Structural Biology and Molecular Biophysics

Structural mechanisms for VMAT2 inhibition by tetrabenazine

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Abstract

The vesicular monoamine transporter 2 (VMAT2) is a proton-dependent antiporter responsible for loading monoamine neurotransmitters into synaptic vesicles. Dysregulation of VMAT2 can lead to several neuropsychiatric disorders including Parkinson's disease and schizophrenia. Furthermore, drugs such as amphetamine and MDMA are known to act on VMAT2, exemplifying its role in the mechanisms of actions for drugs of abuse. Despite VMAT2's importance, there remains a critical lack of mechanistic understanding, largely driven by a lack of structural information. Here we report a 3.1 Å resolution cryo-EM structure of VMAT2 complexed with tetrabenazine (TBZ), a non-competitive inhibitor used in the treatment of Huntington's chorea. We find TBZ interacts with residues in a central binding site, locking VMAT2 in an occluded conformation and providing a mechanistic basis for non-competitive inhibition. We further identify residues critical for cytosolic and lumenal gating, including a cluster of hydrophobic residues which are involved in a lumenal gating strategy. Our structure also highlights three distinct polar networks that may determine VMAT2 conformational dynamics and play a role in proton transduction. The structure elucidates mechanisms of VMAT2 inhibition and transport, providing insights into VMAT2 architecture, function, and the design of small-molecule therapeutics.

eLife assessment

The authors report the cryo-EM structure of human vesicular monoamine transporter 2 (VMAT2) bound to the noncompetitive inhibitor tetrabenazine (in an occluded state). This **important** achievement captures the structure of a major facilitator superfamily (MFS) transporter critical for human neurotransmission. The evidence for the structure is **solid**, but the molecular dynamics aspect of the study is **incomplete**.

Introduction

Neuronal signaling by monoaminergic neurotransmitters controls all aspects of human autonomic functions and behavior, and dysregulation of this leads to many neuropsychiatric diseases. Nearly 60 years ago[1,2], secretory vesicles prepared from adrenal glands were shown to contain an activity that accumulated epinephrine, norepinephrine, and serotonin in an ATP-dependent

manner[3]. Extensive characterization by many different laboratories of synaptic vesicles (SVs) in neurons showed that monoamine transport activity was also dependent on the proton gradient generated by the V-ATPase, exchanging two protons for one cationic monoamine[4–8]. Monoamine transport was shown to be inhibited by non-competitive inhibitors such as tetrabenazine (TBZ)[9] and competitive inhibitors like reserpine which has been used to treat hypertension[3]. Amphetamines were shown to be monoamine transporter substrates, eventually leading to vesicle deacidification, disruption of dopamine (DA) packaging in the SV, and DA release into the synapse[10]. Cloning of the vesicular monoamine transporter (VMAT) in the 1990's[11–13] revealed two different genes, VMAT1 and VMAT2, that were expressed in the adrenal medulla and brain, respectively[12,14]. VMAT2 is expressed in all monoaminergic neurons in the brain, including those for serotonin, norepinephrine, DA, epinephrine, and histamine[3], and is essential for loading these neurotransmitters into SVs. VMAT2 is fundamentally required for neurotransmitter recycling and release[15–17] and changes in VMAT1 and VMAT2 activities either through small-molecule agents or mutations are thought to contribute to many human neuropsychiatric disorders including infantile-onset Parkinson's, schizophrenia, alcoholism, autism, and bipolar depression[18–24].

VMAT1 and 2 are members of the solute carrier 18 (SLC18) family and are also known as SLC18A1 and SLC18A2. The SLC18 subfamily also includes the vesicular transporters for acetylcholine[25] (VAChT, SLC18A3) and polyamines[26] (VPAT, SLC18B1). Sequence alignments also show that SLC18 transporters belong to the major facilitator superfamily (MFS) of membrane transport proteins which use an alternating access mechanism[27,28] to transport substrate across membranes. SLC18 members are predicted to be comprised of 12 transmembrane (TM) spanning helices (TM1-12), which are arranged in two pseudosymmetric halves each with 6 TM helices containing a primary binding site for neurotransmitters, polyamines, and inhibitors located approximately halfway across the membrane[29] (**Fig. 1a**). Conformational changes driven by the proton electrochemical gradient are thought to alternatively expose the binding site to either side of the membrane allowing for transport of neurotransmitter from the cytoplasm to the lumen of SVs[30–32].

VMAT2 and VMAT1 share 62% sequence identity but have distinct substrate specificity and pharmacological properties[33]. Small-molecule ligands such as TBZ and reserpine are high-affinity inhibitors of VMAT2, which prevent neurotransmitters from binding, arrest VMAT2 from cycling, and consequently reduce neuronal signaling. VMAT2 exhibits higher affinity for TBZ, monoamines and amphetamines, whereas reserpine binds equally to both VMAT2 and VMAT1[9]. TBZ is the only drug which is approved for treatment of chorea associated with Huntington's disease and has shown to be effective in various other hyperkinetic conditions such as tardive dyskinesia, dystonia, tics, and Tourette's syndrome[34]. A proposed mechanism for TBZ inhibition of VMAT2 involves two sequential steps, initial low-affinity binding of TBZ to the lumenal-open state of VMAT2 which produces a conformational change, resulting in a high-affinity dead-end TBZ-bound occluded complex[30,35–37].

Here we report a structure of VMAT2 bound to TBZ at 3.1 Å resolution in an occluded conformation using single-particle cryo-electron microscopy (cryo-EM), describing the architecture of VMAT2, identifying the high-affinity TBZ binding site, and revealing the mechanisms of drug and neurotransmitter binding, inhibition, and transport.

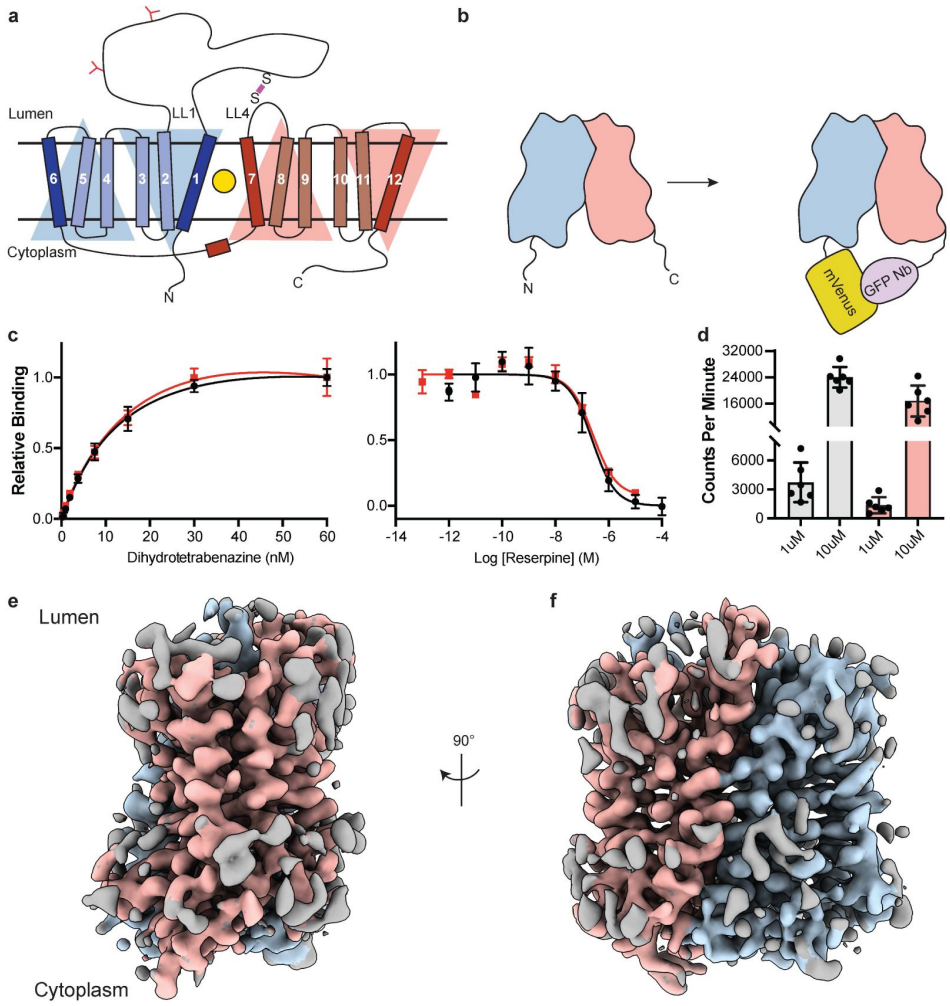

Figure 1.

Cryo-EM reconstruction and functional characterization of VMAT2-tetrabenazine complex.

a, Predicted structural elements of VMAT2. The neurotransmitter substrate is bound at the central site (yellow, circle) between the two repeats comprised of TM1-6 and 7–12. The red and blue triangles depict the pseudo two-fold symmetric repeats. A disulfide bond (purple line) is predicted between lumenal loops LL1 and LL4. The N-linked glycosylation sites in LL1 are shown as red 'Y' shapes. **b,** Intrinsic fiducial strategy involves attachment of mVenus and GFP-Nb to the N- and C-terminus of VMAT2. **c,** Left panel, plots of [^{3}H]-DTBZ saturation binding to wild type VMAT2 (black, circles) and chimera (red, squares). Symbols show the mean derived from n=3 technical replicates. Error bars show the s.e.m. Right panel, graphs of competition binding of ^{3}H-DTBZ with unlabeled reserpine, error bars show the s.e.m. **d,** Plots of transport into vesicles using 1 and 10 μM ^{3}H-serotonin for wild type VMAT2 (grey bars) and chimera (red bars). The bars show the means and points show the value for each technical replicate. Error bars show the s.e.m. **e, f** Occluded map of VMAT2-tetrabenazine complex (3.1 Å resolution, contour level 0.336). The mVenus and GFP-Nb fiducial is not shown for clarity.

Results

Cryo-EM imaging of human VMAT2

Since VMAT2 is a small monomeric membrane protein of approximately 55 kDa, cryo-EM structure determination is challenging. To overcome this, we incorporated mVenus and the anti-GFP nanobody into the N- and C-terminus respectively of human VMAT2 to provide mass and molecular features to facilitate the cryo-EM reconstruction (**Fig. 1b**), this created a hook-like fiducial feature by reconstituting the interaction of these proteins on the cytosolic side of VMAT2[38]. Attachment of both proteins to the termini proved to be ineffective as the unstructured N- and C-terminus of VMAT2 are flexible; to combat this problem, we determined the minimal termini length that would reduce flexibility while maintaining VMAT2 folding. After successive optimizations, our final construct contained mVenus fused to the N-termini at position 17, and the anti-GFP nanobody at position 482 which we denote the VMAT2 chimera (Figure Supplement 1a-e). We investigated the consequences of VMAT2 modification to ensure the chimera maintained functional activity. First, we performed binding experiments with ^{3}H-labeled dihydrotetrabenazine (DTBZ) and found the chimera bound DTBZ with a similar affinity (K_d = 26 ± 9 nM) to the wild type control (K_d = 18 ± 4 nM) (**Fig. 1c**). Competition binding of labeled DTBZ with unlabeled reserpine which stabilizes cytoplasmic-open[39], a state which is incompatible with TBZ binding and produced a K_i of 173 ± 1 nM for reserpine, which was like wild type (161 ± 1 nM). Next, we performed transport experiments using permeabilized cells, initial time course experiments using ^{3}H-labeled serotonin showed clear accumulation (Figure Supplement 1f), and steady-state experiments using 1 and 10 μM serotonin measured within the linear uptake range showed similar transport activity as wild type VMAT2 (**Fig. 1d**). Thus, the functional properties of the chimera are comparable to wild type VMAT2.

To understand the architecture, locate the drug binding site, and assess how TBZ binding influences the conformation of the transporter, we studied the VMAT2 chimera using single-particle cryo-EM (**Fig. 1e,f**, Figure Supplement 2). The resulting cryo-EM map was determined to a resolution of 3.1 Å, the densities of the TM helices were well-defined, continuous, and exhibited density features for TBZ in the primary binding site and most of the side chains (**Table 1**, Figure Supplement 3). This demonstrates the feasibility of our approach and enabled us to build a model of VMAT2.

Architecture of VMAT2

The TBZ bound VMAT2 complex adopts an occluded conformation, with TBZ in a binding site between the central transmembrane helices. The twelve TM helices of the transmembrane domain (TMD) of VMAT2 are arranged in a tight bundle with TM1-6 and TM7-12 each organized into a pseudosymmetrical half (**Fig. 2a**). The cytosolic facing side of VMAT2 is characterized primarily by the unstructured N- and C-termini along with a 20-residue loop that connects the two halves, extending from TM6 to TM7 before terminating in a short α-helix that runs parallel to the bilayer and connects to TM7 with a short linker. TM5 and 11 both contain proline residues near the lumenal face, which break the helical structure and facilitate connections to TM6 and 12 respectively. TM9 and 12 exhibit significant heterogeneity in our cryo-EM reconstructions; we speculate that this is likely due to a dynamic nature intrinsic to the TMs, an aspect that may offer a glimpse into VMAT2 dynamics.

VMAT1 and 2 encode a large lumenal loop (LL) 1 which contains several N-linked glycosylation sites[40] and a disulfide bridge between LL1 and LL4[41]. LL1 and 4 also contain intriguing elements of structure: LL1 extends into the lumenal space in an unstructured loop which is mostly not resolved in our structure, before terminating in a short helix which interacts with the lumenal face of the transporter near TM7, 11 and 12; LL4 extends outward from TM7 into the lumen before connecting back to TM8. A striking feature of LL4 is the location of W318 which positions its indole

	VMAT2-TBZ (EMDB-41269) (PDB 8THR)
Data collection and processing	
Magnification	77,279
Voltage (kV)	300
Electron exposure (e–/Å^2)	60
Defocus range (μm)	-1.0 – -2.5
Pixel size (Å)	0.647
Symmetry imposed	C1
Initial particle images (no.)	~10,000,000
Final particle images (no.)	65516
Map resolution (Å) FSC threshold	3.12 0.143
Map resolution range (Å)*	6.6 – 2.8
Refinement	
Initial model used (PDB code)	
Model resolution (Å) FSC threshold	3.7 0.5
Model resolution range (Å)	20.4 – 3.7
Map sharpening *B* factor (Å^2)	
Model composition Non-hydrogen atoms Protein residues Ligands	 2937 389 1
B factors (Å^2)	

Table 1.

Cryo-EM data collection, refinement and validation statistics.

Protein	28.99
Ligand	55.22
R.m.s. deviations	
Bond lengths (Å)	0.0004 (0)
Bond angles (°)	0.37 (15)
Validation	
MolProbity score	1.23
Clashscore	4.34
Poor rotamers (%)	0.96
Ramachandran plot	
Favored (%)	98.0
Allowed (%)	2.00
Disallowed (%)	0.00

*Local resolution range at 0.5 FSC.

Table 1. (continued)

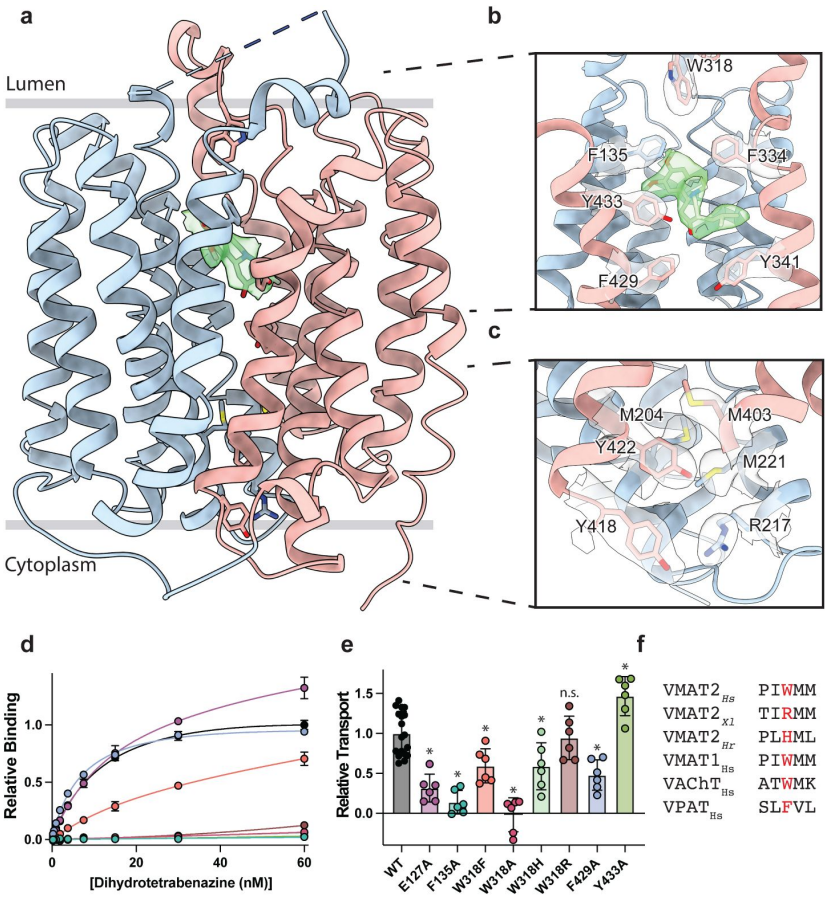

Figure 2.

VMAT2 conformation and residues involved in gating.

a, Overall view of the VMAT2-tetrabenazine (TBZ) complex. TBZ is shown in light green sticks with its map density in transparent surface. **b,** Closeup view of lumenal gating residues and TBZ, shown in stick representation together with transparent surface representation of their map density. **c,** Cytosolic gating residues, same representation as in **b**. **d,** Binding of DTBZ to various VMAT2 mutants in the lumenal and cytosolic gates including wild type (black), W318R (brown), W318H (light green), E127A (purple), W318F (orange), Y433A (forest green), F429A (blue), W318A (red) and F135A (teal). Data were normalized to wild type with error bars denoting standard deviation. **e**, Serotonin transport activity of lumenal and cytosolic gating residue mutants. Symbols show the mean derived from n=6 technical replicates with an identical color scheme to **d**. Asterisks denote statistical significance from wild type, with no significance being denoted with n.s. Data were normalized to wild type transport. Statistics were calculated in Graphpad Prism using a one-way ANOVA with Dunnett's multiple comparison test. Error bars show the standard deviation. **f,** Alignment of five sequential residues of human VMAT2 (two residues on either side of W318) against their counterparts in *Xenopus laevis* (*Xl*), *Helobdella robusta* (*Hr*) and VMAT1, VAChT, and VPAT from humans. The residues which align with W318 in VMAT2 are shown in red.

side chain directly into a lumenal cavity near the TBZ site, acting as a plug to completely occlude the lumenal side of the transporter (**Fig. 2b**). Together, these loops cinch the lumenal side of the transporter closed, locking VMAT2 in an occluded conformation and preventing ligand egress. The conserved nature of LL4 and W318 suggests this motif is necessary for transport function and is a key player in the transport mechanism (Figure Supplement 1b,g, Figure Supplement 4). The conformation of LL1 and 4 is likely also aided by a disulfide bond between cysteines 117 and 324, which is known to be necessary for transporter function[41], our structure was not able to unequivocally place this bond due to the lack of density for residues 48-118 of LL1.

Cytosolic and lumenal gates

The structure of the VMAT2-TBZ complex reveals that both the cytosolic and lumenal gates are closed, which precludes solvent and ligand access from either the cytosolic or lumenal compartments (**Fig. 2a-c**). Previous studies have suggested residues R217, M221, Y418 and Y422 make up the cytosolic gate[32]. We find R217 and Y418 form the outer cytosolic gate with the guanidino group of R217 involved in a cation-π interaction with the aromatic benzyl group of Y418 which seals off cytosolic access to the binding site (**Fig. 2c**). M221 and Y422 form a second set of cytosolic gating residues 'above' the outer cytoplasmic gate through a stable methionine-aromatic interaction which acts to fully seal the cytoplasmic gate (**Fig. 2c**). It is likely that M204 and M403 also contribute to cytosolic gating in this region as their side chains also act to fill this space. On the lumenal side, F135, F334 and W318, form the lumenal gate where they interact with one another to block access to the binding site. W318 acts as 'cap' with the indole side chain facing into a tightly packed hydrophobic pocket consisting of residues I44, V131, L134, L315, I317, and I381 which completely prevent access on the lumenal side. W318 is highly conserved in the SLC18 family, suggesting that SLC18 transporters share a common mechanism of lumenal-gate closure (Figure Supplement 4). E127 of LL1 may play a role in stabilizing the tryptophan in this conformation, with the carboxyl group of the side chain orienting itself near the indole nitrogen potentially forming a hydrogen bond pair. We found that mutation of this residue to alanine did not significantly reduce TBZ binding relative to wild type (**Fig 2d**).

The inner gate is located just below TBZ and comprises residues Y341, F429, and Y433 (**Fig. 2b**)[32]. When probed for their role in inhibitor binding, we found that alanine mutants of F135, Y433, and W318 all greatly reduced DTBZ binding (**Fig. 2d**). Extensive contacts of TBZ with F135 may function to keep the transporter closed on the lumenal side, which would trap VMAT2 in the occluded conformation. F135 and Y433 form π-stacking interactions with TBZ which coordinate the benzene ring of TBZ. F429A did not reduce DTBZ affinity (K_d = 7.7 ± 0.6 nM) compared to the wild type control (K_d = 15 ± 2 nM), showing that while mutation of this residue compromises the inner cytosolic gate (**Fig. 2b**), it is not directly involved in binding TBZ. Conversely, while W318 in LL4 also does not interact directly with TBZ, W318 is required for stabilizing the occluded conformation, and replacement with alanine prevents TBZ from being trapped inside the transporter by preventing closure of the lumenal gate. Sequence alignment with other members of the SLC18 family reveals broad conservation of W318 except for VAChT which contains a phenylalanine in its place (Figure Supplement 4) and a W318F mutation retained some DTBZ binding (K_d = 24 ± 16 nM) (**Table 2**, **Fig. 2d**). Alignment of VMAT2 sequences from other species also show a high degree of conservation of W318 with some notable exceptions which substitute W318 for a large positively charged residue such as an arginine or histidine; and upon investigation we found these mutants all greatly diminish DTBZ binding (**Fig. 2d**).

To further investigate the functional role of the identified gating residues, we performed serotonin transport experiments. We found that mutation of both E127, and F135 to alanine significantly reduced transport activity (**Fig. 2e**). E127A produced a large reduction in transport, resulting in just 34% activity. Replacing F429 with alanine reduced transport as well, but only to about half the level of wild type (**Fig 2e**). Interestingly, the Y433A mutation appeared to enhance transport, and while critical for TBZ binding, this mutant does not prevent transporter cycling (**Fig. 2d,e**). Mutation of W318 to alanine greatly reduced transport, paralleling the effect observed with DTBZ

Mutant	K_d (nM)
WT	15 ± 3
N34A	ND
N34D	ND
N34Q	ND
N34T	ND
E127A	15 ± 6
F135A	ND
R189A	ND
V232L	ND
E312Q	ND
W318A	ND
W318F	24 ± 16
W318H	ND
W318R	ND
F429A	7.7 ± 0.6
Y433A	ND

Table 2.

Calculated K_d values of DTBZ for various single point mutants.

binding (**Fig. 2d,e**) while a histidine mutant at this position maintained a significant amount of transport activity (**Fig. 2d-f**) and the phenylalanine substitution had about half the activity of wild type. The highest activity of the examined W318 mutants was W318R, which fully recapitulated the transport activity of wild type despite being unable to bind DTBZ (**Fig. 2d, e**).

Polar networks

Upon careful inspection of the model, we were able to identify distinct polar networks that we believe may play a role in proton coordination and subsequent transporter conformational change (**Fig. 3a**). The first and largest of these networks lies between TMs 1, 4 and 11, and consists of residues D33, N34, K138, Q142, R189, Q192, S196, S197, S200 and D426 (**Fig. 3b**)[31]. At the center of this network lies D33[32], which makes critical contacts with the side chains of N34, K138, S196, and Q192. Together, the residues comprise a complex hydrogen bond network linking TM 1, 4 and 11. D426[42] lies further toward the cytosol with the side chain carboxyl group facing the bulk of the network, likely forming a hydrogen bond with the hydroxyl group of S200. In the other TMD half there are two distinct groups of interacting polar residues, which bridge between TM 7, 8 and 10 (**Fig. 3a,c,d**). The second group is a pair of residues found on the lumenal side, between residues E312 and N388 with the amide group of the N388 side chain pointed towards the carboxyl group of E312, which could act to stabilize TBZ in the binding site (**Fig. 3c**). The third group is located toward the cytosolic side and consists of N305, Y341, and D399[42], the latter two of which have previously been speculated to form a hydrogen bond pair[31]. The side chains of these residues are positioned toward one another, with the carboxyl group of D399 forming a hydrogen bond with N305 and likely Y314 (**Fig 3d**).

Tetrabenazine binding site

The resolution of our map allowed us to unambiguously place TBZ in the central binding site (**Fig. 4a,b**). TBZ adopts a pose which is predominantly perpendicular to the direction of the TM helices in the lumenal half of VMAT2 near the location of the lumenal gating residues. The TBZ binding site exhibits an amphipathic environment, comprised of both polar and non-polar residues where the tertiary amine of TBZ orients itself towards the negatively charged surface of the binding site near TMs 7 and 11, and toward E312 (**Fig. 4c**, Figure Supplement 5a). We considered that E312 may play an analogous role to the highly conserved aspartate residue present in neurotransmitter sodium symporters such as the serotonin, DA, and norepinephrine transporters[43] which utilize a negatively charged residue to directly bind to amine groups (**Fig. 4d,e** Figure Supplement 4). We thus performed radiolabeled binding experiments to assess the effect of mutating residues in the TBZ binding site by measuring binding of ^{3}H-labeled DTBZ (**Fig. 4f**, **Table 2**). E312 was previously shown to be necessary for substrate transport and inhibitor binding, so we first selected this residue for mutagenesis to probe its importance in TBZ binding[31,44]. The E312Q mutant did not fully abolish DTBZ binding (Figure Supplement 5b) but did greatly reduce DTBZ affinity. This demonstrates that, while not completely essential, E312 is important for inhibitor binding and likely substrate transport by interacting with the amine of the neurotransmitter (**Fig. 4d,e**). Next, we observed that R189 orients its guanidino group towards the methoxy groups of TBZ likely forming hydrogen-bonding interactions and we found that replacement of R189 with an alanine nearly completely abolished DBTZ binding at all concentrations tested (**Fig. 4f**, Figure Supplement 5b). The high degree of conservation of this residue suggests that it plays an important role in transporter function, and even a conservative substitution to a lysine nearly eliminated DTBZ binding (**Fig 4f**, **Table 2**). K138 has been previously shown to play an important role in both TBZ binding and serotonin transport and the primary amine side chain is positioned toward the TBZ binding site[42] (**Fig. 4d,e**). K138 is positioned between two aspartate residues (D426 and D33) and is part of a hydrogen bond network that has been previously hypothesized[42]. Previous experiments found that mutating K138 to alanine resulted in an approximate 4-fold reduction in TBZ binding affinity[31] but did not extinguish TBZ binding and therefore K138 is likely involved in direct interactions with

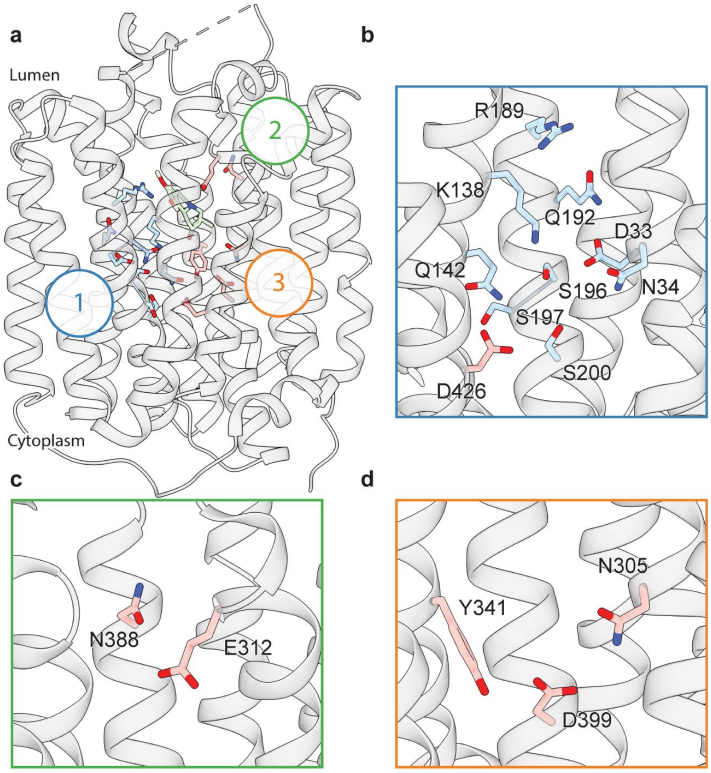

Figure 3.

Polar networks.

a, Overall view showing three distinct polar networks. Polar residues involved in each network and TBZ are shown in sticks. **b,** Cartoon representation showing a zoomed view of polar network 1. **c,** Polar network 2. **d,** Polar network 3.

substrate or inducing conformational changes during proton transport and is not directly involved in TBZ binding. N34 is of particular interest since the amide group of its sidechain appears to form a hydrogen bond with the carbonyl oxygen of TBZ (**Fig 4d,e**) and DTBZ binding was not detectable to N34 mutants of either glutamine, threonine or aspartate while substitution to alanine preserved some binding (**Fig. 4f**, **Table 2**, Figure Supplement 5b).

To investigate the functional role of residues in the binding site, we again performed a series of serotonin transport experiments. We found R189A, E312Q, N34T, N34A and K138A mutations all exhibited reduced transport activity (**Fig. 4g**). R189A and E312Q exhibited the largest change, reducing transport to essentially zero. Substitution of N34 with glutamine had little or no effect on transport activity, opposite of what was noted for DTBZ binding. Replacing N34 with alanine was detrimental, reducing transport to less than half of wild type (**Fig. 4g**). We found N34D and N34T to have opposing effects, with N34D having activity slightly less than wild type and N34T having little to no transport activity at all. R189K greatly affected transport but retained some activity despite lacking measurable DTBZ binding.

Our model of the VMAT2-TBZ complex allowed us to pinpoint two residues which contribute to the specificity of TBZ to VMAT2 over VMAT1. Previous studies have highlighted V232[44], which is a leucine in VMAT1, as being putatively involved in conferring differences in affinity, and our model shows that V232 is positioned closely to the isobutyl of TBZ which is wedged into a small hydrophobic pocket (Figure Supplement 5c). The addition of an extra carbon of the leucine sidechain would produce a steric clash and limit the ability of TBZ to bind (**Fig. 4e**). The V232L mutant in VMAT2 reduces the affinity of DTBZ to VMAT2, confirming its importance in specificity, but the V232L mutant did not show a complete loss in binding (**Fig. 4f**, Figure Supplement 5b). Therefore, we carefully inspected the binding site of VMAT2 and compared it to the predicted structure of VMAT1 to find additional differences in the binding site (Figure Supplement 4), we found that L37 in VMAT2 is a phenylalanine in VMAT1 (Figure Supplement 5c). Given its proximity to TBZ, this substitution would produce a steric clash with the benzene ring (Figure Supplement 5a,c). We found that the L37F mutant resulted in nearly no detectable binding of DTBZ at 60 nM concentration (Figure Supplement 5b). Thus, the combination of these two substitutions likely constitutes the differences in TBZ affinity of VMAT2 *vs.* VMAT1. Interestingly, V232L and L37F both retained some transport activity with L37F producing a more significant reduction in activity (**Fig 4g**).

Our docking simulations suggested that TBZ may sample two different binding poses by small reorientation and movements within the same binding pocket (Figure Supplement 6), both exhibiting similar binding affinities (−9.5 ± 0.2 kcal/mol); the first pose is almost identical to that resolved in our cryo-EM structure (0.4 Å RMSD, Figure Supplement 6e); and the second (3 Å RMSD in TBZ heavy atom coordinates) shows a reorientation of the TBZ methoxy groups toward C430, a residue previously identified to play an important role in binding TBZ[45] (Figure Supplement 6f). These two poses were also observed in molecular dynamics (MD) simulations as illustrated in Figure Supplement 6.a-d and Movie Supplement 1-4. The second pose provides insights into the adaptability of TBZ to the conformational dynamics of VMAT2, while it preferentially positions itself into the pocket resolved in our cryo-EM map. TBZ is thought to enter from the lumenal side of VMAT2 by binding to the lumenal-open conformation[37]. It may interact first with C430 and the other coordinating residues at this pose before R189 moves between the two methoxy groups and allows TBZ to settle into the resolved orientation (Figure Supplement 6f). This result highlights the stepwise events that inhibitors like TBZ may undergo to stably bind to their targets.

The binding stability of TBZ is also influenced by its protonation state. When TBZ is protonated (TBZ^+), it induces the diffusion of 3-4 times more water molecules within the TBZ binding pocket compared to neutral TBZ (**Table 3**, Figure Supplement 7). This flux of water results in the dissociation of TBZ from its binding site as illustrated in Movie Supplement 5 and 6. Several titratable residues, including D33, E312, D399, D426, K138, and R189, line the central cavity of

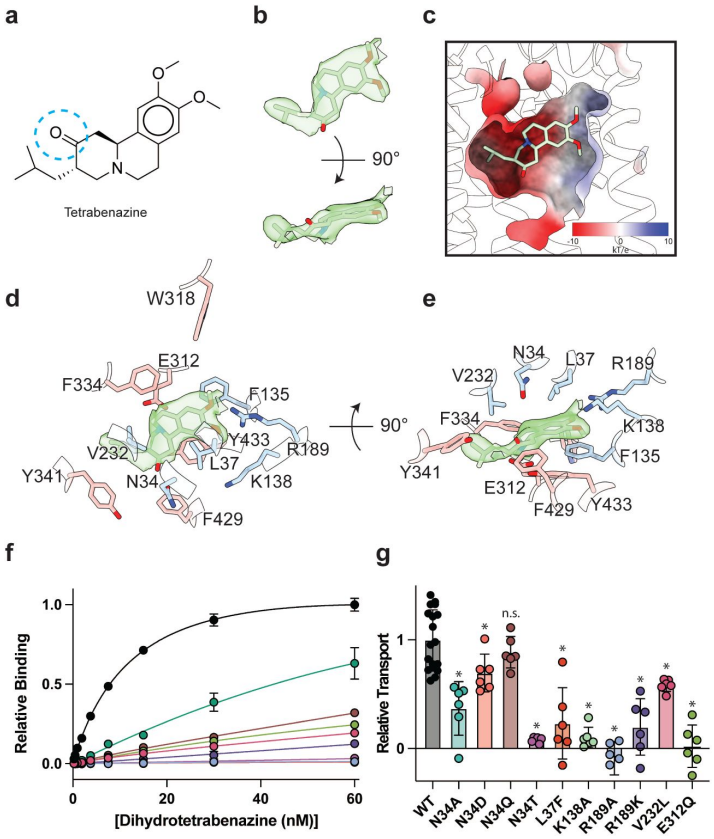

Figure 4.

Tetrabenazine recognition and binding.

a, Chemical structure of tetrabenazine (TBZ). The blue dotted circle indicates the position of the hydroxyl group in DTBZ. **b,** Density associated with TBZ is shown in green transparent surface sharpened with a B-factor of −50 Å^2. TBZ is shown in sticks fit to the density. **c,** Electrostatic potential of the TBZ binding site. **d, e** Binding site of TBZ, residues which are involved in binding are shown in sticks. TBZ is shown in light green sticks and the associated density. **f,** Plots of ^{3}H-DTBZ saturation binding to wild type (black), V232L (pink), R189A (blue), E312Q (forest green), N34D (orange), N34T (light purple), N34A (teal), N34Q (brown) R189K (purple) L37F (red) and K138A (light green). **g,** Serotonin transport activity of mutants in TBZ binding site. Symbols show the mean derived from n=6 technical replicates with an identical color scheme to f. Asterisks denote statistical significance from wild type, with no significance being denoted with n.s. Statistics were calculated in Graphpad Prism using a one-way ANOVA with Dunnett's multiple comparison test. Error bars show the s.e.m.

VMAT2 and impact TBZ binding stability (**Table 4**). We found that maintaining an overall neutral charge within the TBZ binding pocket, as observed in system TBZ_1, most effectively preserves the TBZ-bound occluded state of VMAT2. Residues R189 and E312 in particular are within close proximity of TBZ and participate directly in binding.

Neurotransmitter release

To examine the binding propensity of DA to VMAT2 in the occluded conformation, we constructed five simulation systems with varying protonation states for the four acidic residues (D33, E312, D399, and D426) that line the binding pocket (**Table 5**). For all five systems, DA carried a +1 charge and was initially placed with a pose predicted by docking simulations to be the most favorable binding pose (Figure Supplement 8a,b); and for each system, two MD runs of 100 ns were performed, except for the case where all acidic residues were in a protonated state of which one of the runs was extended to 200 ns to visualize the release of DA to the vesicular lumen. The simulations revealed alterations in DA's binding properties depending on the protonation states of the four acidic residues (Figure Supplement 8c, **Table 5**). Two notable differences were observed when comparing DA to TBZ binding (system DA_2 *vs.* TBZ_1). First, upon binding TBZ, R189 orients its guanidino group toward the methoxy groups of TBZ and forms hydrogen bonds in both the cryo-EM structure and MD simulations; in the case of DA, hydrogen bond formation of the hydroxyl groups of DA was primarily facilitated by K138, D33 or D426, or N34, rather than R189. This resulted in the exposure of R189, triggering a continuous water pathway near the hydrophobic gate residue F135 (**Fig. 5a**). This water path recurred in multiple runs conducted with DA and was lined by the hydrophilic network composed of Y182, R189, Q192, S137, K138, D33, N34, Q142, N146, S200, and D426 (Figure Supplement 8d).

Second, an additional water wire near the hydrophobic gate residue F334 was observed in DA-bound VMAT2 (**Fig. 5a**), but not in TBZ-bound VMAT2 which, in contrast, was minimally hydrated (**Fig. 5b**). The second water wire was lined by the backbone polar groups of hydrophobic residues, e.g., F334, together with a hydrophilic network containing K379, N388, S338, E312, Y433, Y341, N305, and D399. We also observed that the amine group of DA acted to facilitate the water influx from the lumenal side.

We hypothesize that those two water paths may be related to proton transfer and be associated with protonating the pocket-lining acidic residues. Likely, lumenal DA release depends on the number of protonated acidic residues (Figure Supplement 8d-h). When at least two acidic residues were protonated, we observed fluctuations in DA position (run DA_2; Movie Supplement 7) and dislocation of DA from its pocket (run DA_3; Movie Supplement 8). In system DA_4 (**Table 5**), the protonation of E312, D399, D426, and D33 resulted in complete opening of the hydrophobic gates formed by F135, W318, and F334, which led to the release of DA to the vesicle lumen (**Fig. 5c**, Movie Supplement 9). DA was observed to migrate toward a cluster of acidic residues, including E127, E120, D121, and D123, before complete dissociation, and the acidic environment within the vesicle lumen should assist in promoting the release of DA.

Discussion

The VMAT2 – TBZ complex captures the transporter in a fully occluded state with the ligand binding site centrally located between the two repeated substructures TM1-6 and TM7–12. VMAT2 functions by alternating access which involves alternate exposure of the primary binding site to either side of the membrane and isomerization between a cytosolic-open and lumenal-open state in a mechanism known as the rocker-switch (**Fig. 6a,b**)[3,29,46]. Studies have proposed that TBZ first enters VMAT2 from the lumenal side, binding to a lumenal-open conformation[35]. TBZ makes extensive contact with residues in the primary site, likely in a lower affinity state as the

System id	Protonated residues	Bound ligand	Duration (ns)	Waters in binding pocket[a]	RMSD in VMAT2 C^{α}-atoms (Å)[b]	RMSD in TBZ heavy atoms (Å)[c]
TBZ_1	E312 and D399	TBZ	3 x 180	2.8 ± 1.3	2.4 ± 0.3	2.7 ± 0.5
TBZ_2		TBZ^+	2 x 100	8.1 ± 1.4	2.4 ± 0.4	5.9 ± 1.4
TBZ_3	E312, D399, and D426	TBZ	3 x 100	4.6 ± 1.4	2.7 ± 0.5	3.5 ± 1.3
TBZ_4		TBZ^+	2 x 100	14.3 ± 2.4	2.3 ± 0.2	3.7 ± 0.4

[a] number of water molecules within 3.5 Å of TBZ (or TBZ^+) averaged over multiple MD snapshots; [b]RMSDs of VMAT2 C^{α} atoms from their initial (cryo-EM resolved) positions; [c]RMSD of the heavy atoms of TBZ from their cryo-EM resolved positions. All averages and standard deviations were calculated between 50 to 100 ns portion of the MD trajectories.

Table 3.

MD simulation systems of VMAT2 in the presence of TBZ, their properties and simulation durations.

Residue	pKa with TBZ	pKa without TBZ	Model pKa
D33	5.48	5.43	3.8
D121	4.46	4.46	3.8
D123	4.75	4.75	3.8
D213	3.23	3.23	3.8
D214	4.95	4.95	3.8
D262	6.44	6.44	3.8
D291	4.44	4.44	3.8
D399	7.64	7.56	3.8
D411	2.74	2.74	3.8
D426	6.38	6.35	3.8
D460	8.19	8.07	3.8
E120	3.06	3.06	4.5
E127	4.77	4.77	4.5
E215	4.27	4.27	4.5
E216	3.91	3.91	4.5
E244	4.52	4.52	4.5
E278	4.22	4.22	4.5
E312	6.71	7.46	4.5
E321	4.53	4.53	4.5
K138	9.86	10.04	10.5
R189	9.10	10.02	12.5
TBZ	8.57		10

The cryo-EM structure (with or without TBZ) was used for pKa calculations using ProPKA 3.5.0. Key residues of interest are written in boldface.

Table 4.

pKa calculations performed by ProPKA 3.5.0.

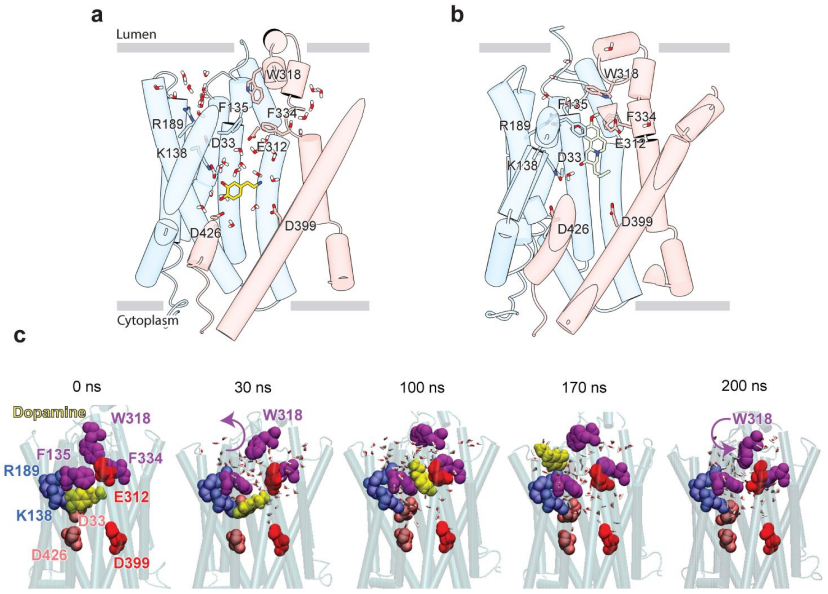

Figure 5.

Release of dopamine from the occluded binding site to the lumen through the lumen-facing vestibule and concurrent conformational changes in VMAT2.

Comparison of **a,** DA (*yellow* sticks) and **b,** TBZ (green sticks) binding to VMAT2, captured by MD simulations. Two water pathways are observed in the MD simulations of VMAT2 bound to DA (run DA_2 in **Table 5**). Water molecules and key residues are shown in sticks. **c,** Positions of dopamine (DA) (*yellow* van der Waals (VDW) spheres) with respect to hydrophobic gate composed of F135, W318, and F334 (*purple* VDW spheres), and charged residues lining the binding pocket at t = 0 ns, 30 ns, 100 ns, 170 ns, and 200ns. W318 side chain isomerization plays a critical role in mediating the opening/closure of the hydrophobic gate, accompanied by the reorientation of F135 side chain, permitting a flux of water molecules eventually giving rise to the destabilization and release of DA to the SV lumen by translocating through a hydrated channel. Waters within 10 Å radius from the center of mass (COM) of the hydrophobic gate residues are displayed.

System #	Protonated residues	Duration (ns)	Waters in binding pocket[a]	VMAT2 RMSD[b] from Cryo-EM (Å)	Observed events
DA_0	none	2×100	7.7 ± 1.5	2.6 ± 0.2	Salt bridge formation between DA and D399; influx of water into the binding pocket.
DA_1	D399	2×100	10.5 ± 2.2	2.2 ± 0.2	Salt bridges formation between DA and E312; influx of water into the binding pocket.
DA_2	E312, D399	2×100	8.8 ± 2.1	2.2 ± 0.3	Fluctuations in DA binding pose while remaining within the binding pocket. Formation of two water wires.
DA_3	E312, D399, D426	2×100	8.8 ± 2.1	2.5 ± 0.2	Fluctuations in DA binding pose; dislocation of DA from its binding site in one of the two simulations.
DA_4	E312, D399, D426, D33	1×100 1×200	11.7 ± 2.3	2.6 ± 0.3	Fluctuations in DA binding pose; dislocation and release of DA in the 200 ns run

[a]number of water molecules within 3.5 Å of dopamine averaged over MD snapshots recorded in the time interval 50-100 ns; [b]RMSDs in VMAT2 C^{α} atoms positions; all averages and standard deviations refer to the portion 50-100 ns of MD simulations.

Table 5.

MD simulations of VMAT2 in the presence of dopamine and the observed events

transporter subsequently closes to forming the high-affinity occluded state. The lumenal gates lock the transporter into an occluded state, preventing displacement by other ligands and producing a so-called dead-end complex[32,34,35,47] (**Fig. 6a**).

Comparison of the transmembrane domain with more distantly related MFS transporters in other conformational states such as the outward-open VGLUT2[48] and inward-open GLUT4[49] models (1.2 Å RMSD) show that the conformational changes involving TM1, 7, 8 and 11 are likely involved in mediating the transport cycle and alternating access (Figure Supplement 10). Comparison with the Alphafold model shows that while the TMD is largely similar (1.1 Å RMSD overall difference in TMD), Alphafold lacks several key features such as the conformations of the LLs and is unable to predict key details that are critical to ligand binding. Hence, computational docking could not identify the TBZ binding site using the Alphafold-predicted model, alluding to the critical importance of our experimental structural data (and simulations based on that structure) for gaining insights into VMAT2 functional mechanisms.

To enable us to solve the structure of VMAT2, we developed an intrinsic fiducial tool consisting of mVenus and the anti-GFP Nb which provides a feature on the cytosolic side of the transporter. GFP and the GFP-Nb are ubiquitous tools used for cell biology, protein biochemistry and structural biology and here we describe another application of this powerful toolkit. In contrast to traditional antibody-based campaigns which may require multiple rounds of screening and take many months or years to discover a suitable binder, our strategy only required us to make between 15-20 constructs to find a suitable chimera (Figure Supplement 1a-c). There are now many other similar methods such as the fusion of the target protein with BRIL and binding of the anti-BRIL Fab[50] and fusions of maltose-binding protein and DARPin[51]. However, since our strategy directly incorporates fluorescent proteins, this enables FSEC based screening of constructs and monitoring fluorescence throughout purification[52]. One obvious disadvantage of all intrinsic fiducial strategies is that the fusion may distort the protein structure, but monitoring the functional activity and comparison with the wild type protein should mitigate these problems.

Among human mutations in VMAT2 causing an infantile-onset form of parkinsonism[53–55], three mutants, P316A, P237H, and P387L, have been shown to extinguish monoamine transport. We find these residues are in LL4 and the lumenal ends of TM5 and 9 respectively (Figure Supplement 9). LL4 is involved in lumenal gating and the P316A variant would likely disrupt the conformation of the loop. In the case of P237H, a histidine would result in not only an insertion of a positively charged residue into the lumenal membrane interface but also in a reduction of the helical bend and would distort the connection of TM5 with TM6. The P387L variant would also disrupt helical connections and the overall architecture of the helix by insertion of a bulky residue into a small hydrophobic cavity. Therefore, we speculate that these SNPs alter the transporter's ability to sample multiple conformations by suppressing transporter dynamics and perturbing VMAT2 stability and folding. Recently, many additional disease variants have been discovered[56] many of which are also found in the lumenal or cytoplasmic ends of TM helices, LL1, and the N- and C-termini and our structure will provide insight into the functional and structural consequences of these variants.

Large aromatic side chains of the lumenal gating residues compartmentalize the transporter, likely ensuring directional transport of substrate. MD simulations revealed little variation in the pose of the aromatic gating residues comprising the inner gates (Figure Supplement 6d). It is interesting to note that substitution of Y433 with alanine resulted in enhanced transport while reducing DTBZ binding. Reduced inhibitor binding is likely due to disruption of π-stacking interactions, but the mechanism for enhanced transport remains unclear. The lumenal gates showed more movement in MD simulations of DA, likely owing to its residue composition and lack of strong interactions (**Fig. 5**). Despite this observation, we assert that the tight hydrophobic

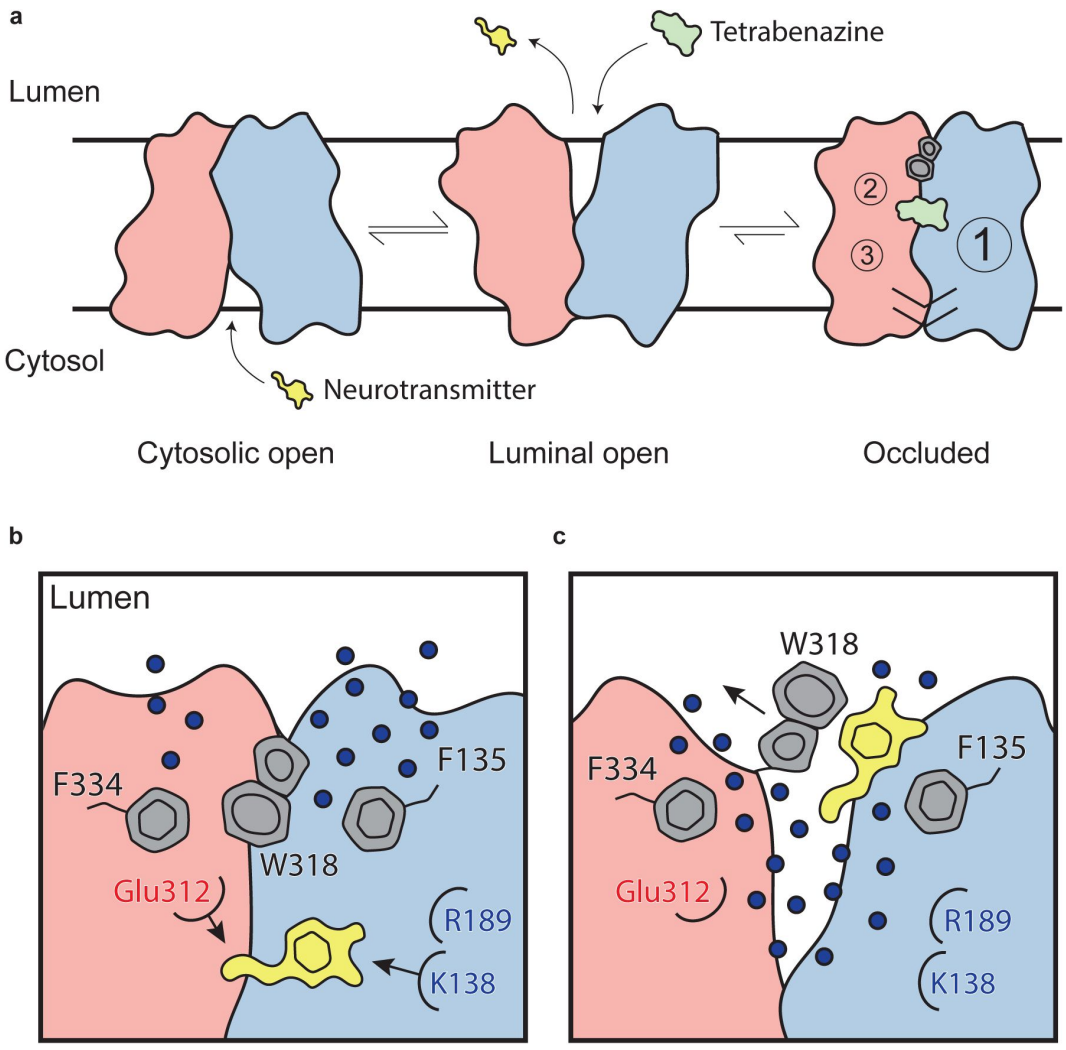

Figure 6.

Mechanism of tetrabenazine inhibition, and the roles of gating residues and polar interactions networks.

a, Cartoon depicting substrate transport and tetrabenazine binding to VMAT2. Neurotransmitter (yellow cartoon) binds to the cytosolic open conformation before being released from the transporter in the lumenal open state. Tetrabenazine (green cartoon) binds to the lumenal-facing state and induces a conformational change to a high-affinity occluded conformation which is the resolved cryo-EM structure reported in this work. The VMAT2 – tetrabenazine complex highlighting significant features including both cytosolic (slashes) and lumenal gates (hexagon and pentagon depicting W318), the three polar networks (numbered circles) and relative location of the tetrabenazine binding site (green). **b,** Water penetrating through two pathways is involved in opening the lumenal gate. Dopamine is shown in yellow cartoon. **c,** Opening of W318 is associated with neurotransmitter release.

environment prevents exchange into the lumenal space. These mechanisms of gating are atypical of MFS transporters which more commonly use salt bridges to gate access to the substrate binding site[46].

Of particular interest among gating residues is W318, which acts as a central plug to prevent solvent access to the transporter. Our mutagenesis analysis suggests that while a large residue is required for maintaining some transporter function, lack of charge also appears to be essential for inhibitor binding. Replacement of W318 with residues present in VMAT2 from other species, except for the phenylalanine mutant, failed to recapitulate any DTBZ binding.

Interestingly, of these substitutions, only an arginine was able to fully recapitulate native transport, suggesting lumenal gating activity requires either a large, charged or a large aromatic residue. Our studies also suggest E127 plays a role in lumenal gating by interacting with LL4 as substitution to alanine reduced transport, and we speculate that large, charged groups at position 318 directly interact with E127 to maintain full transporter function. In contrast, DBTZ binding remained unaffected for the E127A mutant, suggesting a role for this residue in a later step of transporter closure of the lumenal compartment. Positively charged residues at position 318 may stabilize the lumenal gate and promote conformational cycling through enhanced interactions with negatively charged residues such as E127 but may prevent TBZ from entering the lumenal pathway of the transporter in any capacity. Conservation of this residue in the SLC18 family suggests that this is a common mechanism for lumenal gating. It is unclear what other structural adaptations are present to enable W318 replacement with large positively charged residues in other VMAT2 sequences, but we suspect that the overall architecture of the lumenal domain remains similar. We also note that the disulfide bond between LL1 and 4 plays a critical role in transport[40], and the disulfide bond may function to restrict the dynamics of this region to allow W318 to occlude the neurotransmitter binding site during transport.

Residues R189 and K138 stand out as critical for drug binding and inhibition and substrate transport. Simulations highlight the role K138 may play in coordinating with the hydroxy group of neurotransmitters and enabling binding at the central site. These results are consistent with previously observed results demonstrating the critical role K138 plays in substrate transport[31,42]. Remarkably, even mutation to lysine at position 189 abolishes DTBZ binding while still retaining a small amount of transport activity, highlighting the specificity of the DTBZ binding site.

Our structure also provides important clues for understanding the chemical specificity and selectivity of TBZ binding, suggesting that the enhanced affinity of DTBZ is due to preferential interaction with N34. DTBZ is a metabolite of TBZ[47] which differs by only a hydroxyl group *vs.* a double bonded oxygen and binds to VMAT2 with approximately 2-fold higher affinity[56] (**Fig. 4a**). Our structure suggests that the amide of N34 acts as a hydrogen bond donor for TBZ, and in the case of DTBZ the hydroxyl of the ligand may act as a hydrogen bond donor for the carbonyl oxygen of N34. We hypothesize that this interaction is more favorable for DTBZ, leading to a higher binding affinity. Valbenazine, a TBZ analogue with a valine attached to the oxygen of the hydroxyl group of DTBZ, binds VMAT2 with a K_d of 150 nM[58]. We hypothesize that N34 does not form a favorable hydrogen bond with the oxygen of valbenazine and that addition of this larger moiety causes steric clashes in the binding site. Mutation of N34 nearly eliminated DTBZ binding, highlighting its importance in coordinating inhibitor binding. We found N34 is also important for transport, but its precise role remains unclear. Strikingly, replacement with glutamine had no effect on transport whereas aspartate had only a small effect. This suggests that N34 is likely involved in substrate coordination but may not be entirely essential.

This is confirmed with the N34A substitution which still showed significant transport activity. In contrast a threonine substitution was greatly detrimental, but we believe this is likely due to larger active site perturbations.

Comparison of the residues involved in TBZ binding in VMAT2 *vs.* VMAT1 also provides insight into the selectivity of TBZ by demonstrating that key differences in the ligand binding site are likely responsible for the reduction in TBZ binding affinity observed in VMAT1[9]. L37 and V232 are highlighted, and mutation to the equivalent residue in VMAT1, phenylalanine and leucine respectively, eliminated DTBZ binding but only reduced substrate transport. Given these results, we suggest these are the primary residues involved in determining TBZ selectivity for VMAT2 *vs.* VMAT1. We suspect VMAT1 exhibits structural differences compared with VMAT2 to accommodate the larger residues and maintain functional activity.

Through MD simulations, we gained insights into the effects of the protonation states of TBZ and E312. Protonation of TBZ at the amine resulted in an unstable complex, and a structure markedly different from the state resolved with electron microscopy. This suggests TBZ remains unprotonated when bound to the occluded state, as the protonated form will not remain in complex with VMAT2. We believe there may be other TBZ bound states such as the lumenal-open state where TBZ may be protonated. However, in the terminal complex, E312 must remain protonated suggesting that E312 acts as a hydrogen bond donor to stabilize TBZ in the binding site. Our binding and transport studies further confirm this as the mutation of E312 to glutamine greatly reduces serotonin transport and DTBZ binding (**Fig 4f, g**). Moreover, mutation of E312 to an alanine has long been known to completely negate all TBZ binding and transport activities[30,31,44] again highlighting the requirement of hydrogen bond pairing with TBZ.

We also highlight three key polar networks which may be involved in conformational changes induced by proton binding during the transport cycle and are likely also involved in mediating proton transduction (**Fig. 6a**). Taken together, we believe these networks play a critical role in mediating the conformation changes taking place in the transporter upon substrate or inhibitor binding. We hypothesize that the protonation of D33, E312, D399 and D426 would significantly perturb these interactions by breaking crucial hydrogen bond pairs and/or salt bridges, leading to opening of the cytosolic gate. This falls in line with previous work highlighting the role these residues play in transport through a variety of mutagenesis and functional experiments[30,42,44]. Given the known transport stoichiometry of two protons per neurotransmitter, we speculate that two protons may dissociate back into the lumen, perhaps driven by the formation of salt bridges between D33 and K138 or R189 and E312 for example in an cytosol-facing state. The asymmetry between these two networks is also striking, with the first network consisting of TM1, 4 and 11 being substantially larger. This may allow for larger conformational changes on this side and an overall asymmetry in the cytosolic-open state of the transporter. To our knowledge, this is also an atypical feature in MFS proteins[46] and would represent an interesting adaptation upon the rocker-switch mechanism.

Our studies lead us to propose a mechanism by which TBZ inhibits VMAT2 by first entering the transporter from the lumenal open state and subsequently trapping it in an occluded state through a two-step mechanism centered around W318, which acts as a central plug to close off the lumenal compartment. This provides a basis for non-competitive inhibition as endogenous neurotransmitter is unable to compete for binding from the closed off cytosolic compartment (**Fig 6a**). Our results also provide insight into transporter function, and we propose a mechanism for substrate release from the central binding site (**Fig 6b**). The bound substrate coordinates in the binding site predominantly through interactions with E312 and K138. R189 is directed towards the lumenal space where it engages with one of two water channels. Water enters through these channels into the transporter, passing F135 and F334 and inducing conformational change. In our simulations, we find intrusion of water to cause W318 to flip out of the central site which enables neurotransmitter to dissociate from the transporter (**Fig. 6b**).

In summary, we have developed a new fiducial tool incorporating mVenus and the GFP-Nb into the primary sequence of a previously intractable transport protein of great importance to human health. Cryo-EM of VMAT2 allowed us to pinpoint the lumenal and cytoplasmic gates, polar

networks likely involved in conformational change and proton transduction, and the conformation and binding site of inhibitors. Our structure facilitated the discovery and detailed analysis of many residues involved in these key molecular mechanisms and enabled further extension in our understanding of neurotransmitter transport. Thus, by capturing the VMAT2 bound with TBZ along with subsequent mutagenesis, binding, transport, and computational experiments, our work delivers insights into the transporter's mechanism of function and provides a framework for understanding the structural underpinnings of neurotransmitter release, transport, and inhibition in VMAT2 and other related transport proteins in the MFS family.

Endnotes

Acknowledgements

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Author contributions

M.P.D. and J.A.C. designed the project. M.P.D. performed protein purification, cryo-EM analysis, model building, and biochemical analysis. M.P.D., M.H.C., I.B., and J.A.C. wrote the manuscript. M.H.C. and I.B. designed and performed MD simulations. All authors contributed to editing and manuscript preparation.

Author information

The data that support the findings of this study are available from the corresponding author upon request. The coordinates and associated volumes for the cryo-EM reconstruction of the VMAT2-TBZ data set have been deposited in the Protein Data Bank (PDB) and Electron Microscopy Data Bank (EMDB) under the accession codes 8THR and 41269. The half-maps and masks used for refinement have also been deposited in the EMDB. Simulation trajectories are available upon request. The authors declare no competing interests. Correspondence and requests for materials should be addressed to coleman1@pitt.edu

Methods

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Gene (*Homo sapiens*)	Human vesicular monoamine transporter 2	Clone ID 40025175	NCBI Reference Sequence: BC108928	Horizon Discovery
Gene (*Lama glama*)	Anti-GFP nanobody	Plasmid #49172		Addgene
Cell line (*Homo sapiens*)	HEK293S GnTI$^-$	ATCC	Cat # ATCC CRL-3022	Used for expression of VMAT2 (PMID:27929454)
Cell line (*Spodoptera frugiperda*)	Sf9	ATCC	Cat # ATCC CRL-1711	Used in production of baculovirus for transduction (PMID:27929454)
Transfected construct (human)	pEG BacMam	Gouaux lab		PMID:25299155
Affinity chromatography resin	Strep-Tactin Superflow high capacity resin	Iba life sciences	Cat#2-1208-500	Affinity purification resin
Chemical compound, drug	n-dodecyl-β-D-maltoside	Anatrace	Cat # D310	Detergent
Chemical compound, drug	Cholesteryl Hemisuccinate	Anatrace	Cat # CH210	Lipid
Chemical compound, drug	Reserpine	Sigma	Cat # 83580	Inhibitor
Chemical compound, drug	Dihydrotetrabenazine	Cayman Chemicals	Cat # 27182	Inhibitor
Chemical compound, drug	[^{3}H]5-HT	PerkinElmer	Cat # NET1167250UC	Radiolabeled substrate

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Chemical compound, drug	[^{3}H]dihydrotetrabenazine	American Radiolabeled Chemicals	Cat # ART 2119	Radiolabeled inhibitor
Chemical compound, drug	Tetrabenazine	Sigma	Cat # T2952	Inhibitor
Chemical compound, detergent	Digitonin	Sigma	Cat # 300410	Detergent
Software, algorithm	Phenix	PMID:22505256	RRID:SCR_014224	https://www.phenix-online.org/
Software, algorithm	SerialEM	PMID:16182563	RRID:SCR_017293	http://bio3d.colorado.edu/SerialEM
Software, algorithm	cryoSPARC	PMID:28165473	RRID:SCR_016501	https://cryosparc.com/
Software, algorithm	RELION	PMID:23000701	RRID:SCR_016274	http://www2.mrc-lmb.cam.ac.uk/relion
Software, algorithm	UCSF-Chimera	PMID:15264254	RRID:SCR_004097	https://www.cgl.ucsf.edu/chimera/
Software, algorithm	Coot	PMID:15572765	RRID:SCR_014222	https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot
Software, algorithm	MolProbity	PMID:20057044	RRID:SCR_014226	http://molprobity.biochem.duke.edu/
Other	R 2/1 200 mesh Au holey carbon grids	Electron Microscopy Sciences	Cat # Q210AR1	Cryo-EM grids
Other	R 1.2/1.3 200 mesh Cu holey carbon grids	Electron Microscopy Sciences	Cat # Q2100CR1.3	Cryo-EM grids
Other	Copper HIS-Tag YSI	PerkinElmer	Cat # RPNQ0096	SPA beads

Data reporting

No statistical methods were used to predetermine sample size. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

VMAT2 construct design and cloning

Wild type VMAT2 was expressed as a C-terminal eGFP fusion protein containing an 8x His-tag. The VMAT2 chimera consisted of mVenus[59,60] fused to the N-terminus of VMAT2 at amino acid position 17, and the anti-GFP nanobody[38,60] containing both a 10x His-tag and a TwinStrep tag

fused to the C-terminus at position 482 by Infusion cloning. Single point mutants were made using PCR starting from wild type VMAT2 C-terminally tagged eGFP construct, and constructs were initially evaluated using FSEC[52].

Expression and purification

VMAT2 was expressed in HEK293S GnTI⁻ cells[61] using baculovirus mediated transduction[62]. Enriched membranes were first isolated by sonication followed by an initial spin at 10,000g followed by a 100,000g spin and subsequent homogenization. Membranes resuspended in 25 mM Tris pH 8.0 and 150 mM NaCl and frozen at −80°C until use. Membranes were thawed and solubilized in 20 mM n-Dodecyl-β-D-maltoside (DDM) and 2.5 mM cholesteryl hemisuccinate (CHS) with 1 mM DTT and 10 µM TBZ for 1 hr before centrifugation at 100,000g. VMAT2 was purified into buffer containing 1 mM DDM, 0.125 mM CHS, 25 mM Tris, 150 mM NaCl, 1 mM DTT, and 1 µM TBZ pH 8.0 using either a NiNTA column which was eluted in the same buffer containing 250 mM imidazole (for the wild type VMAT2 C-terminally tagged eGFP protein) or a StrepTactin column eluted with 5 mM desthiobiotin. Purified VMAT2 was pooled and concentrated using a 100 kDa concentrator (Amicon) before separating by size-exclusion chromatography on a Superose 6 Increase column in 1 mM DDM, 0.125 mM CHS, 25 mM Tris pH 8.0, 150 mM NaCl, 1 mM DTT, and 1 µM TBZ. Peak fractions were pooled, concentrated to 6 mg/ml with a 100 kDa concentrator before addition of 100 µM TBZ, and ultracentrifuged at 60,000g prior to cryo-EM grid preparation.

Cryo-EM sample preparation and data acquisition

The VMAT2 chimera (1.5 µl) at a concentration of 6 mg/ml was applied to glow discharged Quantifoil holey carbon grids (1.2/1.3 or 2/1 200 mesh copper or gold). Grids were blotted for 4 seconds at 100% humidity, 4°C, with a blot force of 4 using a Vitrobot Mk IV (ThermoFisher) before flash freezing into a 50/50 mixture of liquid propane/ethane cooled to ~170°C with liquid nitrogen. Movies containing 40 frames were recorded on a FEI Titan Krios operating at 300 kV equipped with a Gatan K3 direct electron detector and a Bioquantum energy filter set to a slit width of 20 eV. Images were collected in super-resolution counting mode at a pixel size of 0.647 Å/pixel with defocus ranges from −1 to −2.5 um with a total dose of 60 e/Å^2. Images were recorded using SerialEM[63].

Image processing

Micrographs were corrected using Patch Motion Correction and contrast transfer function estimated using Patch CTF in CryoSPARC v4.2[64]. A total of 24,875 micrographs were collected between two datasets recorded on the same microscope. Particles were initially classified by 2D classification in CryoSPARC to generate an ab-initio model for template picking which resulted in ~10 million picks which were extracted at a box size of 384 binned to 128 and classified multiple times using 2D classification and hetero-refinement using a newly generated ab-initio model, an empty detergent 'decoy' class, and a junk class containing random density. The resulting approximately 500,000 particles from each dataset were re-extracted at with a box size of 384 binned to 256, refined using non-uniform refinement[65], and combined before being subjected to further classification and refinement. The remaining 212K particles were then re-extracted at full box size of 384, refined, and subjected to Bayesian polishing in RELION 3.1[66]. The resulting 3.5 Å map still exhibited significant anisotropy and was subjected to further rounds of 3D classification and refinement in CryoSPARC with a TMD mask to improve features of the peripheral TMs. The final 65,516 particles resulted in a 3.12 A map that was sharpened in DeepEMhancer[67] using the mask setting and utilizing a mask around the transmembrane domain only.

Model building

The resulting EM map was sufficient for modeling most VMAT2 sidechains in the TMD. The Alphafold2[68] model of VMAT2 was used for initial fitting and was further refined using RosettaCM[69]. After successive rounds of RosettaCM, the model was locally fit using Coot 0.98[70].

and Isolde[71] with the majority of the manual rebuilding being done in Isolde. The model was refined in real space using Phenix 1.2[72] and validated by comparing the FSC between the half maps and the refined model.

Radioligand binding assays

Purified VMAT2 (wild type eGFP tagged and VMAT2 chimera) were diluted to 5 nM in 1 mM DDM, 0.125 mM CHS in 20 mM Tris pH 8.0, 150 mM NaCl with 1 mg/ml CuYSi beads (Perkin Elmer). Protein concentration was estimated using FSEC and a standard GFP concentration curve. Protein was then mixed 1:1 to a final protein concentration of 2.5 nM in detergent buffer containing serially diluted ^{3}H-labeled DTBZ (American Radiolabeled Chemicals) starting at 60 nM final concentration. Counts were then measured using a Microbeta2 scintillation counter in 96 well plates with triplicate measurements[73]. Specific counts were obtained by subtracting values obtained by the addition of 100 µM reserpine. Mutants were evaluated similarly from cell lysates of transfected cells. Data were fit to a single-site binding equation using Graphpad Prism.

Competition binding experiments were performed at the same protein concentration in the same detergent buffer. 10 nM ^{3}H-labeled DTBZ was added to buffer and used for nine 1:1 serial dilutions with detergent buffer which initially contained 100 µM reserpine (10 µM for chimera). Measurements were done in triplicates and fit with a one-site competitive binding equation in Graphpad Prism.

Serotonin transport

Cells transduced overnight were spun down and resuspended in 140 mM KCl, 5 mM $MgCl_2$, 50 mM HEPES-KOH pH 7.4 and 0.3 M sucrose. Cells were permeabilized at 30°C for 10 min in the presence of 5 mM MgATP and 0.01% digitonin[32]. Controls additionally included 100 µM reserpine. After 10 min, cells were spun down and resuspended in the same buffer containing 2.5 mM MgATP and incubated at 30°C for 10 min. Cells were then mixed 1:1 with buffer containing ^{3}H-labeled serotonin at a final concentration of 1 or 10 µM and incubated at room temperature for 6 min. Transport was stopped by the addition of ice-cold buffer, and the cells were collected on Glass Fiber C filters. The filters were then counted in scintillation fluid. Time course experiments were performed in the same way using 1 µM serotonin.

To evaluate mutants, ~ 5 million cells were transfected with 5 µg of DNA using Polyjet reagent. 24 hrs after transfection, the cells were harvested into assay buffer containing 130 mM KCl, 5 mM $MgCl_2$, 25 mM HEPES-KOH pH 7.4, 1 mM ascorbic acid, 5 mM glucose and washed once with 1 ml of assay buffer. Cells were permeabilized in 500 ul of assay buffer containing 0.001% digitonin and washed once with 1 ml of assay buffer. Next the cells were resuspended, mixed 1:1 with of 0.2 µM ^{3}H-labeled serotonin with 5 mM ATP in assay buffer, and incubated at room temperature for 15 min. In some cases, 12.5 µM reserpine was added to the cells along with ^{3}H-labeled serotonin for a control. Cells were washed with 2 ml of ice-cold assay buffer and solubilized with 5% Triton-X100 followed by scintillation counting. Mutant expression was assessed with fluorescent microscopy to ensure protein expression levels were comparable.

Molecular dynamics simulations

The initial MD simulation system was prepared using CHARMM-GUI Membrane Builder module[74]. The structure of VMAT2 bound to TBZ was aligned using PPM2.0 webserver[75] and embedded into 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) membrane lipids. The pKa values of titratable residues of VMAT2 in the presence or absence of TBZ were calculated using PROPKA 3.5.0[76]; and the computed pKa values for acidic residues and TBZ are listed in **Table 4**. Of note, D33 and E312, as well as D399 and D426 (yellow highlights in Figure Supplement 8c and bold in **Table 4**) are in proximity to TBZ; thus, their protonation states may impact substantially TBZ binding. Given that D33 may form a salt bridge with its nearby K138, we

assumed it to be deprotonated; and E312 and D399 were assumed to be protonated because of their comparatively higher pKa values. To further assess how the protonation states on TBZ and/or D426 affect the binding propensity of TBZ, we constructed four different simulation systems (see **Table 3**) with either protonated or deprotonated TBZ and with/without protonation of D426 (in addition to E312 and D399), denoted as systems TBZ_1 – TBZ_4. For each system, TIP3P waters and K^+ and Cl^- ions corresponding to 0.1 M KCl solution were added to build a simulation box of 92 ×92 ×108 $Å^3$. The simulated systems contained approximately 86,000 atoms, including VMAT2 and TBZ, 203 POPC molecules, and 17,400 water molecules.

All MD simulations were performed using NAMD[77] (version NAMD_2.13), following default protocol and parameters implemented in CHARMM-GUI[78]. Briefly, CHARMM36 force fields were adopted for VMAT2, lipids, and water molecules[79,80]. Force field parameters for both protonated (carried +1 charge) and neutral-charged TBZ were obtained from the CHARMM General Force Field for drug-like molecules[81]; and proton was added using Open Babel[82]. Prior to productive runs, the simulation systems were energy-minimized for 10,000 steps, followed by 2 ns Nosé–Hoover[83,84] constant pressure (1 bar, 310 K; NPT) simulation during which the constraints on the protein backbone were reduced from 10 to 0 kcal/mol. Finally, the unconstrained protein was subjected to 100-180 ns NPT simulations. Periodic boundary conditions were employed in all simulations, and the particle mesh Ewald (PME) method[85] was used for long-range electrostatic interactions with the pair list distance of 16.0 Å. The simulation time step was set to 2 fs with the covalent hydrogen bonds constrained with the SHAKE algorithm[86]. A force-based switching function was used for Lennard-Jones interactions with switching distance set to 10 Å. Langevin dynamics was applied with a piston period of 50 fs and a piston decay of 25 fs as, well as Langevin temperature coupling with a friction coefficient of 1 ps^{-1}. For each system, 2-3 independent runs of 100-180 ns were performed (see **Table 3**). Snapshots from trajectories were recorded every 100 ps for statistical analysis interactions and structural changes.

For DA bound to the occluded VMAT2, we constructed five simulation systems with varying protonation states for the four pocket-lining acidic residues (denoted as runs DA_0 to DA_4; see **Table 5**). In all systems, the DA carried +1 charge and was initially positioned at the top (lowest energy) docking-predicted binding pose (see Figure Supplement 6a,b); and for each system, two runs of 100 ns were performed, following the same simulation protocols described above for TBZ, except for an extended run of 200 ns conducted to visualize the DA release to the SV lumen, which comprised 170 ns conventional MD simulations, followed by 4 ns of enhanced conformation sampling using the adaptive biasing force method[87,88], and subsequent 30 ns conventional MD simulations.

Docking simulations

The binding of dopamine, serotonin, and TBZ to the AlphaFold2 modeled VMAT2 conformer (AF-Q05940-F1-model_v4) and to the present cryo-EM-resolved structure were simulated using AutoDock Vina[89]. The molecular structures of protonated DA and serotonin were adopted from the previous studies[90,91]. Docking simulations were carried out using a grid with dimensions set to 65 x 65 x 70 $Å^3$ to encapsulate the entire structure of the transporter. The exhaustiveness of the simulation was set to 50, and the algorithm returned a maximum of 20 ligand binding poses.

Computational data analysis

MD trajectory analysis and visualization were performed using VMD[92]. For each snapshot, the TBZ binding affinity was evaluated using contact-based binding affinity predictor PRODIGY-LIG[93]; and the number of waters within the TBZ binding pocket were assessed by counting the number of oxygen (OH2) atoms within 3.5 Å of TBZ. For RMSD calculations, the C^{α} atoms of VMAT2 and the heavy atoms from TBZ were used after alignment of the simulated VAMT2 onto the cryo-EM structure. The binding pockets of VMAT2 were assessed using Essential Site Scanning Analysis

(ESSA)[94] and Fpocket[95], implemented in ProDy 2.0[96]. Sequence conservation of VMAT2 was computed using the ConSurf server with default parameters[97]. Multiple sequence alignment for SLC18 family members was performed using PROMALS3D[98].

Figure supplement legends

Figure Supplement 1. Biochemical characterization, construct design, and sequence conservation of VMAT2. a, The mVenus and GFP-Nb was fused the N- and C-terminus of VMAT2 and the length of the termini are varied to find constructs which can be studied by cryo-EM and retain functional activity. **b,** Sequence and secondary structure prediction. The position of the various transmembrane helices are shown and the position of mVenus and GFP-Nb. **c,** Screening of various constructs by FSEC. **d**, The SEC profile of the 17-481 chimera exhibits a single monodisperse peak. **e,** SDS-PAGE gel showing purified VMAT2 chimera which migrates as a ~75 kDa species. **f**, Time course accumulation of serotonin in vesicles using 1 μM ^{3}H-serotonin for wild type (black trace) and chimera (red trace). **g,** VMAT2 colored by sequence variation from different species, using the Consurf server[1].

Figure Supplement 2. Cryo-EM data processing of the VMAT2-tetrabenazine complex. A representative micrograph (defocus −1.3 μm) is shown (scale bar equals 80 nm). The workflow depicts the data processing scheme used to reconstruct VMAT2. Two datasets were collected comprising 7,742 and 17,133 micrographs respectively. Movies were corrected for drift using patch motion correction in cryosparc[64] and resultant micrographs were used to estimate defocus and pick particles. Blob picking followed by template picking was utilized to select approximately 5 million particles from each dataset. 2D classification was used to sort particles and the sorted particles were subjected to ab-initio reconstructions to obtain initial reference. Next, all of the particles picks from each dataset were subjected to multiple rounds of heterogeneous classification/refinement with the ab-initio VMAT2 map and two 'decoy' classes (yellow, a spherical blob and red, empty detergent micelle) starting with a box size of 128 pixels, followed by subsequent rounds of classification at box size of 256. This resulted in approximately 212k particles after combining both datasets. Particles were then extracted at full box size 384 before Bayesian polishing in Relion[66] and local CTF refinement in Cryosparc[64]. The resulting 3.5 Å map was then subjected to Cryosparc 3D classification using a transmembrane domain (TMD) mask[64]. The final stack of 65k particles was then subjected to local refinement to produce the final unsharpened map. DeepEMhancer was used to locally sharpen the map for interpretation[67].

Figure Supplement 3. Cryo-EM maps and interpretation of VMAT2 reconstruction. a, Cryo-EM density colored by local resolution estimation. **b,** FSC curves for cross-validation, the unmasked map (blue), loose mask (green), tight mask (red) and the reported corrected (purple) curves. The dotted line indicates an FSC value of 0.143. **c,** FSC curves for model versus half map 1 (working, red), half map 2 (free, blue) and model versus final map (black). **d,** Angular distribution of particles used in the final reconstruction. **e,** Cryo-EM density segments of TM1 to TM12.

Figure Supplement 4. Sequence alignment of VMAT1, VMAT2, VAChT, and VPAT. The positions of mutated residues are shown in red boxes. The human variants are shown in blue boxes[97,98].

Figure Supplement 5. Point mutants in tetrabenazine binding site. a, 2D cartoon of the TBZ binding site showing only highlighted residues. Green, red, and blue indicate hydrophobic, negative, or positively charged properties of the side chains[99]a. **b,** Plots of binding of 60 nM of [^{3}H]-DTBZ. The bars show the means and points show the value for each technical replicate. Error bars show the s.e.m. **c,** Binding site showing the positions of L37 and V232 which are a phenylalanine and a leucine in VMAT1 respectively.

Figure Supplement 6. Tetrabenazine docking and molecular dynamics (MD) simulations. a-c, Time evolution of **a,** root-mean-square-deviations (RMSDs) in VMAT2 C^{α} coordinates from those resolved in the cryo-EM structure, **b,** RMSD for TBZ heavy atoms, and **c,** TBZ binding affinities, observed in three different runs conducted for the TBZ_1 system (see **Table 3**). Binding affinities were calculated using PRODIGY-LIG. **d,** The TBZ binding poses and variations of W318 captured in these MD simulations, with a snapshot taken every 4 ns and a total of 50 frames are shown from 0 to 100 ns for each run. The ligand conformations are shown in cyan sticks with blue stick illustrating cryo-EM resolved binding pose. The variations of W318 are displayed in purple sticks with dark purple showing the cryo-EM-resolved orientation. Docking simulations of TBZ onto VMAT2 revealed two poses, shown in the panels **e** and **f**. The former, captured in runs 1 and 3, is almost identical (RMSD of 0.4Å) to the cryo-EM resolved pose, illustrated in panel **e**. The latter, captured in run 2, differs from the cryo-EM pose by 3.0 Å.

Figure Supplement 7. Effects of protonation states of TBZ and selected VMAT2 residues on the time evolution of TBZ (heavy atoms) and VMAT2 (α-carbons) RMSDs from their original (cryo-EM resolved) positions. Results are presented for three different types of simulations (TBZ_2 – TBZ-4; see **Table 3**). In each case, the top panels (**a**, **c**, and **e**) display the RMSDs in the heavy atoms of TBZ and the lower panels (**b**, **d**, and **f**) display the RMSDs in the C^{α}-atoms of VMAT2. Multiple runs are displayed by curves in different colors.

Figure Supplement 8. Comparisons of binding poses of substrate (DA and serotonin) and inhibitor (TBZ) from docking simulations and MD simulations under different protonation states of substrate-coordinating and/or gating residues. a, Docking of dopamine and **b,** serotonin to the cryo-EM-resolved VMAT2. The most energetically favorable poses are shown for both substrates. Residues within 4 Å of the ligand are shown in sticks. Dopamine and serotonin are displayed in violet and cyan as van der Waals (vdW) surfaces. In both cases, the amine group (of either substrate) is in close contact with E312 and the hydroxyl group interacts with R189. **c,** Resolved 3D positions of aspartates and glutamates in VMAT2. **d**, DA binding pose to the cryo-EM resolved VMAT2 conformer (initial). **e - h,** DA binding poses captured by 100 ns MD simulations of system DA_0 **f,** in which none of the four acidic residues D33, E312, D399 and D426 were protonated and DA bound to D399; DA_1 **g,** in which D399 was protonated and DA bound to E312; DA_2 (**i**), in which E312 and D399 were protonated; and DA_3 **h,** in which E312, D399, and D426 were protonated. DA is shown in yellow vdW spheres around 100 ns, along with hydrophobic gate residues F135, W318 and F334 (purple vdW spheres), and charged residues lining the binding pocket. Waters within 10 Å radius from the center of mass (COM) of the hydrophobic gate residues are displayed.

Figure Supplement 9. Human variants of VMAT2. P316A, P237H and P387L localize to LL4 and the lumenal ends of TM5 and TM9, respectively.

Figure Supplement 10. Comparison of the VMAT2-TBZ structure with the predicted Alphafold structure and with other MFS transporters. a, Comparison of the cryo-EM structure (light blue) vs. Alphafold (navy). The position of TM1, 2, 4, 7, 8, 10, and 11 and LL4 in the cryo-EM structure show the most substantial differences and are shown in white for clarity. **b,** Comparison with the outward-open VGLUT2 structure (PDB: 8SBE) shown in navy. **c,** Comparison with the inward-open GLUT4 structure (PDB: 7WSM) shown in navy.

Movie Supplement Legends

Movie Supplement 1 (related to Figure Supplement 6): **Movie_S1.mpg**. Binding and coordination of neutral TBZ (*cyan* van der Waals (vdW) representation observed in a 180 ns MD simulation (*run 1*). Three runs were performed for VMAT2 embedded in a lipid bilayer in the presence of TBZ, where E312 and D399 were protonated. We focus here on the time evolution of TBZ coordination, hence the naming of the runs as TBZ_1 run 1, TBZ_1 run 2, and TBZ_1 run 3. Movie S1 displays

TBZ_1 run 1. For each snapshot, water molecules (in CPK format; ball and stick) within 4 Å of TBZ are shown; and acidic, basic, hydrophilic, and hydrophobic residues within 4 Å of TBZ are displayed in *red, blue, green* and *orange licorice* representations, respectively. The predominant pose adopted by TBZ is closely similar to that resolved in our cryo-EM structure, and illustrated in Figure Supplement 6 panel e. See **Table 3** for the description of the runs.

Movie Supplement 2 (related to Figure Supplement 6): **Movie_S2.mpg**. Binding and coordination of neutral TBZ (*cyan* vdW representation) observed in a 180 ns MD simulation (TBZ_1 run 2), in the same format as Movie Supplement 1. TBZ slightly altered its position within the same binding pocket, to stabilize the pose illustrated in Extended Data Figure 6 panel f.

Movie Supplement 3 (related to Figure Supplement 6): **Movie_S3.mpg**. Binding and coordination of neutral TBZ (*cyan* van Der Waals (vdW) format) observed in a 180 ns MD simulation (TBZ_1 run 3), in the same format as Movie S1. TBZ adopts a pose similar to that resolved in the cryo-EM structure.

Movie Supplement 4 (related to **Table 3**): **Movie_S4.mpg**. Same as Movie Supplement 1-3, with neutral TBZ, but with the additional protonation of D426 (TBZ_3 run1). TBZ adopted the predominant pose similar to that resolved in the cryo-EM structure.

Movie Supplement 5 (related to **Table 3**): **Movie_S5.mpg**. MD simulation of the binding and coordination of protonated TBZ (TBZ^+; *pink* vdW format) to VMAT2 with protonated E312 and D399. This is a 100 ns run, termed TBZ_2 run 1. The same format as Movie Supplement 1-3 is adopted for the coordinating residues. TBZ tends to alter its binding pose to approximate the one observed in Movie Supplement 2.

Movie Supplement 6 (related to **Table 3**): **Movie_S6.mpg**. Same as Movie Supplement 4, except for the protonation of TBZ (TBZ^+ shown in pink vdW representation). This is a 100 ns run, termed TBZ_4 run 1. In the presence of protonation, TBZ preferentially samples the pose observed in Movie Supplement 2 and 5.

Movie Supplement 7 (related to **Table 5**): **Movie_S7.mpg**. Fluctuations in dopamine binding pose (DA; *yellow* vdW spheres) within its binding pocket, observed in a 100 ns MD simulation of VMAT2 in the presence of DA (system DA_2 in **Table 5**). VMAT2 E312 and D399 are protonated; and D33 and D426 are deprotonated. Hydrophobic gate residues F135, W318 and F334 are displayed in *purple* vdW spheres, and acidic and basic residues K138 and R189 are shown in *red* and *blue* vdW representation.

Movie Supplement 8 (related to **Table 5**: **Movie_S8.mpg**. Release of dopamine (DA; *yellow* vdW spheres) from the binding pocket, observed in a 100 ns MD simulation (system DA_3 in **Table 4**). E312, D399 and D426 were protonated and D33 was deprotonated.

Movie Supplement 9 (related to **Figure 5**): **Movie_S9.mpg**. Release of DA to the vesicular lumen (*yellow* VDW spheres), observed after protonating D33, E312, D399 and D426 (*red* vdW spheres). The movie represents a 200 ns MD simulation of system DA_4 in **Table 5**.

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Reviewer #1 (Public Review):

Summary:

This study presents fundamental new insights into vesicular monoamine transport and the binding pose of the clinical drug tetrabenazine (TBZ) to the mammalian VMAT2 transporter. Specifically, this study reports the first structure for the mammalian VMAT (SLC18) family of vesicular monoamine transporters. It provides insights into the mechanism by which this inhibitor traps VMAT2 into a 'dead-end' conformation. The structure also provides some evidence for a novel gating mechanism within VMAT2, which may have wider implications for understanding the mechanism of transport in the wider SLC18 family.

Strengths:

The structure is high quality, and the method used to determine the structure via fusing mVenus and the anti-GFP nanobody to the amino and carboxyl termini is novel. The binding and transport data are convincing and provide new insights into the role of conserved side chains within the SLC18 members. The binding position of TBZ is of high value, given its role in treating Huntington's chorea and for being a 'dead-end' inhibitor for VMAT2.

https://doi.org/10.7554/eLife.91973.2.sa2

Reviewer #2 (Public Review):

As a report of the first structure of VMAT2, indeed the first structure of any vesicular monoamine transporter, this manuscript represents an important milestone in the field of neurotransmitter transport. VMAT2 belongs to a large family (the major facilitator superfamily, MFS) containing transporters from all living species. There is a wealth of information relating to the way that MFS transporters bind substrates, undergo conformational changes to transport them across the membrane and couple these events to the transmembrane movement of ions. VMAT2 couples the movement of protons out of synaptic vesicles to the vesicular uptake of biogenic amines (serotonin, dopamine and norepinephrine) from the cytoplasm. The new structure presented in this manuscript can be expected to contribute to an understanding of this proton/amine antiport process.

The structure contains a molecule of the inhibitor TBZ bound in a central cavity, with no access to either luminal or cytoplasmic compartments. The authors carefully analyze which residues interact with bound TBZ and measure TBZ binding to VMAT2 mutated at some of those residues. These measurements allow well-reasoned conclusions about the differences in inhibitor selectivity between VMAT1 and VMAT2 and differences in affinity between TBZ derivatives.

The structure also reveals polar networks within the protein and hydrophobic residues in positions that may allow them to open and close pathways between the central binding site and the cytoplasm or the vesicle lumen. The authors propose involvement of these networks and hydrophobic residues in coupling of transport to proton translocation and conformational changes. However, these proposals are quite speculative in the absence of supporting structures and experimentation that would test specific mechanistic details.

https://doi.org/10.7554/eLife.91973.2.sa1

Reviewer #3 (Public Review):

Summary:

The vesicular monoamine transporter is a key component in neuronal signaling and is implicated in diseases such as Parkinson's. Understanding of monoamine processing and our ability to target that process therapeutically has been to date provided by structural modeling

and extensive biochemical studies. However, structural data is required to establish these findings more firmly.

Strengths:

Dalton et al resolved a structure of VMAT2 in the presence of an important inhibitor, tetrabenazine, with the protein in detergent micelles, using cryo-EM and with the aid of protein domains fused to its N- and C-terminal ends, including one fluorescent protein that facilitated protein screening and purification. The resolution of the maps allows clear assignment of the amino acids in the core of the protein. The structure is in good agreement with a wealth of experimental and structural prediction data, and provides important insights into the binding site for tetrabenazine and selectivity relative to analogous compounds. The authors provide additional biochemical analyses that further support their findings. The comparison with AlphaFold models is enlightening.

Weaknesses:

The authors follow up their structures with molecular dynamics simulations of the tetrabenazine-bound state, and test several protonation states of acidic residues in the binding pocket, but not all possible combinations; thus, it is not clear the extent to which tetrabenazine rearrangements observed in these simulations are meaningful. Additional simulations of the substrate dopamine docked into this structure were also carried out, although it is unclear whether this "dead-end" occluded state is a relevant state for dopamine binding. The authors report release of dopamine during these simulations, but it is notable that this only occurs when all four acidic binding site residues were protonated and when an enhanced sampling approach was applied.

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Author Response

The authors' responses to the public reviews can be found here

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The following is the authors' response to the most recent recommendations.

> ***Recommendations for the authors:***
>
> ***Reviewer #2 (Recommendations For The Authors):***
>
> *I appreciate the effort that the authors have put into this revised version of the manuscript. Before going into details, I would suggest that, in the future, the authors include enough information in their response to allow reviewers to follow the changes made. Not simply "Fixed", but instead "we have modified the description of these results and now state on lines XXX to XXX (revised text)".*

We greatly apologize, we certainly did not wish to cause more work for the reviewer to find the necessary changes. We will list the line number and our changes in the following response.

> *The authors' response to my comments was confined to the minor points, with no attention to more important questions regarding speculations about mechanism which were (and still are) presented as factual conclusions. I do not consider the responses adequate.*

We responded to each of your comments and where we disagree, we have explained in detail.

> *With respect to the meaning of "above" and "below" in the context of an intracellular organelle, I think that referring to up and down in a figure is fine, provided that the cytoplasmic and luminal sides are indicated in that figure. I think that labeling to that effect in each figure would be immensely helpful for the reader.*

We agree with this point and have updated all the figures to include these labels.

> *The statement on lines 333-335 about non-competitive inhibition is a bit naïve. The only thing ruled out by this type of inhibition is that substrate and TBZ binding do not share the same binding process, in which case they would compete. It doesn't show that TBZ gets to its binding site from the lumen or from the bilayer, or by any other process that isn't shared with substrate. It also doesn't rule out kinetic effects, such as slow inhibitor dissociation, that result in non-competitive kinetics. Please rewrite this sentence to indicate that one explanation of the non-competitive nature of TBZ inhibition would be that TBZ diffuses into the vesicle and binds from the lumen. It's not the only explanation.*

We have changed this sentence lines 334-336 to be more speculative and not include any statement about non-competitive inhibition. Please see, “Studies have proposed that TBZ first enters VMAT2 from the lumenal side, binding to a lumenal-open conformation.”

> *The revised version integrates the MD simulations into a plausible mechanism for luminal release of substrate. A key element in this mechanism is the protonation of D33, E312 and D399, which allows substrate to leave following water entry into the binding site. The acidic interior of synaptic vesicles should facilitate such protonation, but the fate of those protons needs to be considered. Are any of them predicted to dissociate prior to the return to a cytoplasm-facing conformation? If so, are all 3 released in that conformation? Postulating protonation events at one point in the reaction cycle requires some accounting for those protons - or at least recognition of the problem of reconciling their binding with the known stoichiometry of VMAT.*

We completely agree with this point and while we cannot account for all protons with a single structure and simulation of neurotransmitter release, some discussion of the fate of the protons is warranted. We have included a highly speculative statement in the discussion on this point, see lines 462-465, “Given the known transport stoichiometry of two protons per neurotransmitter, we speculate that two protons may dissociate back into the lumen, perhaps driven by the formation of salt bridges between D33 and K138 or R189 and E312 for example in an cytosol-facing state.”

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *On page 13, line 238, the statement "The protonation states of titratable residues D33, E312, D399, D426, K138 and R189, which are in close proximity to TBZ, also impact its binding stability (Table 4)" is misleading. Table 4 only shows that D426 is charged and what the pKa values are. This should be rephrased to separate out which residues are in close proximity from what is known about how their protonation states affect TBZ stability.*

We agree with this statement and have rephrased this on line 290-294 on page 13 to read, “Several titratable residues, including D33, E312, D399, D426, K138, and R189, line the central cavity of VMAT2 and impact TBZ binding stability (Table 4). We found that maintaining an overall neutral charge within the TBZ binding pocket, as observed in system TBZ_1, most effectively preserves the TBZ-bound occluded state of VMAT2. Residues R189 and E312 in particular are within close proximity of TBZ and participate directly in binding.” We note that

given the acidic pH of the vesicle lumen (5.5), it is likely all four residues may be protonated to a significant degree in this state.

> *Typos:*
>
> - *luminal is another name for the drug generically known as phenobarbital, lumenal means in the lumen. (This typo seems to have crept into the published literature now too).*

Thank you for pointing this out. Indeed, we had considered carefully whether to use 'lumenal' or 'luminal' in our revised text. In fact, both are used interchangeably throughout the scientific literature and luminal is the more commonly used term. Please also see: https://www.merriam-webster.com/medical/luminal we do agree that there may be confusion because 'Luminal' is a trademark of phenobarbital. Therefore, we have changed the text to read 'lumenal' throughout.

The following is the authors' response to the original recommendations.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *I congratulate the authors on this study, which I enjoyed reading. Overall, the study reports a novel and exciting new structure for a member of the SLC18 family of vesicular monoamine transporters. Associated MD, binding and transport assays provide support for the hypothesis and firm up the modelled pose for the TBZ drug. The main strengths of the study largely sit with the structure, which, as the authors say, provides additional and essential insights above those available from AF2. The structures also reveal several potentially interesting observations concerning the mechanism of gating and proton-driven transport. The main weakness lies in the limited mutational data and studies into the role of pH in regulating ligand binding. As detailed below, my main comment would be to spend a little extra time expanding the mutational data (perhaps already done during the review?) to enable more evidence-based conclusions to be drawn.*

We thank reviewer #1 for their helpful comments and suggestions. We agree that mutational analysis specifically of neurotransmitter transport would strengthen the mechanistic conclusions of the work. We also agree with reviewer #1 and #3 that the role of pH and the protonation state of charged residues was a weakness in the first version of the manuscript. Therefore, we have expanded our mutational and computational data as detailed below and we believe that this has further solidified our findings.

> *Specific comments & suggestions:*
>
> *It is an interesting strategy to fuse the mVenus and anti-GFP nanobody to the N-/C-termini. The authors should also include in SI Fig. 1 a full model for the features observed in these maps and deposit this in the PDB.*

Great point, we have made a main text panel describing the construct. Figure S1 includes a full description of the construct. The reviewer will note that the PDB entry contains the entire amino acid sequence of the construct and while the GFP and GFP-Nb cannot be well modeled into the density, we have included all of the relevant information for the reader.

> *Difficult to make out the ligand in Fig. 2b, I would suggest changing the color of the carbon atoms.*

Fixed.

> *It is difficult to make out the side chains in ED Fig. 5d.*

This is now its own supplemental figure and is presented larger.

> *ED Figures are called out of order in the manuscript. For example, in line 143 ED Fig.6 is called before ED Fig. 5d (line 152), and then ED 5d is called before ED 5a. This makes it rather confusing to follow the description, analysis, and data when reading the paper. Although there are other examples. I would suggest trying to order the figure callouts to flow with the narrative of the study.*

Agreed. Fixed.

> *It wasn't clear to me what the result was produced by just imaging the ligand-free chimaera protein. It would be useful to say whether this resulted in low-resolution maps and whether the presence of the TBZ compound was essential for high-resolution structure determination.*

The ligand is likely required for structure determination. We have not, however, made such a statement largely because we have yet to determine an apo reconstruction.

> *The role of E127 and W318 on EL1 in gating the luminal side of the transporter is very intriguing. As the authors suggest, this may represent an atypical gating mechanism for the MFS (line 182). I did wonder if the authors had considered providing more insight into this potentially novel mechanism. Additional experiments would be further mutations of W318 to F, Y, V, and I to see if they can identify a non-dead variant that could be analysed kinetically. They may have more luck with variants of E127, as they suggest this stabilises W318. If these side chains are important for gating and transport regulation, one might expect to see interesting effects on the transport kinetics.*

This is a fantastic suggestion. We have done this, and we think that the reviewer will find the results to be quite interesting. Some VMAT2 sequences have an R or an H at position 318 while VPAT has an F at the equivalent position. We have made these mutants including the E127A mutant and analyzed them using TBZ binding and transport experiments. Interestingly the W318R, H, and F mutants preserve activity in varying degrees with the R mutant closely resembling wild type. W318A has no transport activity. Only the W318F mutant retains some TBZ binding. The E127A mutant also has little transport activity but nearly wild type like TBZ binding which we believe suggests a role for this residue also in stabilizing W318.

> *The authors identify an interesting polar network, which is described in detail and shown in Fig. 2d. However, the authors present no experimental data to shed further mechanistic insight into how these side chains contribute to monoamine transport or ligand binding. Additional experiments that would be helpful here might include repeating the binding and competition assays shown in Fig. 1c under different pH conditions for the WT and different mutations of this polar network. At present, this section of the manuscript is very descriptive without providing much novel insight into the mechanism of VMAT transport. I did wonder whether a similar analysis of pH effects on DTBZ binding might also provide insight into the role of E312 and the role of protons in the mechanism.*

Thank you, we have addressed this point in several different ways. The first is that many of these residues have already been characterized in several earlier studies, see refs 31, 32, and 42 and we have incorporated this into our discussion where appropriate. With respect to

E312, the reviewers' comments are again very appropriate. We have addressed this using computational experiments exploring the protonation status of E312 and other residues as well as TBZ. Our simulations and Propka calculations clearly show that E312 must be protonated and TBZ must be deprotonated to maintain TBZ binding. We have also extended these computational studies toward understanding the protonation status of residues which orchestrate dopamine binding and release.

> *The authors then describe the binding pose for TBZ. This section also provides some biochemical characterisation of the binding site, in the form of the binding assay introduced in Fig. 1. However, the insights are again somewhat reduced as the mutants were chosen to show reduced binding. Could the authors return to this assay and try more conservative mutations of the key side chains to illuminate more detail? For example, does an R189K mutant still show binding but not transport? Similarly, what properties does an E312D have? The authors speculate that K138 might play a role in coupling ligand binding/transport to the protonation, possibly through an interaction with D426 and D33 (line 236). Given the presence of D33 in the polar network described previously, I was left wondering how this might occur. I feel that some of the experiments with pH and conservative mutants might shed some light on this important aspect. Please label the data points in Fig. 3d.*

Indeed, alanine mutants at these positions while valuable do not provide the level of detailed insight into mechanism that we also would have liked to obtain. Thus, we have made more conservative and targeted mutants like the R189K mutant and various mutants at N34 for example and tested them in both transport and binding assays. We have also made a mutant at K138 and found that it is not transport competent or able to bind TBZ to a significant degree. With respect to labels and color codes, we have made the color codes consistent between the bar graphs and the curves. We have also labeled the data points in the figure legends.

> *The manuscript currently doesn't present a hypothesis for how TBZ induces the 'dead-end' complex compared to physiological ligands. Does the MD shed any light on this aspect of the study? If the authors place the physiological ligand in the same location as the TBZ and run the simulation for 500ns, what do they observe? 100ns is also a very short time window. I appreciate the comment about N34 in line 303, but is this really the answer? It would be very interesting to provide more evidence on this important aspect of VMAT pharmacology.*

MD with a natural ligand (dopamine) provides substantial insight into why TBZ is a dead-end complex. Since water cannot penetrate into the binding site in the TBZ bound complex, this does not allow for substantial luminal release. In contrast, simulations conducted in the presence of DA bound to the occluded VMAT2 show the propensity of that structure to accommodate an influx of water molecules that promote the release of DA to the lumen. The new results are illustrated in Figure 5 (main text) as well as supplemental figure 8 panels d-h. The new simulations further emphasized the importance of the protonation state of acidic residues near the substrate-binding pocket.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Line 68, "both sides of the membrane" -> "alternately to either side of the membrane".*

Fixed. Thanks.

> *Transmembrane proteins in intracellular organelles present unique issues of nomenclature. I suggest the authors refer to cytoplasmic and luminal faces of the*

> *protein (not intracellular or extracellular (line 124)) and adhere to these names to avoid confusion. This creates problems for loops called IL and EL, but they could be defined on first use.*

We agree with this point and had initially gone with the conventional definitions used in the literature. We have now changed this throughout the text to be luminal and cytosolic.

> *Lines 135-6, are these residue numbers correct? The pdb file lists 126 as Asp and 333 as Ala.*

Thank you. This is fixed.

> *ED Fig. 6 is not clear. A higher-resolution figure is needed.*

We have updated this figure and hope that the reviewer will find it to be much clearer.

> *Lines 158-9, Is there any data to support effects on dynamics or folding? If not, please indicate that this is speculation.*

Fixed.

> *Line 174, Should "I315" be "L315"?*

Fixed.

> *Line 179, Please indicate what is meant by "inner" and "below" (also lines 183 and 258).*

We have added Figure calls here where needed.

> *Line 192, S197 is listed as part of polar network 1, but not discussed further. Is it actually involved, or just in the neighborhood?*

It is part of the network, but we did not discuss in further detail because we do not have data indicating its precise function and thus have left this as a description.

> *Line 199, E312, and N388 are fairly distant from each other. Do you want to clarify why they represent a network?*

While they are not within hydrogen bonding distance, we nevertheless include them as part of the same network because they may come into closer proximity in a different conformational state.

> *Line 206, Protonation of all 3? VMAT2 doesn't transport 3 protons per cycle. Please clarify.*

We believe that these residues may be protonated, but they may not necessarily all be involved in proton transport.

> *Line 219, Do you mean the aspartate unique to DAT, NET, and SERT? This is Gly in all the amino acid transporters in the NSS family. Please be specific.*

Fixed. Thank you.

> *Line 224, "mutation of E312 to Q" or "mutation of Glu312 to Gln".*

Fixed. Thank you.

> *Fig. 3d, Normally, one would expect full saturation curves for each mutant. How can a reader distinguish between low affinity or a decrease in the number of binding sites? Would full binding curves be prohibitive for the mutants because of the cost or availability of the ligand? These points should be addressed. A couple of the curves are not visible. Would an expanded scale inset show them more clearly? Also, would it be possible to include chemical structures for all ligands discussed?*

Many if not most of these mutants bind TBZ with such low affinity that it is not possible to measure a full saturation curve either because of ligand availability (radioactive ligand concentration is only in μM) or due to technical issues with being able to measure such low affinity binding. We have changed the presentation of the curves and have split the gating and binding site mutants into their own figures. We feel this improves the readability of these curves. We have also included a table with the respective Kd values determined for each of the mutants where possible.

> *Line 235, The distances are long for a direct interaction between K138 and the TBZ methoxy groups. The unusual distances should be mentioned if an interaction is being proposed.*

We do not think that K138 is directly involved in TBZ binding, however this was written in a confusing way and has been now changed.

> *Line 243, Please give a quantitative estimate of the affinity difference. "modestly" is vague.*

It is an approximately 2-fold difference. Fixed in the text.

> *Line 248, 150 nM is, at best, a Kd, not an affinity.*

Agreed, this is changed.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *The (3 x ~100ns-long) molecular dynamics simulations provided suggest some instability of the pose identified by cryo-EM. While it is not unreasonable that ligands shift around and adopt multiple conformations within a single binding site (in a reversible manner), the present results do raise questions about the assumptions made when starting the simulations, in particular (1) the protonation states of charged residues in the TBZ binding sites; (2) the parameters used for tetrabenazine; (3) the conformations of acidic side chains that are notoriously difficult to resolve in cryoEM maps; and (4) any contributions of the truncated regions truncated in the simulated structure, namely the cysteine cross-linked loop and the terminal domains. The authors should examine and/or discuss these contributions before attributing mechanistic insights into the newly observed binding orientation.*

In order to estimate the effects of protonation states on TBZ binding, we now added three new systems with altered protonation on TBZ and binding pocket lining residues (see Table 3 in the revised vision); and for each system, we performed multiple MD runs to address the question and concerns raised by reviewer.

> *Regarding the protonation states: Propka3.0 was used to determine the protonation states, finding that E312 and D399 should be protonated. If I am not mistaken, this*

> *version of ProPka cannot account for non-protein ligands (https://github.com/jensengroup/propka). Given their proximity to the binding site, these protonation states will be critical factors for the stability of the simulations. The authors could test their assumption by repeating the calculations with Propka 3.1 or higher, to establish sensitivity to the ligand. Beyond this, showing the resultant hydrogen bond networks will help to reassure the reader that the dynamics in the lumenal gates do not arise from an artifact.*

We thank the reviewer for suggestion of using higher version of Propka. We used the most recent Propka3.5 and carried out protonation calculations in the presence and absence of TBZ. The new calculations are presented in Table 4 and SI Figure 8c of the revised version.

> *It should be possible to assess whether waters penetrate the ligand binding site during the simulations if that is of concern.*

We now added the number of waters within the ligand binding pockets for all MD simulations we performed, which are presented in Table 3 and Table 5 of the revised version.

> *Finally, I didn't fully understand the conclusion based on the simulations and the "binding affinity" calculations: do they imply that the pose identified in the EM map is not stable? What is the value of the binding affinity histogram?*

We apologize for this confusion. For each MD snapshot, we calculated TBZ binding affinity using PRODIGY-LIG (Vangone et al., Bioinformatics 2019), which is a contact-based tool for computing ligand binding affinity. The binding affinity histogram shown in the original submission was the histogram of those binding affinities calculated for MD snapshots. In the revision, we replaced binding affinity histogram by time evolution of binding affinity changes (SI Fig 6c in the revision). The simulations confirmed that the pose identified in the EM map is stable, with a flattened binding affinity of -9.4 ± 0.3 kcal/mol in all three runs.

> ***Recommendations regarding writing/presentation:***
>
> *The authors use active tense terminology in attributing forces to elements of structure (cinching, packing tightly, locking). While appealing and commonplace in structural biology, this style frequently overstates the understanding obtained from a static structure and can give a rather misleading picture, so I encourage rephrasing.*

We appreciate this point; the use of these words is not meant to overstate or provide a misleading picture but rather to aid the reader in mechanistic understanding of the proposed processes.

> *I would also recommend replacing the terms "above" and "below" for identifying aspects of the structure; the protein's location in the vesicular membrane makes these terms particularly difficult to follow.*

These terms refer specifically to the Figures themselves which we have always oriented with the luminal side at the top of the page and the cytosolic on the bottom. We have indicated in Figure 1 the orientation of VMAT2. The Figures are the point of reference which we refer to, and the ‘above’ and ‘below’ terms have been used to assist the reader to make the manuscript easier for a more casual or non-expert reader to follow.

> *Minor corrections:*

- *the legend in Figure 2 lacks details, e.g. how many simulation frames are shown, how were the electrostatic maps calculated?*

We revised Figure 2 and moved simulation frames to SI figure 6e. A total of 50□3 simulation frames are shown.

- *how were the TBZ RMSDs calculated? using all atoms or just the non-hydrogen atoms?*

For TBZ RMSDs, we used non-hydrogen atoms. This information is presented in the Methods section.

MD simulation snapshots and input files can be provided via zenodo or another website.

We will upload snapshots and input files to Zenodo upon acceptance of the manuscript.

Reviewing editor specific points:

Specific points

L.97: Remove "readily available"

Fixed.

L.99: The authors are not measuring competition binding. It is well known that reserpine and substrates inhibit TBZ binding only at concentrations 100 times higher than their respective KD and KM values. It is, therefore, surprising that the authors use this isotherm and refrain from commenting on the significance of the finding. Moreover, the presentation of results as "Normalized Counts" does not provide any information about the fraction of VMAT molecules binding the ligand. At least, the authors should provide the specific activity of the ligand, and the number of moles bound per mole of protein should be calculated.

The point was not to infer any details about the conformations that TBZ and reserpine bind but merely to point out that both constructs have a similar behavior with respect to their Ki for reserpine. We have added a sentence to say that reserpine binding stabilizes cytoplasmic-open so the reader is aware of the significance of this competition experiment.

L.102: The characterization of serotonin transport activity needs to be more satisfactory. The Km in rVMAT2 is 100-200 nM, so why are the experiments done at 1 and 10 micromolar? Is the Km of this construct very different? The results provided (counts per minute at the steady state) need to give more information.

The Km of human VMAT2 varies somewhat according to the source but has generally been reported to be between 0.6 to 1.4 μM for serotonin according to these references.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3019297/ https://www.cell.com/cell/pdf/0092-8674(92)90425-C.pdf https://www.pnas.org/doi/abs/10.1073/pnas.93.10.5166

Fig 1B could be more informative. I suggest adding a cartoon model with TMs labeled, similar to ED Fig6a.

This panel is to aid the reader in accessing the overall map quality and thus we do not wish to add additional labels/fits which would distract from that point. Instead, we have added overall views of the model in Figs 2,3.

> *L.179: The authors claim that the inner gate is located "below" (whatever this could mean) the TBZ ligand. In L.214, they claim that TBZ adopts a pose.....just "below" the location of the luminal gating residues. Please clarify and use appropriate terminology.*

This refers to the position of these residues in the Figures themselves. We have added figure calls where appropriate here.

> *Fig. 4: The cartoon could be more informative.*

We have added more information to the mechanism cartoon which is now Figure 6. This incorporates some of our new data and we believe it will be more informative.

> *L. 213: The paragraph describes residues involved in TBZ binding. Mutagenesis is used to validate the structural information. However, the results (ED fig. 5B) must be corrected for protein expression levels. In the Methods section, the authors state (L.444), "Mutants were evaluated similarly from cell lysates of transfected cells." Without normalization of protein expression levels, the results are meaningless even if they agree with predictions.*

In fact, we have normalized the concentrations of protein in our binding experiments. This was noted in the methods section. And to account for these differences, experiments were conducted using 2.5 nM of VMAT2 protein as assessed by FSEC.

> *L.220: The referral to ED Fig.7 is not appropriate here. The figure shows docking-predicted poses of dopamine and serotonin.*

Figure call has been changed.

> *L.226: The referral to Fig. 3b needs to be corrected. The figure shows TBZ and not the neurotransmitter.*

This has been corrected.

> *L. 337: "The neurotransmitter substrate is bound at the central site." What do the authors mean in this cartoon? Do they have evidence for this? Tetrabenazine is not a substrate.*

This cartoon drawing is meant to illustrate the elements of structure. Similar drawings are presented throughout the literature such as here: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5940252/ Figure 3 and here: https://pubs.acs.org/doi/10.1021/acs.chemrev.0c00983 Figure 2.

> *The same compound is mentioned with different names: 3H-dihydrotetrabenazine and 3H-labeled DTBZ.*

Fixed.

> *ED fig 1d is illegible.*

The high-resolution figure is completely legible. We will provide this to the journal upon publication.

> *Figure 2d: A side view would be more visual.*

We have updated this figure and believe that it is much easier to understand now.

> *L. 179: The inner gate is located 'below' the TBZ ligand*

Please see above response, this refers to the figures themselves. The figures are our point of reference.

> *L. 213-215: Tetrabenazine binding site just 'below' the location of the luminal gating residues.*

See above.

> *Throughout the paper, results are given as cpm or counts. The reader can only estimate the magnitude of the binding/transport by knowing the specific activity of the radiolabel. I recommend switching to nano/picomoles or supplying enough information to understand what the given cpm values could mean.*

Binding experiments were done using scintillation proximity assays and therefore converting the CPMs to values in pmol of bound ligand is simply not possible. For the transport experiments (now Fig 1d) the point was to show that the wild type was similar in activity to the chimera. In our new transport experiments we have presented for the mutants, many experiments were combined together and therefore, we have normalized the counts to the relative activity of wild type VMAT2.