also directly interacts with neurotransmitter transporters (Dipace *et al*. 2007) and inhibits excess currents through SERT (Quick *et al*. 2004) and GAT1 (Wang *et al*. 2003). Surprisingly DAT-mediated efflux, however, is enhanced upon co expression of syntaxin 1a (Binda *et al*. 2008). These data are difficult to interpret because (i) current is a prerequisite for transporter-mediated efflux (see above) and (ii) the apparent disparity to the experiments performed by Wang *et al*. (2003) where the presence of syntaxin 1a clearly reduces the efflux of GABA via GAT1. It would be crucial to see, if this action can be recapitulated in neuronal cells that had been treated with for instance clostridial toxins (of *C. tetani* and *C. botulinum*) to elicit the predicted opposite effect.

Multiple targets of amphetamine and its congeners within the presynaptic specialization

As outlined earlier, the monoamine transporters DAT, NET and SERT are the primary site of action of amphetamine and its congeners, [e.g., 3,4-methylene-dioxy-met-amphetamine (MDMA), ‘ecstasy’], methamphetamine [‘ice’] or amphetamine [‘speed’] (Seiden *et al*. 1993; Sulzer *et al*. 2005). Individual compounds differ by their affinity to the three transporters but the mechanism of action is the same. In the absence of monoamine transporters (or upon inhibition of the transporters), the compounds do not elicit their biological actions. However, amphetamines have additional targets that contribute to their mechanism of action: (i) inhibition of VMAT1 and VMAT2 and (ii) MAO-A, MAO-B. Under physiological conditions, there are two processes that keep the cytosolic concentration of monoamine neurotransmitters low in the presynaptic specialization: (i) the reserpine-sensitive, proton-driven VMAT1 and VMAT2 retrieves neurotransmitters into the synaptic vesicles (Liu *et al*. 1992). (ii) Monoamines that escape the action of VMAT are rapidly degraded by the action of mitochondrial MAO-A and MAO-B and to a lesser extent by catechol *O*-methyltransferase (Seiden *et al*. 1993). Amphetamines inhibit MAO and are substrates for VMAT. The concerted action on all targets can be summarized as follows: (i) charged amphetamines are recognized by DAT, NET and/or SERT as substrates and enter the cells. More amphetamine molecules will be in their protonated form, because the intracellular pH is lower than the extracellular pH. Nevertheless, the remaining unprotonated amphetamine molecules will diffuse back into the synaptic cleft through the membrane and be quickly available for another round of transporter-mediated uptake (Rudnick 2002). Hence, one molecule of amphetamine can, in principle, cycle repeatedly between the interior of the cell and the extracellular space; this cycle is thought to underlie the accumulation of Na^+, Cl^- and H^+ in the cytoplasm (Rudnick 1999). (ii) Amphetamines interfere with vesicular uptake of monoamines by competing for VMAT and, upon accumulation in the vesicles, by their buffering action, which dissipates the pH gradient. This has lead Sulzer and colleagues to hypothesize a succinct model, termed the ‘weak base hypothesis’ (Sulzer *et al*. 1993, 1995): here, the amphetamine action leads to the elevation of cytosolic substrate which is then available for reverse transport. (iii) Amphetamines also inhibit the degradation

of monoamines by MAO (Robinson 1985). Accordingly, these actions explain why amphetamines readily induce reverse transport while natural substrates do not.

Shortcomings of the present hypotheses (weak base/facilitated exchange diffusion): futile cycling, phosphorylation and Zn^{2+}

There are several observations that the currently held models fail to explain:

(i) The facilitated exchange diffusion hypothesis suffers from one conceptual shortcoming. If amphetamine resides in the inner vestibule of the transporter and has raised intracellular Na^+ by multiple rounds of inward transport, why should it be released and replaced by the physiological substrate (which is not yet in the vestibule), rather than not *per se* be subject to reverse transport? In other words, there must be a mechanism that prevents futile cycling of amphetamines in the transporter. This conceptual problem can be solved in two ways: (a) amphetamines have a negligible affinity for the internal vestibule such that they cannot be subject to reverse transport in the presence of (competing) substrate on the cytosolic side. (b) The actions of amphetamines are contingent on the oligomeric nature of the transporters (see below).

(ii) The cogent framework outlined above serves to integrate several actions of amphetamines in the presynaptic terminal and explains how substrates become available for outward transport in the nerve terminal. However, any model on the action of amphetamines must account for the requirement of protein phosphorylation: inhibition of protein kinase C (PKC) or of calmodulin-dependent kinase-2 blunts the action of amphetamines but does not eliminate substrate-induced efflux (i.e., exchange diffusion) (Kantor and Gnegy 1998; Kantor *et al*. 2001; Gnegy 2003; Seidel *et al*. 2005; Fog *et al*. 2006). These observations can also be recapitulated upon genetic ablation of PKCβ (Chen *et al*. 2009).

(iii) Any model on the action of amphetamines must explain why amphetamines can, in principle, give rise to bell-shaped concentration-response curves. This can readily be seen in superfusion experiments, where the concentrations can be varied appropriately over a large range (Seidel *et al*. 2005).

(iv) Last but not least, the observations obtained with Zn^{2+} are difficult to reconcile with a model that treats efflux and influx as coupled processes (see above for the quotation of Fischer and Cho, which can be distilled into ‘the faster in, the faster out’): Zn^{2+} binds to an endogenous allosteric site in the DAT (Norregaard *et al*. 1998). The residues for the coordination of Zn^{2+}-binding are His189, Glu377 and Glu396 (Norregaard *et al*. 1998; Loland *et al*. 1999). Upon binding to this tridentate coordination sphere, Zn^{2+} inhibits substrate uptake by DAT (Norregaard *et al*. 1998; Scholze *et al*. 2002b). This non-competitive effect on uptake indirectly suggests that Zn^{2+}, by binding to the transporter, constrains relative movements between ECL2, TM7 and TM8 critical for the inward translocation process (Loland *et al*. 1999). However, Zn^{2+} stimulates

amphetamine-induced efflux (Scholze *et al*. 2002b; Pifl *et al*. 2004). The asymmetrical effect is obvious, the Zn^{2+}-induced inhibition of uptake is accompanied by an increase in efflux and this may be accounted for by the fact that Zn^{2+} promotes an additional ion current (Meinild *et al*. 2004; Pifl *et al*. 2009). Regardless of the underlying mechanistic details, the observations stress two pertinent aspects: influx and efflux do not necessarily occur via the same route and enhanced inward currents favor outward transport.

Amphetamine-induced activation of protein kinases

Originally, reverse transport was defined as a carrier-mediated, Ca^{2+}-independent non-exocytotic release of neurotransmitter (Levi and Raiteri 1993). However, Gnegy *et al*. (2004) showed that removal of internal Ca^{2+} by the membrane-impermeant Ca^{2+}-chelator BAPTA reduces amphetamine-induced dopamine efflux in cells expressing DAT. This requirement for low calcium concentrations can be rationalized by the fact that the action of amphetamines is – in part – contingent on PKC-mediated (Kantor and Gnegy 1998) and Ca^{2+}/calmodulin-dependent phosphorylation (Kantor *et al*. 1999; Fog *et al*. 2006). This was first observed for DAT but subsequently confirmed for NET (Kantor *et al*. 2001) and SERT (Seidel *et al*. 2005). It is at present not clear, how amphetamines cause activation of PKC (Giambalvo 1992a,b) and of Ca^{2+}/calmodulin-dependent protein kinase, but the stimulation is dependent on intracellular Ca^{2+} (Giambalvo 2003). The pleiotropic actions of kinases may allow for the recruitment of many different mechanisms that afford transport reversal, e.g. phosphorylation-dependent inhibition of Na^+/K^+-ATPase (Kazanietz *et al*. 2001) which would directly favour intracellular accumulation of Na^+. Initially, activators of PKC such as 12-*O*-tetra-decanoyl phorbol-13-acetate, diacylglycerol and arachidonic acid, have been shown to enhance both DAT-mediated efflux of dopamine (Pozzan *et al*. 1984; Davis and Patrick 1990; L'hirondel *et al*. 1995). However, there is circumstantial evidence for a direct action of PKC-isoforms on transporters: truncation of the 22 N-terminal residues of DAT, eliminates the ability of DAT to undergo PKC-dependent phosphorylation (Granas *et al*. 2003) and suppresses reverse transport induced by amphetamine (Khoshbouei *et al*. 2004). The relevant PKC isoform has been identified as PKC beta(II), interacting with DAT in rat striatum (Johnson *et al*. 2005); however, there is no formal proof that this PKC isoform actually phosphorylates DAT. Foster *et al*. (2002) detected DAT phosphorylation after stimulation of PKC-isoforms with phorbol myristate (which activates typical and novel isoforms, e.g. PKCs α, β, γ, δ, ε). The N-terminus of human DAT can be phosphorylated by PKCα (Fog *et al*. 2006), and this is also observed with the N terminus of rat-DAT (Gorentla *et al*. 2009). However, PKCα does not associate with DAT (Johnson *et al*. 2005). Hence, the role of the different PKC isoforms has not yet been clarified. Nevertheless, amphetamine-induced reverse transport is blunted after mutational replacement of the five serine residues in this segment of DAT by alanine (Khoshbouei *et al*. 2004). From these mutagenesis experiments, the authors concluded that amino terminal phosphorylation by PKC shifts the DAT from a 'reluctant' to a 'willing' state for efflux (Robertson *et al*. 2009). This was further underscored by mutation of the same five serine residues to aspartate and thereby restoring the efflux property of the transporter (Khoshbouei *et al*. 2004). It must be conceded, though, that there is no direct evidence for phosphorylation of these sites.

There are many discrepancies between heterologous expression systems and endogenously expressed transporters and these are evident when examining the actions of protein kinases on transporters: Sorkina *et al*. (2006) and Eriksen *et al*. (2009) did neither find any appreciable effect of PKC by activation with phorbol myristate nor by inhibition with staurosporine or GF109203X on dopamine uptake by dopaminergic neurons. However, inhibition of uptake (presumably by down-regulation of surface transporters) had been described many times for heterologous expression systems (see Foster *et al*. 2006) and this has also been recapitulated in rat striatal synaptosomes (Vaughan *et al*. 1997; Chi and Reith 2003; Hoover *et al*. 2007). Hence, to date it is unclear if PKC exerts a direct effect on transporter activity.

Protein kinase C's are not the only intracellular kinases postulated to phosphorylate neurotransmitter transporters. As mentioned above, calmodulin-dependent kinase-2α phosphorylates DAT (but not NET or SERT) and the mechanistic details are understood: first, the enzyme engages the carboxyl terminus of DAT and to subsequently phosphorylates the amino terminus (Fog *et al*. 2006). However, it is unclear to date if the two kinases exert their effects independently from each other or in strictly hierarchical order. Furthermore, other kinases (e.g., Moron *et al*. 2003; Zhu *et al*. 2005; Ramamoorthy *et al*. 2007) impinge on neurotransmitter transporters: this causes changes in trafficking (Moron *et al*. 2003) and/or velocity of substrate influx (Zhu *et al*. 2005). It is not clear if these kinases also affect amphetamine-induced outflow. However, it may be worth considering this possibility: amphetamines are used to treat attention-deficit hyperactivity disorder (ADHD; Mazei-Robinson and Blakely 2006). The effectiveness of amphetamine is counter-intuitive, because amphetamines elicit psycho stimulant effects (an action which is sought in their illicit consumption). However, a (rare) mutation in the dopamine transporter allows for rationalizing the paradoxical effect of amphetamines in ADHD: a substitution of alanine^{559} by valine (found in an affected family) results in a transporter which is targeted to the cell surface and mediates dopamine uptake in a manner indistinguishable form the wild type. However, DAT-A559V has augmented substrate basal efflux, which is blocked by amphetamine (and methylphenidate) (Mazei-Robison *et al*. 2008). It is conceivable that in other individuals affected by ADHD, it is not the transporter that is mutated but that the defect is in a regulatory pathway which switches the transporter into a mode analogous to that adopted by DAT-A559V.

An oligomer-based counter-transport model as a unifying concept

Neurotransmitter transporters are oligomers (Kilic and Rudnick 2000; Schmid *et al*. 2001; Kocabas *et al*. 2003; Sorkina *et al*. 2003; Torres *et al*. 2003a). Oligomerization has been recognized as an important prerequisite for exit from the endoplasmic reticulum (Farhan *et al*. 2004, 2007, 2008). It has furthermore been shown that – throughout the secretory pathway from the endoplasmic reticulum to the plasma membrane – DAT is an oligomer and this is also true for the endocytosed transporter (Sorkina *et al*. 2003). For endoplasmic reticulum export, oligomeric assembly has been proposed to facilitate efficient recruitment of the transporter into endoplasmic reticulum exit sites by the assembling coat protein complex II (COPII)-coat (Farhan *et al*. 2006). This may also be true for other cargo receptors (e.g., ARF-GAP1; Reiterer *et al*. 2008) and proteins that regulate trafficking

through the secretory pathway (e.g. GTRAP3-18, Liu *et al*. 2008; Ruggiero *et al*. 2008; Maier *et al*. 2009). In addition, we posit that oligomerization is also relevant to understand the mechanism of action of amphetamines. We refer to this model as oligomer-based counter-transport model, in which the releaser is taken up by one moiety and transporter-mediated substrate efflux is achieved by the neighbouring moiety. There are several arguments that support this concept:

(i) It is evident that a single transporter moiety can hardly allow for permeation of amphetamine and simultaneously support the efflux of substrate. It is much more plausible to assume that amphetamine triggers the channel mode in an adjacent transporter moiety within the oligomeric complex. If this were the case, amphetamine ought to induce maximum substrate release at a concentration where it occupies about 50% of the available transporters. A further increase of amphetamine is predicted to cause a decline in substrate release, because progressive occupation of transporters with inward moving amphetamine reduces the number of those available for outward transport of substrate resulting in a bell-shaped concentration-response curve. Bell-shaped concentration-response curves have actually been observed with amphetamine and several times reported (Ross and Kelder 1977; Langeloh and Trendelenburg 1987; Pifl *et al*. 1999; Seidel *et al*. 2005).

(ii) The role of an oligomeric arrangement has been tested by using a concatemer composed of SERT and the GAT1. In this SERT-GAT1 concatemer, the SERT-selective amphetamines employed (pCA; MDMA) elicited [^{3}H]GABA efflux (Seidel *et al*. 2005). This effect demonstrates that – in the concatemer – GAT1 can sense the action of amphetamines on the adjacent SERT. It is likely that the signal that is sensed is the Na^+ influx in the vicinity of the inner vestibule. This finding is most parsimoniously explained by the oligomer-based counter-transport model (see Fig. 2). It is impossible to formally rule out other mechanisms, e.g. amphetamine-induced changes in second messenger-triggered cascades or alterations of the membrane potential propagated through ion channels. But, based on our current understanding of the biological actions of amphetamine, these alternative possibilities cannot readily account for driving the GAT1-moiety of the concatemer into the reverse-transport conformation.

(iii) It is worth noting that pCA-induced efflux in this concatemer is still sensitive to PKC-inhibition. This observation indicates that accumulation of internal Na^+ *per se* does not suffice to explain amphetamine-induced efflux and again stresses the importance of amphetamine-induced protein kinase activation (see above).

It is not clear, how individual subunits communicate within the oligomer. A testable model, however, can be developed by examining the recently solved structure of the bacterial betaine transporter BetP (Ressl *et al*. 2009). BetP is only distantly related to neurotransmitter transporters; however, the pseudo symmetrical fold is conserved. The topology is somewhat different from $LeuT_{Aa}$, the bacterial homolog of neurotransmitter transporters. In $LeuT_{Aa}$ where the pseudo symmetric fold comprises transmembrane helices TM1–TM10 (with symmetrical positions held by TM1 and TM6, TM2 and TM7 etc.), while TM11 and TM12

make up the crystallographic dimer interface (Yamashita *et al*. 2005; Singh 2008) and a candidate interaction site in neurotransmitter transporters (Just *et al*. 2004). In BetP, the trimer interface is comprised by the two N-terminally located transmembrane helices TM1 and TM2 and the pseudo symmetrical fold is comprised by the TM3–TM12. BetP is an osmolyte transporter and senses (hyper)osmotic stress by the ensuing change in the intracellular K^+ concentration. Interestingly, the stress signal is communicated via the C-terminus to a partner within the trimer to trigger inward transport. Based on the topology, a speculative model can be developed that posits that in the monoamine transporters the Na^+-triggered signal is communicated from one transporter moiety to the adjacent moiety by the N-terminus which is topologically equivalent to the C-terminus in BetP. Efficient communication requires a priming action of protein kinase-dependent phosphorylation of the N-terminus. This model is consistent with the observation that the N-terminus of DAT contains the phosphorylation sites essential for amphetamine-induced substrate efflux (Khoshbouei *et al*. 2004; Fog *et al*. 2006). It is also consistent with the intermolecular distances of N- and C-termini (Just *et al*. 2004): the distance between C-termini within oligomers is smaller than that of N-termini. Last but not least, the C-terminus of one BetP monomeric moiety communicates via a network of ionic bonds with charged residues in loop 2 and loop 8 of the adjacent monomer (Ressl *et al*. 2009). In GAT1, the N-terminus apparently binds to the intracellular loop 4 (equivalent to loop 8 in BetP) via interaction of charged residues (Hansra *et al*. 2004). It is obvious that phosphorylation of the N-terminus may have a substantial effect on these ionic interactions. Thus, while the model is highly speculative, it can account for most findings and – most importantly – it provides a starting point to locate the latch by which for amphetamine-induced substrate efflux is triggered (Fig. 3). It is obvious that at this stage, we cannot discriminate between the sequence of events that follow the conformational change induced by binding of amphetamine, i.e. whether the N-terminal latch is simply triggered by binding of amphetamine, whether it requires ‘channel mode’ and/or the accompanying Na^+ influx or amphetamine-induced protein kinase activation.

In an oligomer-based counter-transport model, the substrate is taken up by one moiety and transporter-mediated release is achieved by the neighbouring moiety (Fig. 2). However, from a pharmacological perspective, the occupancy of the single transporter moieties in an oligomeric complex by substrate must reduce the probable efflux; in other words, the more substrate available at the cell exterior, the fewer transporters can be available for efflux – hence, amphetamine-induced SERT efflux ought to subside when the amphetamine concentrations rises above a certain level; a biphasic concentration response curve again reflected these predictions (Seidel *et al*. 2005).

Most importantly, we hypothesized that carrier-mediated efflux relies on the spatial proximity within a transporter oligomer. To verify this conjecture, we examined the amphetamine effects on GABA-preloaded SERT-GAT1-expressing cells. A tiagabine sensitive, concentration dependent GABA efflux was triggered by amphetamine application. This finding confirmed the oligomer-based counter-transport model. Most importantly, increasing the doses of amphetamine to induce efflux did not result in a bell-shaped concentration-response curve but reached saturation: this was to be expected since GAT1 is

resistant against amphetamine and therefore, the amphetamine uptake was not able to prevent GAT1 phosphorylation needed for efflux.

How to test the oligomer-based counter-transport model

Our model predicts that monomeric transporter molecules cannot support amphetamine induced efflux. The model cannot readily be tested, because monomeric transporters are not exported (Scholze *et al*. 2002a). However, the export defect may be remedied by linking an engineered FK506-binding domain that can be reversibly dimerized/oligomerized by using a pharmacologically inert analogue of tacrolimus.

Truncation of the N-terminus is known to abolish amphetamine-induced efflux. It is difficult though to differentiate between the loss of (potential) protein phosphorylation sites and the absence of the putative amphetamine-operated latch. An alternative approach is to tether the N-terminus and thus to restrict its mobility.

This strategy can be further developed by creating various SERT concatemers (SERT is the molecule of choice because concatemers of DAT and/or NET are not readily inserted into the plasma membrane): if the first moiety (of SERT) is truncated at its N-terminus, the concatemer is predicted to be amphetamine-resistant. The approach with SERT concatemers may also be combined with engineered Zn^{2+}-binding sites: if the first SERT moiety carries the Zn^{2+} binding site, the oligomer-based counter-transport model predicts that Zn^{2+} ought to depress amphetamine-induced release. Conversely, if the engineered Zn^{2+}-binding site is in the second moiety, the amphetamine action will be enhanced by Zn^{2+}. The Zn^{2+}-experiment has the advantage that it provides a handle to model the propagation of conformational changes through the protein by providing a fixed geometrical constraint for molecular dynamics studies.

As mentioned earlier, the actions of amphetamines are thought to be in part mediated via protein kinases. These observations must be accounted for by mechanistic hypotheses on the action of amphetamines. The oligomer-based counter-transport model is not superior to the facilitated exchange diffusion model when rationalizing the effects of the protein kinases. The facilitated exchange diffusion model and the oligomer-based counter-transport model do also not differ in their ability to accommodate the peculiar actions of Zn^{2+}.

Concluding remarks

Monoaminergic neurotransmitter transporters are the prime target of the amphetamines; they comprise a large variety of widely abused, psychotropic substances including D-amphetamine (= speed), methamphetamine (= ice) and methylene-dioximethamphetamine (MDMA; = ecstasy). Amphetamines were originally marketed as appetite suppressants (before they were withdrawn on a worldwide basis because of their addictive potential and because some compounds caused pulmonary hypertension). At present, the amphetamine derivative methylphenidate is used in hyperactive children; this compound acts in a manner similar to cocaine because it blocks uptake and also inhibits transporter-mediated current (Sonders *et al*. 1997). All commonly used and abused amphetamine-like compounds induce carrier-mediated efflux (Levi and Raiteri 1993), with the notable exception of

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methylphenidate. Regardless of the underlying molecular mechanism, all amphetamine-like compounds elevate synaptic monoamine concentrations. Hence, a prolonged stimulation of postsynaptic receptors ensues. Amphetamines differ in their affinities for monoamine transporters: for instance, D-amphetamine predominantly targets DAT while MDMA acts mainly on SERT (Seiden *et al*. 1993; Green *et al*. 2003). Many insights have been generated that shed some light on the mechanism of action of amphetamines. However, the mechanistic basis for their effect has remained enigmatic at the molecular level. The strength of the oligomer-based counter-transport model is that it attempts to integrate different observations: (i) the quaternary structure of the monoamine transporters, (ii) the unique action of compounds that act as releasers *in vivo*, (iii) the electrophysiological properties of transporters, and (iv) the putative regulation by intracellular kinases. By proposing the N-terminus as a latch, it also provides for testable hypotheses. Like any hypothetical model, it may not withstand the test of experimental scrutiny.

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Abbreviations used

ADHD	attention-deficit hyperactivity disorder
DAT	dopamine transporter
GAT1	GABA-transporter-1
MAO	monoamine-oxidase
MDMA	3,4-methylene-dioxy-met-amphetamine
NET	norepinephrine transporter
NSS	neurotransmitter:sodium symporters
PKC	protein kinase C
SERT	serotonin transporter
VMAT	vesicular monoamine transporter

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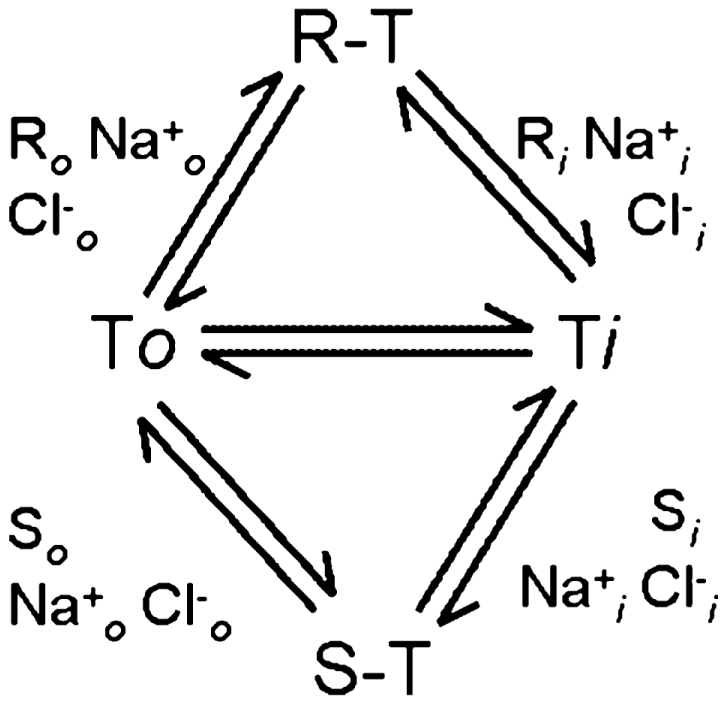

Fig. 1.

A kinetic scheme of substrate (S) and/or releaser (R) interaction with either the outward (o) or inward (i) facing transporter (T; adapted from Jones *et al*. 1999).

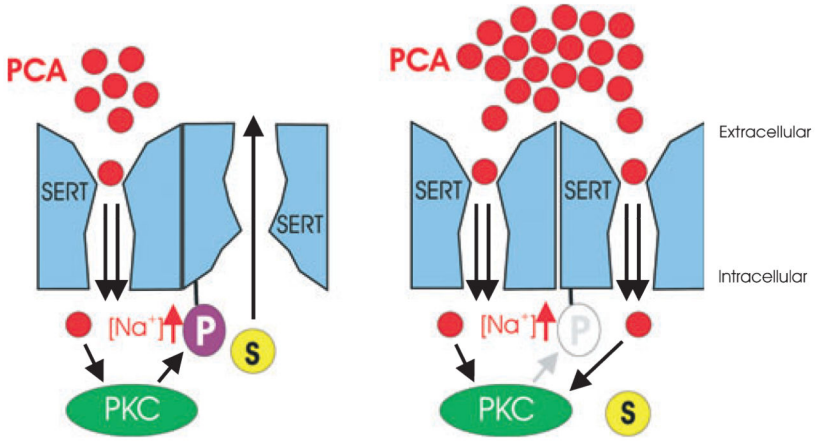

Fig. 2.

Schematic illustration of the oligomer-based counter-transport model (described in more detail in Seidel *et al*. 2005). The left side illustrates the effect of low concentrations of amphetamine (in this figure, pCA stands for para-chloroamphetamine); only a fraction of SERT moieties is occupied by pCA in the oligomeric complex (shown here for the sake of simplicity as a dimer). Occupancy by pCA precludes phosphorylation by PKC (Ramamoorthy and Blakely 1999). The other SERT moieties in the oligomeric complex that has not been occupied by pCA are subject to phosphorylation and thereby primed for outward transport of substrate. Right, at high pCA concentrations, all transporter subunits are occupied by pCA, which impairs the action of PKC and thus prevents the accumulation of inward-facing conformations: efflux of substrate is impaired.

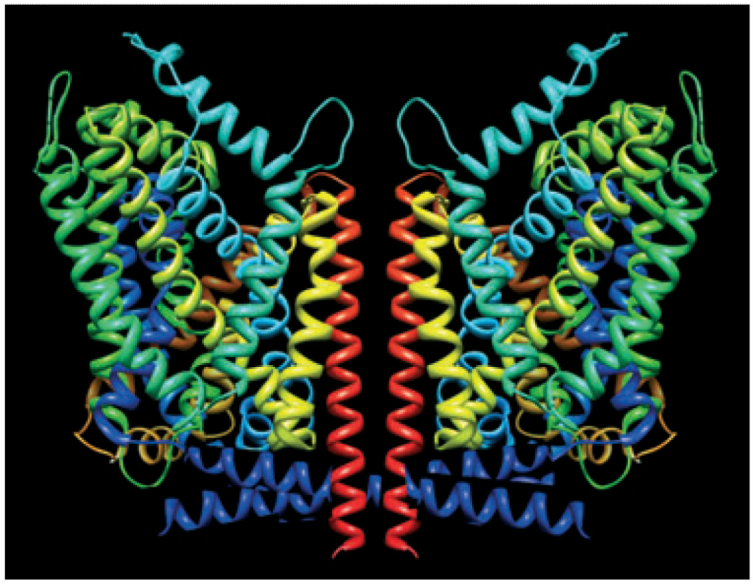

Fig. 3.

Hypothetical representation of a possible cooperative interplay within a transporter oligomer (for the sake of simplicity, the transporter oligomer is shown as a dimer only). The amino-termini drawn with 11 helical turns suffice to reach the centre of the putative oligomeric partner to locate the latch by which for amphetamine-induced substrate efflux is triggered. The scheme is based on the published coordinates of $LeuT_{Aa}$ (Yamashita *et al*. 2005) and the artwork created by using UCSF Chimera (http://www.cgl.ucsf.edu/chimera; Pettersen *et al*. 2004).

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