Peer Review File

Manuscript Title: Structural insights into vesicular monoamine storage and drug interactions

Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

The manuscript by Ye et al. reports the high-resolution cryo-EM structures for the human vesicular monoamine transporter VMAT1. From a physiological and pharmaceutical perspective, VMAT1 is an important solute carrier transporter responsible for loading monoamine neurotransmitters into storage vesicles in neurons and neuroendocrine cells. Considerable biochemical and physiological studies have been conducted on VMAT1 (neuroendocrine) and VMAT2 (neuronal) homologues, and a clear picture of their mode of action is known. This study addresses the important question of ligand recognition and transport mechanism, focusing on using high-resolution structures to capture VMAT1 in both cytoplasmic and vesicular-facing states. Additional structures in complex with an inhibitor (Resperine - cytoplasmic open) and substrates (dopamine, norepinephrine, serotonin and histamine - vesicular open) enable the authors to map out the binding site (Fig. 3) suggest a mode of action of neurotoxins (MPP+) and propose a transport model (Fig. 5h).

Overall, the study presents an excellent and comprehensive set of structures for an essential transporter and establishes the structural basis for ligand recognition. The main strengths of the study are the number and quality of the structural data, for which the authors should be congratulated. The main weakness is the lack of biochemical characterisation and reliance on past literature, which the authors naturally have to pick and choose from to support their hypotheses. In my opinion, the study would be strengthened substantially by including in-depth structure-function data. Much of the insights gained about the mechanism are based on superposition rather than experimental data, and the study leaves open several important questions concerning the mechanism unanswered. Although the structural work is impressive, the study is very descriptive, highlighting the absence of supporting functional data to provide insight into the many observations recorded. This is a shame, as I feel this study could significantly contribute to the SLC literature with sufficient biochemical and mutational data.

Below, I have highlighted what I think are the main areas for strengthening.

- line 232-234. The data in Fig. 3h cannot be used to support this statement. The data simply show these mutants are non-functional in your assay. However, the assay cannot distinguish between binding and transport. You need to develop a binding assay before ascribing a function to these side chains. As a general comment, a binding assay is necessary to assist in interpreting these structures.

- lines 280-285. I didn't understand this statement or what the authors mean by a 'common binding site'. The statement appears to say that the 'common' binding site is identical between the cytoplasmic vs. vesicle open states. In that case, at least in theory, reserpine should be able to bind both, at least biochemically. I didn't understand what the authors wished to convey about VMAT function or reserpine action with this statement.

- lines 299-301. What is the evidence for this statement? This statement appears to rely on conjecture rather than experimental or computational evidence concerning the protonation state of E320.

- lines 308-311. Similarly, much of this section is conjecture. I'm not opposed to the hypotheses concerning the roles of D222, E320 or D419, which are very sensible given the structures. However, the study presents no experimental evidence or computational data supporting the proposed roles.

- lines 313-315. This was an interesting observation by the authors. My understanding from the literature was that D34 was considered essential for the proton-coupling mechanism. The authors appear to dismiss this work relatively quickly in the text. There are several plausible explanations for the inconsistency with regard to this previous study, consistent with the location of D34 and H422 in the structures. Unfortunately, the inability of the current data to shed light on the function of D34 and H422 and correlate with previous studies suggests the model in Fig. 5 is likely too simplistic.

- Transport model, Fig. 5. I am not convinced the study provides sufficient data to support this model and not for a main figure in the paper. Whilst the conformations are supported, as are the interactions, the mechanism is conjecture at this point. As noted above, the study provides no direct evidence for protonation/deprotonation of the side chains, or the impact this might have on conformational changes or ligand binding. The authors also need to consider previous data on this family (for example D34 above). The alternating access model for MFS proteins is very well reported in the literature, and the current structures are fully consistent with the structural changes observed in other systems.

- line 391-393. Can the authors expand on this statement? This strikes me as a key part of the mechanism of reserpine that is not fully explained in this study. Which factors contribute to the very

low off rate for this drug in VMAT isoforms, or VMAT2 in particular. This is central to the main conclusion of the study, which is that the structural work as presented lays the foundation for more targeted drugs against members of the SLC18 family.

- line 209-210. This statement is now incorrect. The recent OAT1 structure (NSMB 2023) shows that MFS folds can accommodate multiple distinct binding sites.

- lines 165-171. The authors analyse the structures and produce Table 1, which they use to suggest that an asymmetry of interactions in the two gate regions of the transport explains how VMAT1 is held in a predominantly vesicular open state. However, the description of the cryo-EM data suggested the transporter was very mobile prior to the addition of the drug reserpine (line 113). Please explain this disparity between the conclusion drawn and the experimental data from the EM more clearly.

Minor comment: I would change the figures to place the transporter in one orientation, either cytoplasm up or down. Convention seems to prefer the cytoplasm facing up, but I think the key is be consistent.

Referee #2 (Remarks to the Author):

The vesicular monoamine transporters pack the monoamines into the vesicular are several therapeutic targets. In the manuscript "Structural insights into vesicular monoamine storage and pharmacological interactions". The authors found that reserpine (Res) could induce the dimerization of VMAT1; although the dimerization of VMAT1 may not be physiologically related, it is excellent for structural studies usage. The authors reported eight structures of VMAT1 with inhibitor Res bound at cytoplasmic open sate, the apo, four monoamines bound, and 3 neurotoxicants bound at vesicular (or lumen) open state. The quality of maps and models is high, and the mechanism interpretation is also comprehensive through detailed structural analysis and incorporation with former biochemical studies.

Moreover, the authors also explored the possible substrate-binding behavior of other members of the SLC18 family based on modeling and published lectures. This manuscript is of broad interest and will impact the field significantly. The manuscript is well written and overall suited for publication. However, the following concerns should be addressed to ensure the rigor of the manuscript.

Major concerns:

1: As in lines 142-143, the Apo state represents the state just after substrate release or before protonation-induced antiport. No matter which one, it is an essential functional state. The authors also mentioned this conformation is essentially the same as monoamines bound conformation at lines 203-204. I can't find the structure comparisons between the Apo state and other bound states in the manuscript. Any comparisons would help to understand the transport cycle.

2: Lines 158-165 and Figure 2d. The authors analyze the disease mutation (T137I), but how to model this mutation is not mentioned. I can't see how the NTD/CTD interaction is enhanced from Figures 2c and 2d. Please provide a detailed description. Even the interaction between T137I and A450 enhances the NTD/CTD interaction, facilitating the transition towards the cytoplasmic-open state; it is unnecessary to increase the transport activity, as the transport cycles are composed of several states. Do the authors have any other explanations?

3: I have carefully checked the maps and models. The modelling is at high quality. What is the contour level in Figure 3c-d? Are they at the same or similar level? I found the density is weaker for some of the monoamine ligands; for example, the HSM density is weaker than the surrounding amino acids and the density of RSP in protomer B. Does it reflect the lower occupancy of the HSM?

4: For the uptake experiments, it would be nice if the authors show the time course of substrate uptake in the WT and transfected cell and check whether the 25-minute points correspond to the linear regime.

Minor points:

1 What does the yellow color mean in the TM2,5,8,11 (center part) of the overall structures in Figure 2b?

2: Is the lines 155-156 related to Figure 2c?

3: The methods of FSEC should be more detailed, such as the column type. It is better if the X axis is volume instead of time.

4: Does figure 1d show the antiparallel RSP/Apo dimer? Please clarify it in the figure legend; it may cause misunderstanding when the protein opens in the same direction but labeled with cytoplasmic open and Vesicular open.

5: Why does the F437A affect the uptake of Serotonin but not Dopamine in figure 3g.

6: The authors should mention how the right part of Figure 5b is modeled.

7:It could be better if Figure 5g is shown in a similar form as the upper part of Figures 5c and d.

8: The authors should explain the role of BFA in figure legend or method.

9: which model is used for the surface presentation in supplementary 8g and 9b?

Referee #3 (Remarks to the Author):

The Vesicular MonoAmine Transporter (VMAT) is an essential protein that plays a central role in neurotransmission. VMATs have two isoforms, VMAT1 and 2, that exhibit high sequence similarity but distinct tissue distributions and inhibitor sensitivity.

The valuable study by Bin Liu and collaborators reveals the first structure of the mammalian VMAT1 transporter. The authors report eight cryo-electron microscopy structures of human VMAT1 in unbound form and in complex with substrates, toxicants, and inhibitors.

This report is an impressive achievement that was possible thanks to the tinkering of the protein that dramatically increased the expression of a stable and functional transporter. Moreover, the fortuitous finding that Reserpine, the potent inhibitor of both isoforms, induces dimerization of this relatively small 52 kDa protein paved the way for the extensive structural analysis by cryoEM. Furthermore, in this non-physiological dimer, the monomer occupied by Reserpine is in cytoplasmic facing conformation while the other is in the lumenal facing, allowing for simultaneous capture of the ligand-free and Reserpine bound conformation.

Although the structures elegantly presented in this MS are important, I feel that the authors have overstated several of the conclusions that can be drawn from them. The study's main weakness is the need for in-depth mechanistic insights into the transport mechanism, which would make the conclusions less speculative. There is a wealth of information about how MFS transporters, VMAT specifically, bind inhibitors and substrates, undergo conformational changes to transport them across the membrane, and couple these events to the transmembrane movement of ions. Additional analysis of the literature, together with more in-depth studies of some of the mutants generated, could greatly benefit this milestone study.

The structures provide strong evidence for the central role of E320 and D407 in substrate and inhibitor binding. However, there is little or no evidence that they are the target of protonation, as pointed out in the movie and Fig 5H. A study of the D400 mutants in the rat VMAT1 isoform (D407 in the human protein) suggested a role in substrate binding and predicted that D400 interacts with Y342 (Y349 in human) via a hydrogen bond. However, a detailed study of the mutant protein D400E in both rat isoforms showed a robust, although somewhat weaker, transport activity. Even though the authors are aware of these studies (L.319), they report here that the hVMAT1 D400E mutant is inactive (L.233, Fig 2h). Is the difference in the reported results likely due to poor expression of the mutant? The Methods section does not state whether and how the results are standardized relative to expression levels.

Previous studies show that position 427 of rVMAT2 (D434 in hVMAT1) does not tolerate conservative replacements. Notably, however, the negative charge in the cluster is not required for

either inhibitor or substrate binding. All the D427 mutants bound Tetrabenazine with similar affinities, and MPP+ can displace it. In a preprint recently deposited at Bioxrv (https://www.biorxiv.org/content/10.1101/2023.09.05.556211v1)

the authors identified a distinct polar network in hVMAT2, also previously suggested in biochemical studies, that may play a role in proton coordination and subsequent transporter conformational changes. The network lies between TMs 1, 4, and 11 and consists of residues D33, N34, K138, Q142, R189, Q192, S196, S197, S200, and D426 (hVMAT2). Is protonation of D33, as previously suggested for rVMAT2 and other MFS transporters, rather than D407, the best candidate for playing a role in proton coordination and subsequent transporter conformational change?

Regarding the catalytic cycle proposed by the authors. Defining the lumenal-facing conformation as the resting state is misleading. It may be the resting state in solution, in the crystal, and even in the membrane in the absence of a proton gradient, but this is not the case when the protein functions. The intravesicular pH is acidic, and the protein would undergo protonation and most likely isomerize to the cytoplasmic facing state, release the protons, and be ready for the following substrate molecule. Could this be the "resting state" of the functional protein? The substrate is more likely to bind with high affinity to the non-protonated species and induce the conformational transition. Protonation at the lumenal side may decrease the substrate affinity and facilitate release.

Specific points:

Transmembrane proteins in intracellular organelles present unique issues of nomenclature. I suggest the authors refer to the cytoplasmic and luminal faces of the protein (not vesicular-open) and adhere to these names to avoid confusion.

I am somewhat surprised that there is no discussion on the possible reasons for the insensitivity of this isoform to Tetrabenazine. Since they provide information about the binding of so many substrates/inhibitors, could they, at least, speculate about Tetrabenazine?

L.40: The authors state: "Reserpine binding stabilizes a cytoplasmic-open conformation resembling a protonated state, while other structures capture a vesicular-open conformation stabilized by extensive gating interactions." I propose a different explanation. Reserpine binds to the non-protonated state of the cytoplasmic-open conformation. Without a proton motive force (lumen not acidified), the resting state is the lumen-facing conformation; therefore, the Reserpine binding site is unavailable.

L.112: Was Reserpine added during or before solubilization? Solubilized VMAT2 does not bind Reserpine since its binding requires the proton motive force. Does hVMAT1 behave differently?

L.265: The appropriate reference to MPP+ is nine rather than 10.

L.295 and 296: The appropriate reference is 12 rather than 41 and 27.

The paragraph summarizing the mechanistic findings needs some clarification:

L.324: "The vesicular-open state, stabilized by extensive gating, is energetically favored and predominates as the resting state ." Again, this is only in the absence of a proton gradient and is irrelevant when describing the catalytic cycle.

L.330: "This deprotonation triggers a return to the vesicular-open state" should be: This deprotonation AND substrate binding triggers the return to the lumenal-facing conformation.

L.337: Regarding this suggestion, please see reference Maron et al. J. Biol. Chem 1983 Vol. 258 Pages 11476-81. I wonder whether this is relevant but may connect with the authors' suggestions.

L.347-352: References 27 and 45 are for VAChT, not VMAT. Please insert appropriate references.

Fig 3 legends: Please provide the values of 100% uptake.

Fig 5h and Movie: Unless more support is provided that E320 and D407 are the targets of protonation, the authors should consider refraining from pointing them specifically.

Extended Data Fig 7: Since most of the published mechanistic VMAT studies are on the rat homologs, it would be helpful for the reader to add them to the alignment so that the residue numbering can be quickly understood.

Author Rebuttals to Initial Comments:

We greatly appreciate the highly insightful comments from reviewers and their deep knowledge of the field. Please see our point-by-point responses below and tracked changes in the revised manuscript.

Reviewer 1:

1. This study could significantly contribute to the SLC literature with sufficient biochemical and mutational data.

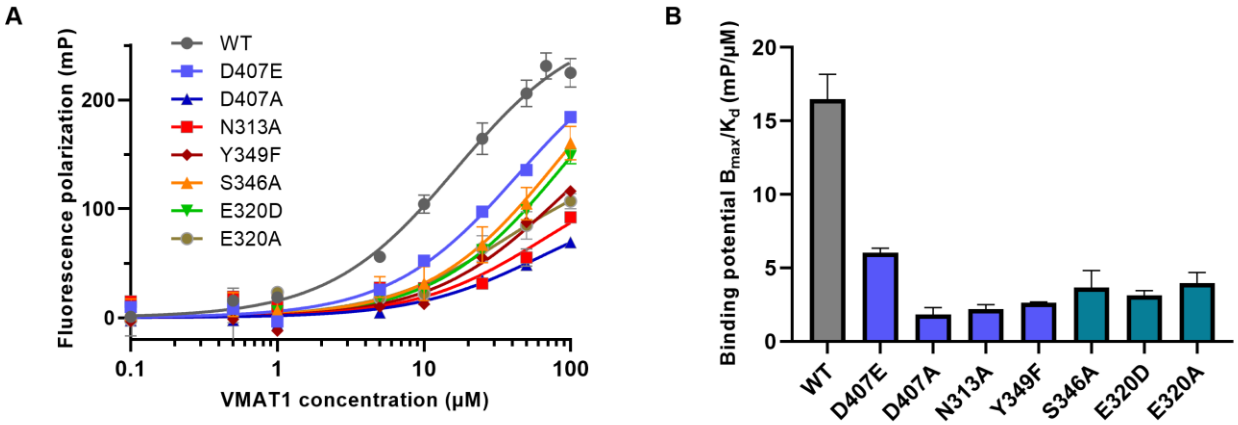
Response: We have now conducted binding experiments demonstrating that mutations at the monoamine binding site result in decreased affinity. We have also characterized potential protonation sites in human VMAT1 by responses of neutralization mutants to proton gradient and by MD simulation. In other VMATs, these sites have been suggested by separate reports[1-4] but lacked a systematic comparison. These new data are presented as Fig. 5e, g, Extended Data Fig. 7f, 8c, and Movie S1 in the manuscript, in addition to transport activities of mutants at the monoamine-binding site and putative protonation sites (Fig. 3g, h, 5f).

We appreciate the reviewer's encouragement for these extensive biochemical and computational studies, while noting that structural reports in leading journals typically present limited data in this regard. In agreement with the reviewer, we believe these new data substantially advance the knowledge of VMATs and the broader SLC field.

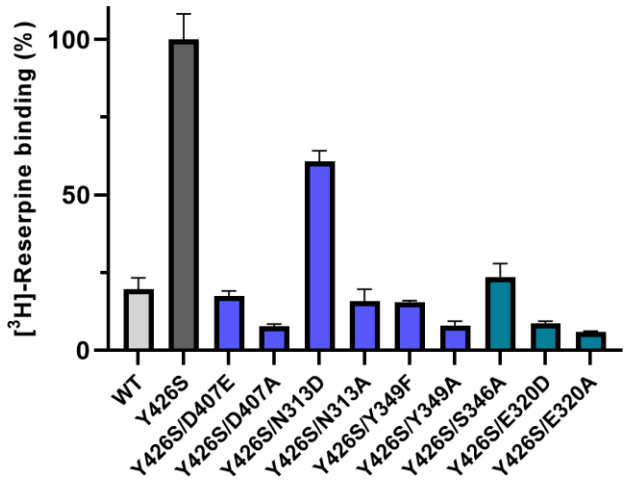
2. Line 232-234. The data in Fig. 3h cannot be used to support this statement. The data simply show these mutants are non-functional in your assay. However, the assay cannot distinguish between binding and transport. You need to develop a binding assay before ascribing a function to these side chains. As a general comment, a binding assay is necessary to assist in interpreting these structures.

Response: To our knowledge, transport-based binding assays have been consistently documented in the literature[5-9]. In our case, however, the K_M of transport or competition of transport cannot be compared because many mutants have lost activity. On the other hand, direct binding assay (not based on transport) for monoamines is challenging because of their relatively low affinities (μM range) for VMATs, unlike the binding assays for VMAT inhibitors (e.g., reserpine and tetrabenazine), whose high affinities (nM range) allow their retention on VMATs during a binding assay. After extensive trials with multiple approaches, including monoamine competition using radiolabeled reserpine and isothermal titration calorimetry (see below), we have successfully developed a fluorescence polarization-based assay to assess the binding of a false fluorescence neurotransmitter, FFN206, to VMAT1 mutants. False fluorescence neurotransmitters are well documented for VMAT transport assays[10,11], and here we show change of their fluorescence polarization can be used to assess VMAT binding.

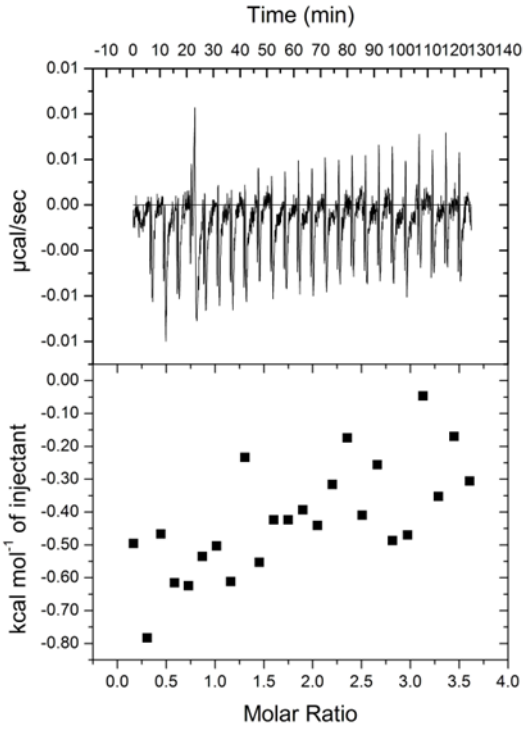
With this new binding assay, we find that mutating key residues at the monoamine binding site drastically decreases FFN206 binding (See Figure below; included as Extended Data Fig. 7f in the manuscript). Most mutant proteins do not achieve saturation in FFN206 binding even at very high protein concentration (100 μM) (Panel A in the Figure below; FFN206 concentration remains constant to maintain the same fluorescence level). Due to this limitation, their relative affinities are estimated through binding potential (B_{max}/K_d) (Panel B)[12]. As expected, disrupting the hydrogen bonding triad with mutations Y349F, N313A, and D407A results in the highest loss of affinity. The mutation D407E, which retains the negative charge, appears more tolerated. Disrupting the other polar site with S136A and E320A/D mutations also interferes with binding. Reduced binding of these mutants is consistent with their decreased dopamine and serotonin transport activities (Fig. 3g-h in manuscript).



Alternatively, we investigated if the binding affinities of VMAT1 mutants could be measured by monoamine competition of reserpine binding. We first made the Y426S mutation to shift VMAT1 to the cytoplasmic-open conformation for reserpine binding[2], and then introduced mutations at the monoamine binding site. However, further analyses of monoamine competition were untenable because most of these mutations also severely disrupt reserpine binding (See Figure below).



We also explored the use of isothermal titration calorimetry (ITC) to detect monoamine binding, and titrated 10 μM wildtype VMAT1 by 100 μM serotonin. However, serotonin binding is not discernible by ITC (see Figure below), likely because the small heat change induced by binding is below the detection limit of ITC.



3. Lines 280-285. I didn't understand this statement or what the authors mean by a 'common binding site'. The statement appears to say that the 'common' binding site is identical between the cytoplasmic vs. vesicle open states. In that case, at least in theory, reserpine should be able to bind both, at least biochemically. I didn't understand what the authors wished to convey about VMAT function or reserpine action with this statement.

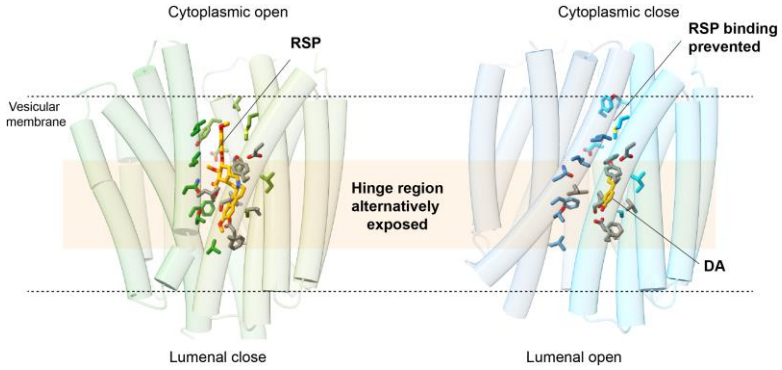
Response: Sorry about the confusion. To clarify, monoamines, amphetamine, and MPP^+ share 'common binding site' in the lumenal-open conformation (Fig. 3 and 4 in the manuscript). Reserpine occupies the substrate-binding site but involves more interactions (see Figure below). Nevertheless, the resulting 'protein conformation' could still be similar to a conformation that occurs during substrate import. This 'common protein conformation' may represent a protonation-induced state, because reserpine binding and substrate transport both require a proton gradient[13,14]. Moreover, MFS transporters typically maintain their substrate-binding site (see explanation to Q7 below) during the rocker-switch transition between alternate conformations. In support of these ideas, a recent VMAT2 structure in a mutation-induced cytoplasmic-open conformation with bound monoamine[15] resembles the reserpine-bound state and uses the same monoamine-binding site as VMAT1 in the lumenal-open conformation.

Taken together, the logic we try to convey is the following. Structural superposition (Fig. 5a) shows that the substrate-binding pocket is maintained between lumenal-open, substrate-bound conformation (A) and cytoplasmic-open, reserpine-bound conformation (B). The cytoplasmic-

open, substrate-importing conformation (C) is expected to maintain the same substrate-binding pocket. Since the substrate pocket is shared between VMAT1 conformation A and B, and between conformation A and C, we propose that conformation B should resemble C. i.e., the reserpine-bound conformation resembles a protonation-induced, cytoplasmic-open conformation during substrate import.

To clearly convey this logic, we have reworded the text (lines 281-286) to the following. “Remarkably, the substrate-binding pocket observed in the lumenal-open conformation is preserved in the cytoplasmic-open conformation bound by reserpine (Fig. 5a). Thus, this cytoplasmic-open conformation may resemble a conformation for substrate import, which is expected to be cytoplasmic-open and maintain the same substrate pocket. Consistently, biochemical data show that reserpine recognizes a protonation-induced, cytoplasmic-open conformation, as presented during the transport cycle”.

As to the reviewer's additional question, reserpine cannot bind to both conformations because part of this large molecule is bound toward the cytoplasmic end (see Figure below). This part is closed in the lumenal-open conformation, thus preventing reserpine binding. In contrast, monoamines are bound around the hinge region, which is alternatively exposed in both conformations. Please refer to the Figure below, in which residues binding both reserpine (RSP) and monoamines (e.g., dopamine, DA) are colored grey, and residues in other colors provide additional interactions to reserpine. The hinge region is shaded in orange.



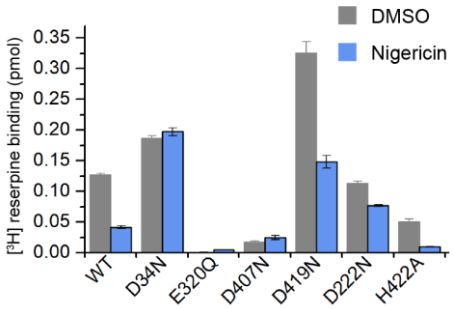
4. Lines 299-301. What is the evidence for this statement? This statement appears to rely on conjecture rather than experimental or computational evidence concerning the protonation state of E320. Lines 308-311. Similarly, much of this section is conjecture. I'm not opposed to the hypotheses concerning the roles of D222, E320 or D419, which are very sensible given the structures. However, the study presents no experimental evidence or computational data supporting the proposed roles. Lines 313-315. This was an interesting observation by the authors. My understanding from the literature was that D34 was considered essential for the proton-coupling mechanism. The authors appear to dismiss this work relatively quickly in the text. There are several plausible explanations for the inconsistency with regard to this previous study, consistent with the location of D34 and H422 in the structures. Unfortunately, the inability of the current data to shed light on the function of D34 and H422 and correlate with previous studies suggests the model in Fig. 5 is likely too simplistic.

Response: Agreed. While our previous hypotheses about these potential protonation sites are reasonable, their exact roles require further elucidation. Thus, we have systematically analyzed

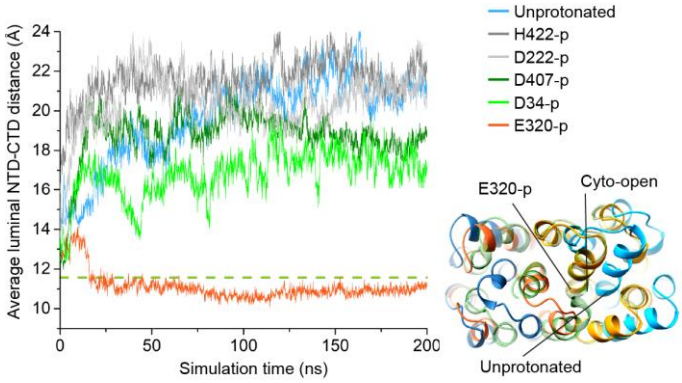
these sites through experimental and computational approaches (new Figure 5e-g and Extended Data Fig. 8).

We adopted an experimental approach from the Schuldiner group[2] with the following rationale. Protonation of at least one of the two sites should induce the cytoplasmic-open conformation recognizable by reserpine. A neutralization mutant at the protonation site may mimic protonation and induce the cytoplasmic-open transition without a proton gradient. Hence, neutralization mutant displaying protonation-independent reserpine binding is suggestive of a protonation site.

We investigated all the potential pronation sites suggested in literature[1-4] and by our new structures (see Figure below). We found that D34N in human VMAT1 shows protonation-independent reserpine binding (proton gradient collapsed by nigericin). This observation is consistent with the data suggesting D34-corresponding residue as the protonation site in rat VMAT2[2] and in a bacterial VMAT homolog[1]. In contrast, the reserpine binding of D419N, D222N, and H422A changes with the proton gradient. However, this experiment cannot determine the roles of E320 and D407, as their neutralization mutants lose reserpine binding affinity.



To further understand the roles of these potential sites, we conducted MD simulations to investigate the conformational flexibility upon their protonation, using the lumenal-open conformation as the starting point. Interestingly, E320 protonation (E320-p) triggers NTD-CTD association at the lumenal side, which may initiate the cytoplasmic-open transition. The Figure below (added as new Fig. 5e and Movie S1 in manuscript) compares the average NTD-CTD distances at the lumenal side, which show lumenal NTD-CTD proximity in E320-p (orange) as in the cytoplasmic-open state (dashed line). The figure inset shows a representative MD state of E320-p, whose conformation at the lumenal side resembles that of the cytoplasmic-open state. In contrast, this lumenal association does not form in unprotonated VMAT1 (blue) or with most other residues protonated, except that the protonated D34 exhibits relatively close lumenal NTD-CTD association during the MD simulation. Overall, these computational and experimental data suggest E320 and D34 as the two potential protonation sites in VMAT1.

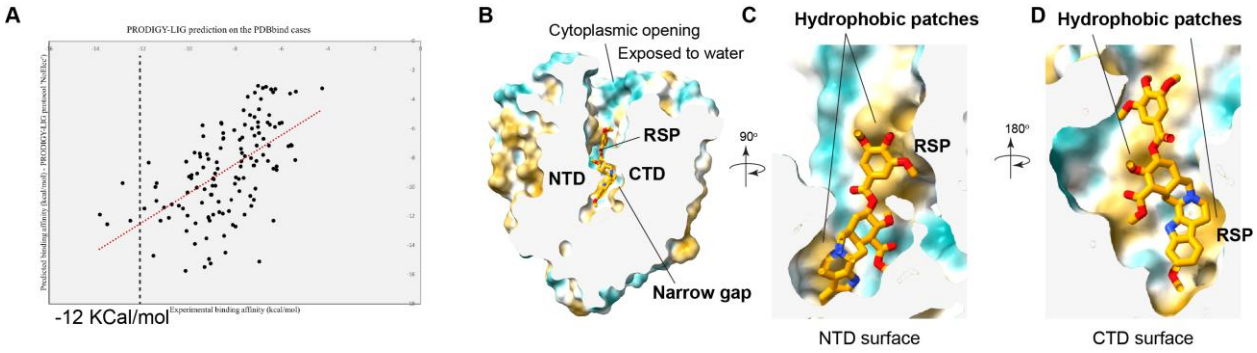


5. Transport model, Fig. 5. I am not convinced the study provides sufficient data to support this model and not for a main figure in the paper. Whilst the conformations are supported, as are the interactions, the mechanism is conjecture at this point. As noted above, the study provides no direct evidence for protonation/deprotonation of the side chains, or the impact this might have on conformational changes or ligand binding. The authors also need to consider previous data on this family (for example D34 above). The alternating access model for MFS proteins is very well reported in the literature, and the current structures are fully consistent with the structural changes observed in other systems.

Response: Removed this model as suggested.

6. line 391-393. Can the authors expand on this statement? This strikes me as a key part of the mechanism of reserpine that is not fully explained in this study. Which factors contribute to the very low off rate for this drug in VMAT isoforms, or VMAT2 in particular. This is central to the main conclusion of the study, which is that the structural work as presented lays the foundation for more targeted drugs against members of the SLC18 family.

Response: This is an excellent suggestion. The free energy of reserpine binding is estimated to be -12 kcal/mol (by the PRODIGY server[16]), placing it among the strongest inhibitors (see black dashed line in panel A of the Figure below, which is from the PRODIGY website that surveys 124 inhibitors; or Fig. 3 in ref [17]). The binding of this large molecule buries a substantial area (630 Å^2). Importantly, hydrophobic interactions account for a major portion (67%, as estimated by PLATINUM[18]) of this buried area, resulting in a pronounced hydrophobic effect because the central cavity of VMAT1 is otherwise aqueously exposed. The bottom of the central cavity forms a narrow gap, and the molecular structure of reserpine fits well into this gap (see panel B below). The NTD and CTD sides of this gap both contain hydrophobic patches that match well with the molecular shape of reserpine. Panel C and D below show surfaces at both sides of this gap, in which the yellow color represents the most hydrophobic areas and cyan stands for the most hydrophilic. In conclusion, future drug development should consider designing sizable molecules that align with the hydrophobic patches within the narrow gap to achieve high binding affinities.



We have added panels B-D as Extended Data Fig. 5c and described these observations in the Results (lines 182-187). "Reserpine binding buries a 630 Å^2 area and occupies the narrow gap along the central cavity (Fig. 2g). Both the NTD and CTD sides of this gap contain hydrophobic patches that match with the molecular shape of reserpine (Extended Data Fig. 5c), resulting in a substantial hydrophobic effect because the central cavity is otherwise aqueously exposed.

Combining hydrophobic interactions and shape complementarity, reserpine acts as a tight-binding inhibitor for the cytoplasmic-open conformation".

We also changed the Discussion (lines 398-404) to: "Reserpine competitively inhibits both VMAT isoforms by binding at their cytoplasmic-open state with near-irreversible affinity, resulting in prolonged pharmaceutical effects. To achieve such high affinities, future drug development should consider designing sizable molecules that align with the hydrophobic patches within the narrow gap of the central cavity (Fig. 2g and Extended Data Fig. 5c). Moreover, compounds with reduced side effects may be developed to specifically target each VMAT isoform based on their distribution of hydrophobic patches and unique hydrophilic interactions".

7. line 209-210. This statement is now incorrect. The recent OAT1 structure (NSMB 2023) shows that MFS folds can accommodate multiple distinct binding sites.

Response: Thanks for pointing this out. The OAT1 structure shows binding site 1 for the α-ketoglutarate and site 3 for tenofovir, and the uptake of this anionic drug is driven by the export of α-ketoglutarate. This observation may conform to the canonical MFS transport mechanism in the way that each substrate is not expected to move to the other site between alternate conformations. Instead, each substrate should stay at its own binding site during the rocker-switch transition, with the alternating exposure facilitating their opposed binding and release. Considering this exception, we have reworded the text to "MFS transporters 'typically' use a single substrate pocket for transport".

8. lines 165-171. The authors analyse the structures and produce Table 1, which they use to suggest that an asymmetry of interactions in the two gate regions of the transport explains how VMAT1 is held in a predominantly vesicular open state. However, the description of the cryo-EM data suggested the transporter was very mobile prior to the addition of the drug reserpine (line 113). Please explain this disparity between the conclusion drawn and the experimental data from the EM more clearly.

Response: We have revised the EM data descriptions for clarity. The coexistence of cytoplasmic- and lumenal-open states is not due to their high mobility. In our experiment, reserpine was incubated with cells for 10 min before membrane disruption. Because proton gradient is present at this stage, a fraction of VMAT1 (Extended Data Fig. 2b) can bind reserpine. The reserpine-bound fraction adopts a cytoplasmic-open conformation, while the unbound fraction is preferably lumenal-open. During membrane solubilization, the molecule at the cytoplasmic-open conformation is prone to dimerize, either with another reserpine-bound molecule or with an unbound, lumenal-open molecule. The membrane solubilization also eliminates the proton gradient, and under this condition, the lumenal-open monomer maintains a stable conformation (see paragraph below). The reserpine-bound monomer is also stable because this inhibitor is nearly irreversible. Consequently, we observe reserpine/unbound or reserpine/reserpine dimers in alternate conformations.

Biochemical data support the stability asymmetry in VMAT conformations, i.e., the lumenal-open state is preferred over the cytoplasmic-open state (recognized by reserpine). Without a proton gradient, reserpine binding takes over 24 h to reach equilibrium[19], indicating a very slow transition from the lumenal-open state to cytoplasmic-open state. Even with a proton gradient, this transition

is the rate-limiting step[14], because the K_M for substrate transport is 10-100 times lower (better) than the substrate-binding K_D[20]. In other words, the uptake with a substrate-occupied binding site (K_M) is much faster than the reappearance of the unoccupied binding site (K_D), i.e., the cytoplasmic-open transition[14]. We have elaborated these explanations in the Supplemental Discussion.

9. Minor comment: I would change the figures to place the transporter in one orientation, either cytoplasm up or down. Convention seems to prefer the cytoplasm facing up, but I think the key is be consistent.

Response: All figures have been changed to cytoplasm facing up (except Fig. 1d,e and Extended Data Fig. 4, 5a, b to show the experimental dimers).

Reviewer 2:

1. As in lines 142-143, the Apo state represents the state just after substrate release or before protonation-induced antiport. No matter which one, it is an essential functional state. The authors also mentioned this conformation is essentially the same as monoamines bound conformation at lines 203-204. I can't find the structure comparisons between the Apo state and other bound states in the manuscript. Any comparisons would help to understand the transport cycle.

Response: We have added comparisons of the apo and dopamine-bound states in the Extended Data Figure 7a (overall structure) and 7b (monoamine-binding site). Substrate binding slightly changes the sidechain conformations.

2: Lines 158-165 and Figure 2d. The authors analyze the disease mutation (T137I), but how to model this mutation is not mentioned. I can't see how the NTD/CTD interaction is enhanced from Figures 2c and 2d. Please provide a detailed description. Even the interaction between T137I and A450 enhances the NTD/CTD interaction, facilitating the transition towards the cytoplasmic-open state; it is unnecessary to increase the transport activity, as the transport cycles are composed of several states. Do the authors have any other explanations?

Response: We have added a zoomed-in view in Fig. 2d to better illustrate the interaction. The T137 sidechain is polar and also too short to reach A450, whereas the nonpolar and larger I137 can interact with A450. This substitution is modeled by maintaining the mainchain position (till C_β) and adjusting the rotamer (Chi2 angle) of the Ile sidechain.

We changed the text (lines 160-163) to: “Structurally, the substitution of threonine to a nonpolar and larger isoleucine sidechain may promote an interaction with A450. This interaction may enhance NTD/CTD association in the cytoplasmic-open state and facilitate the rate-limiting transition to this state (Supplementary Discussion), thereby increasing the transport activity”.

Biochemical data show that transition to the cytoplasmic-open state is the rating-limiting step in the transport cycle. Therefore, facilitating this transition and accelerating the rate-limiting step may increase the transport activity. We added Supplemental Discussion to explain the following

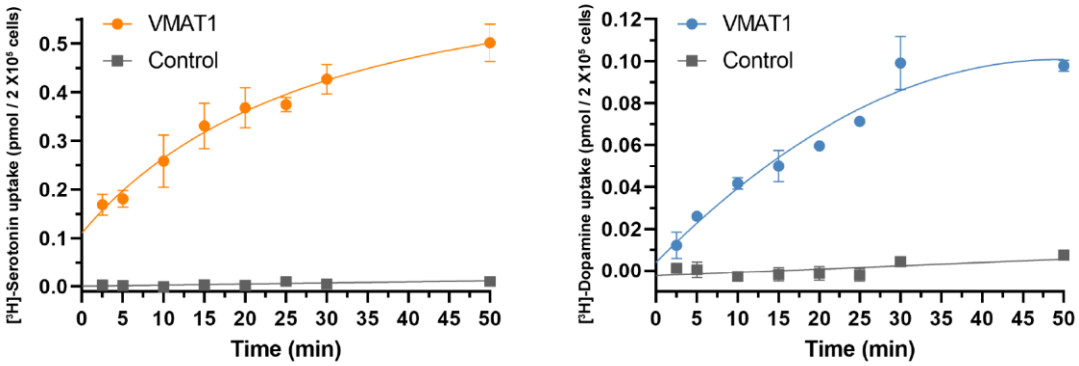
biochemical data. The transport of VMATs (and VAChT) shows an interesting behavior of $K_M << K_D$, suggesting that substrate-occupied transport (K_M) is much faster than the exposure of the empty, cytoplasmic-open site (K_D)[14]. In other words, the transition to cytoplasmic-open state is rate limiting. Additionally, the transport V_{max} is similar with different substrate types[21,22], suggesting that the conformational transition, instead of substrate binding/release, is rate limiting[14]. Consistently, the structures show that the cytoplasmic-open state has only half as many gating interactions compared to the vesicular-open state. This disparity in stability implies a lower propensity for VMAT1 to transition to the cytoplasmic-open state.

3: I have carefully checked the maps and models. The modelling is at high quality. What is the contour level in Figure 3c-d? Are they at the same or similar level? I found the density is weaker for some of the monoamine ligands; for example, the HSM density is weaker than the surrounding amino acids and the density of RSP in protomer B. Does it reflect the lower occupancy of the HSM?

Response: Thanks for all the efforts! Yes, the contour levels in Fig. 3c-f are similar, ranging from 0.06 to 0.1 in ChimeraX (these numbers appear to be software dependent). The affinity of histamine (HSM) for VMAT1 is considerably weaker than that of other monoamines and also much lower than reserpine[23]. Nevertheless, with excess amount of histamine added, we were able to observe density of the bound histamine in the cryo-EM structure. To estimate its occupancy, we re-refined the structure with this parameter included in the Phenix real space refinement. The result shows occupancy=1.00 for both histamine and reserpine.

4: For the uptake experiments, it would be nice if the authors show the time course of substrate uptake in the WT and transfected cell and check whether the 25-minute points correspond to the linear regime.

Response: We have added the time course data to Extended Data Figure 1e (also see below). The serotonin and dopamine uptake at 25 min are within the roughly linear range. For this experiment, we need to allow sufficient time to ensure reliable measurements of the [^{3}H] signal.



Minor points:

1. What does the yellow color mean in the TM2,5,8,11 (center part) of the overall structures in Figure 2b?

Response: The yellow color indicates NTD-CTD contacting regions (last sentence in the Fig. 2b legend).

2. Is the lines 155-156 related to Figure 2c?

Response: Yes. Corrected this.

3. The methods of FSEC should be more detailed, such as the column type. It is better if the X axis is volume instead of time.

Response: Changed the axis and added more descriptions of FSEC in the Method.

4. Does figure 1d show the antiparallel RSP/Apo dimer? Please clarify it in the figure legend; it may cause misunderstanding when the protein opens in the same direction but labeled with cytoplasmic open and Vesicular open.

Response: Added the following clarification in the legend: "the expected orientation (cytoplasmic- or lumenal-open) of each monomer in storage vesicles is indicated."

5. Why does the F437A affect the uptake of Serotonin but not Dopamine in figure 3g.

Response: The cause of this difference is not obvious from the structures. Our speculation is that the F437A mutation may permit a portion of serotonin molecules to bind in a different conformation, thereby lowering the overall transport activity. Dopamine may not be affected because its two hydroxyl groups allow for more specific recognition.

6. The authors should mention how the right part of Figure 5b is modeled.

Response: Added descriptions in the Fig. 5b legend. The structures and DA are superimposed as in Fig. 5a and shown in surface representation. In Fig. 5a, the lumenal- and cytoplasmic-open structures are superimposed by the CTD. The dopamine (DA) molecule is shown as presented in the lumenal-open state.

7. It could be better if Figure 5g is shown in a similar form as the upper part of Figures 5c and d.

Response: We have systematically assessed the potential protonation sites using experimental and computational approaches (new Fig. 5e-g and Movie S1). The previous Fig. 5g (D407) has been removed since we find that D34 and E320 are the most probable protonation sites in VMAT1. The D34N neutralization mutant shows largely increased reserpine binding (i.e., cytoplasmic open) without a proton gradient, as expected for a protonation site. In VMAT2, the D34N corresponding mutation shows a similar pattern[2]. Although E320Q mutation abolishes reserpine binding and cannot be analyzed experimentally, repeated MD simulation shows that E320 protonation induces NTD-CTD association at the lumenal side, as expected during the cytoplasmic-open transition.

8. The authors should explain the role of BFA in figure legend or method.

Response: Added the following explanation in Method (lines 701-703). "BFA specifically inhibits the V-type H^+-ATPase activity and collapses the proton gradient across the vesicular membrane, which is required for the monoamine uptake activity of VMAT1."

9. Which model is used for the surface presentation in supplementary 8g and 9b?

Response: Added descriptions in the legends. The surface presentation in previous Extended Data Fig. 8g (now Fig. 7i) used the reserpine-bound structure, and Fig. 9b used the modeled VAChT structure.

Reviewer 3:

1. Additional analysis of the literature, together with more in-depth studies of some of the mutants generated, could greatly benefit this milestone study.

Response: We are grateful for the reviewer's compliments and helpful suggestions. We have now systematically investigated the potential protonation sites by biochemical experiments and molecular dynamics (MD) simulation (see new Fig. 5e-g, Extended Data Figure 8, and Movie S1). Similar to VMAT2[2], the D34N mutant in VMAT1 shows reserpine binding (recognizing cytoplasmic-open state) independent of a proton gradient, a pattern expected for a protonation site. Because E320Q mutation directly abolishes reserpine binding, we investigated E320 protonation by MD simulation. Compared to the deprotonated state, the protonated E320 exhibits a significant difference: it induces NTD-CTD association at the lumenal side, as expected for initiating the cytoplasmic-open transition. In addition to these new data, more literature has been studied and referenced in this revision (main text and Supplementary Discussion).

2. The structures provide strong evidence for the central role of E320 and D407 in substrate and inhibitor binding. However, there is little or no evidence that they are the target of protonation, as pointed out in the movie and Fig 5H. A study of the D400 mutants in the rat VMAT1 isoform (D407 in the human protein) suggested a role in substrate binding and predicted that D400 interacts with Y342 (Y349 in human) via a hydrogen bond. However, a detailed study of the mutant protein D400E in both rat isoforms showed a robust, although somewhat weaker, transport activity. Even though the authors are aware of these studies (L.319), they report here that the hVMAT1 D400E mutant is inactive (L.233, Fig 2h). Is the difference in the reported results likely due to poor expression of the mutant? The Methods section does not state whether and how the results are standardized relative to expression levels.

Response: We have added new data suggesting D34 and E320, rather than D407, as potential protonation sites (see detailed response to reviewer 1, Q4). For D407E, we have conducted this experiment two more times (each with three replicates). The activities of D407E are indeed higher than our previous measurements: ~20% and 30% of WT for dopamine and serotonin uptake, respectively (Result and updated Fig. 3h). However, the relative activities of D400E in rat VMAT1

are still higher, possibly due to subtle differences from human VMAT1. We have also developed a binding assay that shows D407E is more tolerated than other mutations (new Extended Data Fig. 7f). The expression level of D407E is comparable to other mutants (see Supplementary Fig. 1). We have specified in Methods that the relative activities shown in the figures have not been adjusted for expression levels.

3. Previous studies show that position 427 of rVMAT2 (D434 in hVMAT1) does not tolerate conservative replacements. Notably, however, the negative charge in the cluster is not required for either inhibitor or substrate binding. All the D427 mutants bound Tetrabenazine with similar affinities, and MPP+ can displace it. In a preprint recently deposited at Bioxrv (https://www.biorxiv.org/content/10.1101/2023.09.05.556211v1) the authors identified a distinct polar network in hVMAT2, also previously suggested in biochemical studies, that may play a role in proton coordination and subsequent transporter conformational changes. The network lies between TMs 1, 4, and 11 and consists of residues D33, N34, K138, Q142, R189, Q192, S196, S197, S200, and D426 (hVMAT2). Is protonation of D33, as previously suggested for rVMAT2 and other MFS transporters, rather than D407, the best candidate for playing a role in proton coordination and subsequent transporter conformational change?

Response: Indeed, our new reserpine binding experiments of D34N (new Fig. 5g) suggest that D34 is probably a protonation site in human VMAT1, as D33 in rat VMAT2. We also compared D34 interactions between the alternate conformations of VMAT1 (new Extended Data Fig. 8). However, this experimental approach is not conclusive for D407 because D407N hinders the reserpine binding. For MD simulation, the protonated D407 behaves similarly as the deprotonated form, unlike the dramatic change induced by protonated E320 (new Fig. 5e). Please also see our detailed response to Reviewer 1, Q4.

3. Regarding the catalytic cycle proposed by the authors. Defining the lumenal-facing conformation as the resting state is misleading. It may be the resting state in solution, in the crystal, and even in the membrane in the absence of a proton gradient, but this is not the case when the protein functions. The intravesicular pH is acidic, and the protein would undergo protonation and most likely isomerize to the cytoplasmic facing state, release the protons, and be ready for the following substrate molecule. Could this be the "resting state" of the functional protein? The substrate is more likely to bind with high affinity to the non-protonated species and induce the conformational transition. Protonation at the lumenal side may decrease the substrate affinity and facilitate release.

Response: We have changed “resting state” to “preferred state” in contexts involving protonation to provide a more accurate description. Biochemical evidence suggests that, even with a proton gradient, the lumenal-facing conformation remains a preferred state. The transport of VMATs (and VAChT) shows an interesting behavior of $K_M << K_D$ (10-100 times)[20], suggesting that substrate-occupied import is much faster than the exposure of the empty, cytoplasmic-open site[14]. In other words, protonation-induced transition to the cytoplasmic-open state is rate limiting. Moreover, the transport V_{max} is similar for different substrate types[21,22], suggesting that a conformational

transition, instead of binding/release, is rate limiting. Due to the slow rate of the cytoplasmic-open transition, VMATs spend more time in the preferred lumenal-open state. Consistently, the structures show that the lumen-open state is stabilized by twice as many gating interactions. This rate and stability difference contribute to the preferred import of monoamines, as expected for their dramatic accumulation in storage vesicles.

Specific points:

Transmembrane proteins in intracellular organelles present unique issues of nomenclature. I suggest the authors refer to the cytoplasmic and luminal faces of the protein (not vesicular-open) and adhere to these names to avoid confusion.

Response: Changed all 'vesicular-open' to 'lumenal open' in the text and figures.

I am somewhat surprised that there is no discussion on the possible reasons for the insensitivity of this isoform to Tetrabenazine. Since they provide information about the binding of so many substrates/inhibitors, could they, at least, speculate about Tetrabenazine?

[This has been redacted.]

In the Discussion, we added “as to VMAT2, this isoform shares 78% sequence similarity with VMAT1 and is specifically inhibited by tetrabenazine. Conformationally sensitive mutations in VMAT2 lead to tetrabenazine resistance, whereas chimeric constructs of VMAT1 possessing TM5-8 and TM9-12 from VMAT2 exhibit tetrabenazine sensitivity. Thus, difference in the structural flexibility of these TM regions, along with variations in the substrate-binding pocket, may account for the distinct tetrabenazine sensitivity of these VMAT isoforms”.

L.40: The authors state: "Reserpine binding stabilizes a cytoplasmic-open conformation resembling a protonated state, while other structures capture a vesicular-open conformation stabilized by extensive gating interactions." I propose a different explanation. Reserpine binds to the non-protonated state of the cytoplasmic-open conformation. Without a proton motive force (lumen not acidified), the resting state is the lumen-facing conformation; therefore, the Reserpine binding site is unavailable.

Response: This explanation is certainly plausible too. We changed the sentence to “reserpine binding captures a cytoplasmic-open conformation, while other structures show a vesicular-open conformation stabilized by extensive gating interactions.”

The substrate binding is to a deprotonated state of the cytoplasmic-open conformation[26], but we are uncertain whether reserpine binds before or after the deprotonation. In the reserpine-bound structure, E320 is surrounded by nonpolar groups of the protein and reserpine (Fig. 5c) and may have an unusual pKa in this hydrophobic environment[14]. Protonated E320 neutralizing its negative charge might be energetically preferable in this environment.

L.112: Was Reserpine added during or before solubilization? Solubilized VMAT2 does not bind Reserpine since its binding requires the proton motive force. Does hVMAT1 behave differently?

Response: Corrected in lines 110-111, as reserpine was added before detergent solubilization. Reserpine was incubated with cells for 10 min before membrane disruption (i.e., in presence of the proton gradient), and therefore can bind to hVMAT1 at this stage. Please see Supplementary Discussion for elaborated explanations.

L.265: The appropriate reference to MPP+ is nine rather than 10.

Response: Changed. Thanks for noticing this.

L.295 and 296: The appropriate reference is 12 rather than 41 and 27.

Response: Changed.

The paragraph summarizing the mechanistic findings needs some clarification:

L.324: "The vesicular-open state, stabilized by extensive gating, is energetically favored and predominates as the resting state." Again, this is only in the absence of a proton gradient and is irrelevant when describing the catalytic cycle.

Response: Changed to "the lumenal-open state, stabilized by extensive gating interactions (Fig. 2b), is an energetically favored state". Please see our explanation above in Q3.

L.330: "This deprotonation triggers a return to the vesicular-open state" should be: This deprotonation AND substrate binding triggers the return to the lumenal-facing conformation.

Response: Agreed and changed. The substrate binding can play a major role for this transition according to the canonical MFS mechanism.

L.337: Regarding this suggestion, please see reference Maron et al. J. Biol. Chem 1983 Vol. 258 Pages 11476-81. I wonder whether this is relevant but may connect with the authors' suggestions.

Response: Thanks for pointing this out. The paper shows low pH in the vesicular lumen hinders monoamine leaking. This is consistent with our proposal in the Discussion that the protonation-precluded export and preferred monoamine import together ensure monoamine enrichment.

L.347-352: References 27 and 45 are for VAChT, not VMAT. Please insert appropriate references.

Response: Ref. 27 estimates the vesicular K_D of VMATs and states that they use very similar mechanism as VAChT. We have not found other literature discussing this topic. Ref. 45 removed.

Fig 3 legends: Please provide the values of 100% uptake.

Response: Added to Fig. 3 and Fig. 5 legends.

Fig 5h and Movie: Unless more support is provided that E320 and D407 are the targets of protonation, the authors should consider refraining from pointing them specifically.

Response: Agreed. Removed Fig. 5h and removed these labels in the Movie.

Extended Data Fig 7: Since most of the published mechanistic VMAT studies are on the rat homologs, it would be helpful for the reader to add them to the alignment so that the residue numbering can be quickly understood.

Response: Added rat VMAT1 and VMAT2 to the sequence alignment.

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Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

In my opinion, the revised Ms by Ye et al. is much improved. The authors have undertaken several additional experiments to support the structural work and flesh out the mechanism. In particular, the binding assay (ED Fig. 7f) provides information on the role of the side chains in ligand recognition, and the assay data in Fig. 5e-g and ED Fig. 8 support an important role for D34 in proton-binding in VMAT1. The updated discussion and transport model are also much improved and present a more balanced assessment of the implications for this data in understanding VMAT1 function and the SLC18 family more generally.

Referee #2 (Remarks to the Author):

The authors have satisfactorily addressed the concerns in the early manuscript. The newly added experimental and computational data solidifies the structure interoperation and proton antiport mechanism. I don’t have further concerns. Thank you, and congratulations.

Referee #3 (Remarks to the Author):

The authors have effectively addressed the issues I previously raised and implemented the necessary modifications. However, I would like to stress the importance of the terminology issue, specifically the terms' preferred state' and' resting state.' I suggest using' lumenal open state' instead of' preferred state.' The evidence presented in their rebuttal does not convince me enough to label it as a' preferred state.' The conformation is likely to shift to the cytoplasmic open state in the acidic pH lumen, even if this transition is rate-limiting. It's important to note that conformational transitions typically occur at a slower pace than binding and release.

Author Rebuttals to First Revision:

We greatly appreciate the highly favorable comments from all the reviewers. The response below is to address a remaining terminology issue raised by reviewer 3.

Reviewer 3: I would like to stress the importance of the terminology issue, specifically the terms' preferred state' and' resting state.' I suggest using' lumenal open state' instead of' preferred state.' The evidence presented in their rebuttal does not convince me enough to label it as a' preferred state.' The conformation is likely to shift to the cytoplasmic open state in the acidic pH lumen, even if this transition is rate-limiting. It's important to note that conformational transitions typically occur at a slower pace than binding and release.

Response: We removed the word "preferred" in this sentence (line 151) and now only state the "luminal open state." However, we remain respectfully disagree with this opposing viewpoint. Even if binding and release are faster than conformational changes, rendering them comparatively negligible, it remains imperative to consider the relative rates between the alternate conformational changes, i.e., rate of lumenal-open transition (k_{lum}) vs. rate of cytoplasmic-open transition (k_{cyto}). k_{cyto} is the rate-limiting step for the entire transport cycle that includes k_{lum}. Thus, $k_{cyto} < k_{lum}$. Because the equilibrium constant $K = k_{lum} / k_{cyto}$, the balance shifts in favor of the lumenal-open state.