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Molecules in focus

# An unfolding story: Small molecules remedy misfolded monoamine transporters

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ABSTRACT

The key role of monoamine transporters is to take up neurotransmitters from the synaptic cleft and rapidly terminate neurotransmission. Monoamine transporters begin their journey by folding in the endoplasmic reticulum. Upon achieving their natively-folded state, the oligomerized transporters engage the coat protein complex II machinery and exit the endoplasmic reticulum compartment in a concentrative fashion. The transporters are subsequently sorted in the endoplasmic reticulum-Golgi intermediate complex and the Golgi apparatus, prior to reaching their pivotal site of action at the plasma membrane. Stringent quality-control mechanisms ensure that only the correctly-folded protein cargo departs the endoplasmic reticulum. Genetic point mutations in the coding sequences of monoamine transporters can trigger severe physiologic deficiencies by inducing folding defects. Protein misfolding precludes the delivery of functional monoamine transporters to the cell surface. Chemical- and/or pharmacological-chaperone molecules, which facilitate folding, have proven effective in restoring the activity of several misfolded pathological variants of monoamine transporters.

## 1. Introduction

The seminal work by Otto Loewi and Henry Dale established that neurons communicate via neurotransmitter release (Dale and Dudley, 1929). The presynaptically released neurotransmitters act on the postsynaptically located receptors to elicit biological responses. An efficient neurotransmitter clearance is necessary for terminating the signal and preparing the system for the next neurotransmission event. In the 1960s, Julius Axelrod and Georg Hertting showed that the monoamine neurotransmitter norepinephrine is selectively taken up by the nerve terminals, which paved the way to neurotransmitter transporter studies (Hertting and Axelrod, 1961). Their work also showed that uptake was a major route of neurotransmitter clearance from the synaptic cleft (Axelrod, 2003). Monoamine transporters (MATs) belong to the solute carrier 6 (SLC6) family and rapidly retrieve monoamine neurotransmitters from synaptic and extrasynaptic sites. The MAT protein family encompasses the transporters for serotonin, dopamine and norepinephrine (SERT, DAT and NET, respectively). MATs have a rich pharmacology and are the targets of action of therapeutic drugs (e.g. citalopram and methylphenidate), and illicit substances (e.g. cocaine and amphetamines).

## 2. Structure

The closely related SERT (SLC6A4), DAT (SLC6A3) and NET (SLC6A2) share many common structural features: twelve transmembrane (TM) helices, connected by 5 intra- and 6 extracellular loops. A large extracellular loop between TMs 3 and 4 (EL2) harbors the motifs for N-glycosylation and is stabilized by disulfide bonds. Both termini are cytosolic and carry specific motifs and conserved residues, which play crucial roles in trafficking, signaling and protein folding (e.g. El-Kasaby et al., 2010, 2014; Sucic et al., 2011). This topology was originally inferred from the primary sequence, but more recently confirmed by the crystal structures of the bacterial leucine transporter (LeuT), *Drosophila* DAT (dDAT) and human SERT (Zhou et al., 2007; Penmatsa et al., 2013; Coleman et al., 2016, respectively). Fig. 1 shows a SERT-based homology model of DAT and visualizes the co-substrate ions, i.e. two $Na^+$ (shown as blue spheres) and one $Cl^-$ (shown as orange sphere): translocation of the substrate is driven by symport of two $Na^+$ ions. The involvement of $Cl^-$ is less understood; recent evidence suggests that there is no net $Cl^-$ inward transport and that the transporter requires $Cl^-$ to translocate the substrate across the membrane, but it then returns it to the outward-facing state with $Cl^-$ bound (Hasenhuetl et al., 2016). Similarly, while it was originally thought that only SERT required an antiport of $K^+$ (Nelson and Rudnick, 1979),

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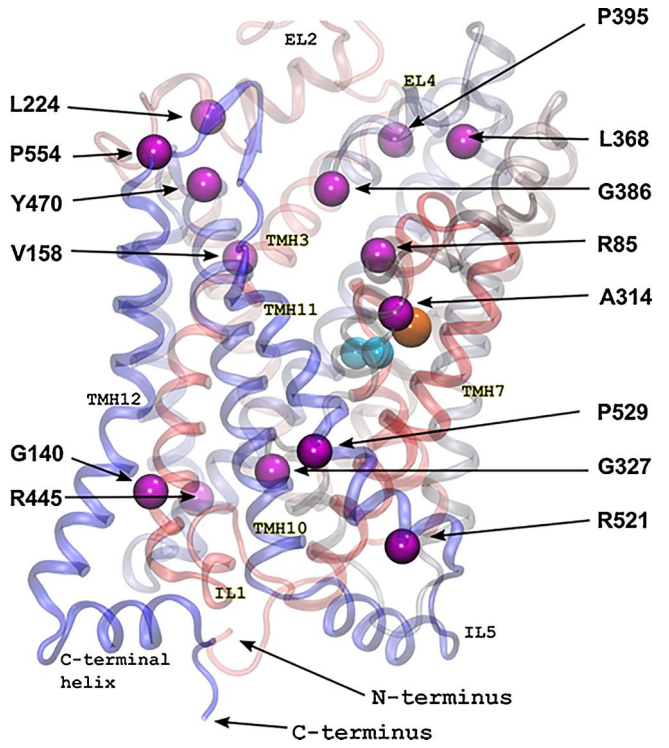


**Fig. 1.** Homology model of hDAT highlighting the position of mutations associated with infantile/juvenile dystonia.
A homology model of the human DAT based on the human SERT structure (PDB ID: 5I71) (Coleman et al., 2016) was developed using Modeller (version 9.17) by applying the automodel procedure. Human DAT is shown from the membrane, with residue numbers color coded from red (N-terminus) to blue (C-terminus). The co-transported sodium ions are shown in blue, the chloride ion in orange. The magenta spheres mark the position of the Cα atoms of those residues, which – when mutated – lead to a folding defect and thus cause infantile/juvenile dystonia and parkinsonism. G140 is also marked: although it has only been found in Drosophila melanogaster (Wu et al., 2008) rather than in people, it results in the equivalent phenotype and it allowed for providing a proof-of-principle for pharmacochaperoning *in vivo* (Kasture et al., 2016).

there is now evidence that antiport of $K^+$ is a universal feature of monoamine transporters (Hasenhuetl et al., 2016).

## 3. Protein folding and quality control mechanisms in the endoplasmic reticulum (ER)

Like all other membrane proteins, MATs are co-translationally inserted into the endoplasmic reticulum (ER) membrane, where they acquire their natively-folded state under the influence of both, lumenal and cytosolic components. After reaching the natively-folded state, MATs form oligomers, required for their concentrative ER-export (Chiba et al., 2014). There are two major steps in the ER-quality control: the proximal/primary quality control, which accounts for protein folding and oligomerization, and the distal/secondary quality control, which encompasses factors associated with ER-export. Misfolded proteins and amorphous aggregates are subjected to ER-associated degradation (ERAD) (Fig. 2).

Some forty-five years ago, Anfinsen put forward the thermodynamic hypothesis (Anfinsen, 1973), stating that natively-folded proteins have the lowest Gibbs free energy in the entire protein folding landscape (Fig. 2). To attain this energetically stable protein conformation, all hydrophobic residues in the protein must be shielded from the aqueous solution, either by the protein itself or by the membrane. In the case of SLC6 transporters, the protein is exposed to the ER lumen, the ER membrane and the cytosolic environment. The folding process of complex proteins such as MATs, which are comprised of twelve TM domains, follows a non-linear progression. Native folding of some parts of the protein may thrust other parts into higher energy states, resulting in partially-folded states, which may stall at different states of the folding trajectory and require chaperones to attain the native, correctly-folded, conformational state (Sucic et al., 2016). The lipid environment of the membrane represents a particular challenge: this can be gauged from Fig. 1, which highlights the position of DAT mutations causing misfolding and give rise to infantile parkinsonism/dystonia: the vast majority of the mutations are situated at the protein-lipid interface. In the absence of guided chaperone assistance, the folding-intermediates form amorphous aggregates and/or eventually undergo ERAD-degradation (Fig. 3).

Calnexin acts as one of the most important folding sensors in the ER lumen (Hebert et al., 1995). Release of the folding-intermediate from calnexin allows for cleavage of the third glucose by α-glucosidase II and marks the protein for anterograde trafficking. If a stable minimum energy is not reached, UDP-glucose:glycoprotein glucosyltransferase re-glycosylates the protein and thus drives its re-association with calnexin or calreticulin (Hebert et al., 1995). If the folded-state is not reached, the protein becomes destined for ERAD degradation. Calnexin and calreticulin share the glycan-binding lectin domain; however, calnexin also has a transmembrane domain, which is *per se* capable of engaging client proteins: interestingly, DAT is very prone to association with calnexin, and substantially more so than the closely related SERT (Korkhov et al., 2008). This may also explain, why infantile/juvenile dystonia, which results from mutations in DAT (see below), is transmitted recessively (Chiba et al., 2014). Folding of SLC6 transporters must also be monitored on the cytosolic side. In fact, SERT carries a HSP70-binding motif RLIIT on its cytosolic C-terminus; HSP70-1A recruitment is observed specifically in the ER and acts as part of a heat-shock protein/HSP-relay (El-Kasaby et al., 2014). Folding-deficient SERT and DAT mutants are stalled in complex with HSP70-1A (and other components of the HSP-relay). The C-terminal domains of MATs all harbor an ER-export motif responsible for recruiting their cognate SEC24 isoforms and their ER-export by COPII-vesicles (Sucic et al., 2011, 2013; El-Kasaby et al., 2010). HSP-release licenses these ER-export motifs for SEC24 binding: if the protein is incorrectly folded, the continuous association with the components of the HSP-relay sterically precludes COPII-components binding and the subsequent ER-export. Hence, the cytosolic HSP acts as a folding sensor, which prevents the ER-export of incorrectly folded SLC6 proteins.

## 4. Chemical- and pharmacological-chaperone molecules

Folding of membrane proteins can be envisaged as a search in conformational space. The folding of membrane proteins is a complex process involving a finely-tuned sequence of events with stringent quality control mechanisms. A single point mutation can impede this search by imposing an insurmountable block in the folding trajectory, generating non-functional misfolded protein (Fig. 2). Evidently, in this case, endogenous chaperones fail to correct the misfolding.

The action of HSP inhibitors can be rationalized within the framework of chaperone/COPII exchange model (Chiba et al., 2014) and assuming that the stringency of the quality control is set to err on the safe side: proteins are monitored for the stability of their final conformation and – in case of doubt – targeted for ER-degradation rather than licensing them for ER-export. Inhibitors of HSP70 (e.g. pifithrin-μ, Fig. 3) and HSP90 (e.g. radiciol, geldanamycin) relax the stringent ER quality control and thus promote ER-export of mutant SLC6 proteins (El-Kasaby et al., 2014).

The mechanism of action of chemical- and pharmacological-chaperones is poorly understood. Pharmacological chaperones differ from chemical chaperones in substrate specificity. Chemical chaperones, such as DMSO, non-selectively facilitate refolding of many client proteins by buffering free water. In contrast, pharmacological chaperones bind selectively to their cognate target proteins. This binding reaction is thought to lower the energy barrier between conformational states and