nature portfolio

Peer Review File

Structures of native SV2A reveal the binding mode for tetanus neurotoxin and anti-epileptic racetams

Corresponding Author: Professor Janine Brunner

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript reports the structure of native SV2A purified from sheep brain. This work is significant because SV2A is an essential protein found in synaptic vesicles of neurons and is targeted by anti-epileptic drugs. SV2A-specific nanobodies were used to purify SV2A from brain and tetanus toxin (TeNT) was mixed with the purified proteins to form a large complex with the luminal domain of SV2A. The structure shows that Nb and TeNT sandwich the LD resulting a well defined structure in this region and describe a unique binding site for TeNT as compared with botulinum neurotoxin.

The density maps of the LD and toxin are of high-quality in particular the local TeNT map provides details of interactions not previously seen including with gangliosides.

The TM regions are not as high resolution but still allow for fitting of most of the residues. The LEV drug molecule is small and this makes detailed interpretation of the binding pose difficult at this resolution.

The lack of biochemical data supporting the binding site of LEV by mutagenesis and direct ligand binding studies is a weakness. While the authors show that addition of LEV to the protein thermostabilizes SV2A, it would be important to show using biochemical experiments that 1) the natively purified SV2A can bind LEV 2) that when key residues involved in binding are disrupted, that LEV binding is lost. Along these lines, several hypotheses are proposed in the discussion about how LEV is selective to SV2A but none of these ideas have been explored experimentally.

It is not appropriate to report the resolution of the Phenix density modified map as being higher resolution than the locally refined map.

Please also remove any reference to 'high-resolution' throughout the manuscript. This is subjective, for instance a crystallographer may consider anything better than 2 A to be high-resolution. Thus, the resolution is whatever value that has been calculated and should simply be stated. Please also do not refer to interaction strength in the context of structure as this cannot be directly derived without biochemical experiments.

Please report all of the Nb sequences that were used in the study in the supplemental. This is essential for reproducibility. Why does the description of the method of how the Nbs were made differ so significantly between the Nat Comm manuscript and the Biorxiv?

Line 390 - SV2C does not bind LEV, please remove this speculation that it does. There are many reports showing that LEV binds only to SV2A.

Line 399-402 - the disulfide may be present also in the recombinant protein but not well-resolved. Since the LD in these structures is sandwiched by Nb and TeNT this may reduce the flexibility in this region which would allow the disulfide to be resolved. This possibility should not be excluded.

The authors report that their structure is outward-open. As this protein is found in vesicles, this should be changed to luminal-open to reflect that SV2s are residents of the SV. Throughout the paper, 'out' should changed to 'lumen'. In addition, other groups have reported that this conformation is luminal-occluded due to the conformation of the ditryosine motif. Are

there any conformational differences between this structure and other recent reports?

The writing of the paper is rough and I would encourage the authors to polish the writing further as it reads like a draft. Many sentences are understandable but awkwardly written. This is important work so I would appreciate if the authors could improve this as it will make the manuscript difficult for non-experts to understand. I will highlight several sentences from the abstract/introduction where this is an issue, but the authors should go through the entire manuscript to fix these problems:
Line 15: bona fide - please choose a more appropriate word here.
Line 18: as receptor - as a receptor
Line 20: we subjected native SV2A to cryo-EM - please define that native SV2A means that it has been purified from brain tissue.
Line 24: Further, a LEV-bound structure - Further, our LEV-bound..
Line 29-30: Synaptic vesicles (SVs) that store neurotransmitters for release into the synaptic cleft upon fusion with the presynaptic membrane are among the best studied organelles in the cell. - Synaptic vesicle store neurotransmitter...and are among...

Reviewer #2

(Remarks to the Author)
This manuscript describes a cryo-EM structural study of native SV2A in complex with TeNT HC and an antiepileptic drug, LEV. The authors developed Pro-Macrobodies (PMbs) recognizing SV2A and successfully purified the SV2A from sheep brains. Further, TeNT-HC binding to SV2A greatly improved the quality of the cryo-EM images and allowed the authors to determine the tripartite complex of SV2A-PMb-TeNT HC. The cryo-EM structure reveals that TeNT HC binds to the N-terminal β-strand of the SV2A LD4, which is the opposite side from the binding interface with BoNT/A. Thanks to the native SV2A, the authors first identified the endogenous gangliosides that allow TeNT (and BoNT) to target the neuronal cell membrane in the cryo-EM map. In addition, the LEV-bound SV2A structure was also determined. The LEV in this report was placed differently from the other group's structure.

Overall, the manuscript is well-written, and the experimental data appear to be of good quality. Although similar structural studies for the SV2 proteins have been reported recently (Yamagata et al. 2024; Mittal et al. 2024), the TeNT-bound structure is a great advance. In addition, the structures of the native SV2A provide several new findings, which were not identified in the other structures using the recombinantly expressed protein. However, I have some concerns, particularly about the bound LEV structure.

Major
1) The authors placed LEV differently from the other group's report. The authors proposed that Cys297 forms a hydrogen bond with the carbonyl of LEV, which may explain why SV2B does not bind to LEV. However, it is difficult for me to tell if the current modeling of LEV is reasonable or not from the cryo-EM densities shown in Fig. 5C. Please provide the cryo-EM densities in different orientations. I am concerned that the authors modeled LEV to satisfy that SV2C does not bind to LEV, rather than the experimental cryo-EM densities. Did the authors test the refinement with the same orientation of LEV as the other group's report? The authors improved the cryo-EM map with local refinement, followed by the density modification with Phenix. Although I agree that these techniques are already established and highly reliable, I am a little concerned that these modifications reduce the detail of the densities of the bound LEV and induce some artificial effects. Therefore, I ask the authors to show the cryo-EM maps of the global refinement, the local refinement, and the density modification so that the readers can understand how these modifications improved the cryo-EM densities of the bound LEV. Also, cryo-EM densities of the LEV-binding site in the apo state should be shown.

2) The previous mutational studies indicated that the mutation of Cys297 to alanine showed only a limited effect on the binding affinity with the racetam derivative (Shi J., et al. 2011 Biochem. Soc. Trans. Doi:10.1042/BST0391341), which is controversial to the structure in this paper, in which Cys297 likely plays a critical role in binding. Please consider the additional biochemical assays with the associated mutations, which could strengthen the paper and alleviate these concerns.

3) The authors performed a thermal shift assay to verify the binding of SV2A to LEV. However, 250 μM LEV used in this assay seems unusually high, considering a few micromolar levels of the binding affinity of LEV. Did the authors observe a similar thermal shift with the lower concentration of LEV (such as 50, 100 μM)?

4) The native SV2A structure reveals the disulfide bond between Cys198–Cys583, which was not observed in the previous studies using the recombinantly expressed proteins. Consider examining the disulfide bond formation of the native SV2A protein using mass spectrometry. This would provide an important resource for future research.

Others
1) The model of the full-length TeNT bound to SV2A in Supplementary Fig. 4E is interesting. The horizontal orientation of the translocation helices (colored in magenta in this figure) seems similar to those of the full-length BoNT/A model bound to SV2A. However, the distance of translocation helices from the membrane of TeNT might be longer than that of BoNT (~40Å). Could the authors discuss how these structural similarities and differences are related to the functional differences of these toxins?

Minor
1) As two similar structural studies have already been published and their structures are already available from the PDB, the

part of the figures should be updated accordingly. For example, in Fig. 4D, the BoNT/A2 HC–SV2A structure is recommended instead of the SV2C LD4–BoNT/A1 HC structure.

2) Please cite Yamagata et al. 2024 in line 299.

3) I am a bit confused with the right panel of the Supplementary Fig.8. Is it necessary?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I am satisfied with the revised experiments/figures in this manuscript. However, there are two major issues that need to be addressed in a revised manuscript.

I thank the authors for providing the sequences of the Nbs. However I would point out that the sequence of Nb2 is the same sequence as Nb-8783 reported in Mittal et al, 2024 and the other Nbs appear to be from the same Nb discovery campaign. My understanding is that these Nbs are the IP of UCB Pharma, as this is how Mittal et al have obtained them. I understand that the Nbs were raised likely before many of the authors began studying these proteins. However, the authors should cite Mittal et al for Nb2 and possibly clarify how these Nbs were generated and their ownership.

The other problem is that the writing is still quite rough and the authors have not done an adequate job to fix these problems. I have now gone through the manuscript and have listed each line where I feel could be improved. As I have said in my earlier review, the authors could do more than this to improve how the manuscript is communicated:

Line 15: genuine < change to 'resident'
Line 19: potentially tetanus neurotoxin < this has been shown in many studies, it is not 'potentially' the biochemical studies that have been cited are definitive.
Line 23: central neurons < neurons in the brain or central nervous system
Line 35: early proposed < In early studies it was proposed..
Line 36: further considered as a regulator of presynaptic Ca2+ levels < this is unspecific. Some studies proposed it was a Ca2+ transporter but it does have a clear function in Syt regulation which involves Ca2+.
Lines 37-38: However, a substrate, potentially even a neurotransmitter or modulator, could not be assigned to SV2A 37 (but see7), and a Ca2+ uptake activity could not be supported. < this is awkwardly written.
Line 40: On electrophysiological level < Electrophysiological studies show..
Line 45-46: but no clear molecular explanation could be suggested derived from these experiments < I don't think this is accurate. Syt trafficking is normal in these experiments so this is suggests another function.
Line 48: at the core of the protein in the center of the membrane < at the primary binding site.
Line 48: SV2 proteins are interacting with < SV2 proteins interact with
Line 53: SVs through attached keratan sulfates < this has been debunked and did not make sense to begin with. Please see Bradberry et al 2023, this is discussed later on in the manuscript.
Line 59-60: of which the latter two were previously identified as essential in rescue experiments of the SV2A KO < of which the Trps were shown to be essential for SV2A function in neurons.
Line 64: To settle questions < To address the binding site of LEV
Line 67: together with < and
Line 70: for the largest part of < for the majority of
Line 75: the peculiar way of TeNT to enter neurons < the peculiar pathway of entry of TeNT in neurons
Line 75-77: The toxin enters first motoneurons, to reach the postsynaptic endings thereof where signal input from central neurons is received, by retrograde trafficking inside endosomal structures. < TeNT first enters into motor neurons which receive inputs from the CNS, reaching the postsynaptic terminals by retrograde trafficking via endosomal pathways.
Line 77: TeNT is then released into the synaptic cleft and enters the presynaptic central neurons, but different from the entry, now by endocytosis into SVs < TeNT is released into synapses and enters CNS neurons by endocytosis into SVs.
Line 107: valid < conserved
Line 115: thus not < thus could not
Line 117: that the LD4 is by itself a flexible element, too < LD4 is also inherently flexible.
Line 140: roofed < capped?
Line 154-155: as judged from the clear density in our map < subjective, please remove and its also not necessary. The density is present and I agree with the modeling of the disulfide.
Line 157: transmembrane part < transmembrane domain
Line 164-165: but whether the LD4 would serve the perception of such molecules is currently difficult to state. < whether LD4 is a binding site for hydrophobic molecules is not known.
Line 166: We noticed that SV2A is not residing in the center of the micelle < We observed that SV2A does not..
Line 168-169: cause the asymmetric micelle < result in asymmetry in the position of SV2A in the detergent micelle
Line 169: but a loose association with SV2A might cause this. < this is speculation and I would remove this.
Line 177: We do not see indications < We do not see any densities for
Line 197: TeNT is also binding to SV2B (but not SV2C) < TeNT binds SV2B but not SV2C.

Line 213: is apparently only possible with full-length protein due to the requirement of all windings < this does not make sense, please rephrase.
Line 220: TeNT binds to gangliosides as nearly all CNTs < TeNT like nearly all CNTs binds to gangliosides.
Line 224: and could, fit < and could fit
Line 235-236: A possible explanation for this is that in the previously reported of the X-ray structure only the oligosaccharide moiety has been applied while in our study we report the interactions with native gangliosides which are further embedded in a detergent micelle. < Only the isolated oligosaccharide was added to the protein during crystallization?
Line 259: results in precise levelling of the height < Rephrase, this is not understandable.
Line 263-273: This idea of exchanging the binding sites of TeNT and BoNTA I do not understand. It seems like the main point is that TeNT needs to bind where it does because it also needs to bind the gangliosides. It would be simpler to say that.
Line 293: For a molecule with the size of Padsevonil < For larger molecules like padsevonil..
Line 300: In SV2B that is only very weakly targeted by LEV (Ki ~1mM) < LEV binds very weakly to SV2B, Ki ~1 mM.
Line 312: This circumstance speaks also for the chosen < This mutagenesis result explains the pose of..

I have noted a few more lines in the discussion but this list is not exhaustive:

Lines 326-327: Their apparently indispensable involvement in the neurotransmission of vertebrates even exacerbates this state of knowledge. < Their indispensable involvement in neurotransmission suggests that their function is essential.
Lines 359-360: Rephrase.
Lines 364: motor neurons
Lines 381-385: These sentences are awkward, please rephrase.
Lines 378-379: Rephrase.
Lines 381-385: Rephrase.
Lines 397-427: I would consider rewriting this to leave a bit more possibility that the other pose reported by Yamagata is the correct pose. While I understand the authors reasoning, the densities are not definitive. Moreover, it is possible that the fitting of Yamagata is correct and that the result of Mittal et al mutating G240C in SV2B which facilitated LEV binding could have occurred through a water at the binding site that cannot be observed at this resolution.

Reviewer #2

(Remarks to the Author)
It is somewhat disappointing that the authors have not provided the biochemical data for LEV-binding and the experimental evidence for the disulfide bond formation in their sample. However, I agree that they have made faithful efforts to detect the disulfide bond with MS spectrometry analysis despite the potential technical difficulties. I also understand the technical challenges of mutational analysis in their experimental system using the native protein. Overall, I have no major concerns. I think it might be necessary for the authors to read through the manuscript and polish it up. The followings are additional suggestions that the authors should consider and discuss with the editor.

Line124: Apparently, TeNT-Hc… substantially. -> TeNT-Hc appears to stabilize … substantially.
Line152: The LD4 is connected to the TM parts through the N-terminal helix 7 and on the C-terminal end by TM8. -> The LD4 is connected to the TM parts through the C-terminus of helix 7 and the N-terminus of TM 8.
Line191: ..side chain interactions are strengthening… -> … side chain interactions strengthen…
Line194: …with Asn1280TeNT outside of LD4. (“out side of LD4” should be removed)
Line197: TeNT is also binding to… -> TeNT also binds to…
Line212: Consequently, revealing the binding of … -> Consequently, the binding of CNT to the N-terminal end of LD4 is only possible with the full-length protein.
Line237-239: The carbohydrate moiety is complexed mainly… -> I recommend re-editing for clarification.
Line308: the lack of LEV-binding in SV2B -> very weak binding to LEV by SV2B
Line432: Higher resolution structures… -> High-resolution structures, possibly better than 2 A resolution, will be needed… (please note that the previous SV2A structures’ resolutions are higher than those in this manuscript.)

Reply to the author’s comment (for Minor comments #2 in the previous revision);
The authors questioned my comments in the revision (Minor comments #2). I intended to cite the reference about the contribution of Y461 and I663 on binding to BRV. It has been cited in the revised version (line 318 in the revised manuscript).

We thank the reviewers for their invested time and efforts to evaluate our study and address all their remarks below.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript reports the structure of native SV2A purified from sheep brain. This work is significant because SV2A is an essential protein found in synaptic vesicles of neurons and is targeted by anti-epileptic drugs. SV2A-specific nanobodies were used to purify SV2A from brain and tetanus toxin (TeNT) was mixed with the purified proteins to form a large complex with the luminal domain of SV2A. The structure shows that Nb and TeNT sandwich the LD resulting a well defined structure in this region and describe a unique binding site for TeNT as compared with botulinum neurotoxin.

The density maps of the LD and toxin are of high-quality in particular the local TeNT map provides details of interactions not previously seen including with gangliosides.

The TM regions are not as high resolution but still allow for fitting of most of the residues. The LEV drug molecule is small and this makes detailed interpretation of the binding pose difficult at this resolution.

The lack of biochemical data supporting the binding site of LEV by mutagenesis and direct ligand binding studies is a weakness. While the authors show that addition of LEV to the protein thermostabilizes SV2A, it would be important to show using biochemical experiments that 1) the natively purified SV2A can bind LEV 2) that when key residues involved in binding are disrupted, that LEV binding is lost. Along these lines, several hypotheses are proposed in the discussion about how LEV is selective to SV2A but none of these ideas have been explored experimentally.

We agree that biochemical data that supports the structure of a protein with bound small molecules is necessary to make claims for an uncharacterized interaction, however for SV2 proteins and Levetiracetam (and derivatives) a substantial body of binding studies has already been published through scanning mutagenesis. These results are very much in line with our LEV-bound structure and the corresponding structure of Yamagata et al. (NatComms, 2024) and binding experiments of Mittal et al. (NatStructMolBiol, 2024). The missing evidence and insight for the findings of the earlier binding studies was a structure, and this is what we provide here. The thermostabilization experiment would ideally be repeated with mutant SV2A proteins to observe a loss of stabilization by LEV, but with our approach to purify SV2A from tissue we cannot choose this option, and such experiments thus remain important for future studies. We want to point out here that the purification from native tissue was essential regarding other aspects of our study such as the binding of TeNT (gangliosides, glycosylation and potentially the disulfide bond between LD4 and the TM part). The DSF experiment that we performed to see if our brain-purified SV2A has LEV-binding capacity is a biochemical experiment that shows qualitatively binding of the ligand, independent of the structural results. We thus wonder why the reviewer does not count a thermostabilization assay as a biochemical experiment. Radioligand binding assays would not provide us with more information in this case. Regarding the selectivity of LEV for SV2A we provide an argumentation in the discussion part that takes the observed density, the conservation of SV2A/B and the binding experiments of Shi et al.

(Biochem Soc Trans, 2011) into account. In the revised manuscript we discuss now also results from Mittal et al. where the binding of LEV to a mutant of SV2B was measured (G240C). This mutation increases the binding to LEV almost to the levels of WT SV2A (where this residue is Cys297). Our initial interpretation of Cys297 as a critical difference for the binding of LEV in SV2A vs. SV2B is strongly supported by this experiment. We also include now different views of the LEV ligand and corresponding density in the binding pocket. Within the limitations of the maps' resolution the density favors the chosen placement of LEV with the ethyl group pointing away from Cys297. Of course, this cysteine could also provide hydrophobic interactions, but so would Ile273, which we see in vicinity of the ethyl group of LEV.

It is not appropriate to report the resolution of the Phenix density modified map as being higher resolution than the locally refined map.

We thank the reviewer for this comment and have changed this in our new manuscript version.

Please also remove any reference to 'high-resolution' throughout the manuscript. This is subjective, for instance a crystallographer may consider anything better than 2 A to be high-resolution. Thus, the resolution is whatever value that has been calculated and should simply be stated. Please also do not refer to interaction strength in the context of structure as this cannot be directly derived without biochemical experiments.

The reviewer is right that the term "high-resolution" is subjective. We have used the terminology to distinguish it from the initial structures of us (SV2A-PMb1 and SV2A-PMb2), but we agree that this is not required anymore in the current manuscript and have removed the term accordingly. We also looked over the use of "interaction strength" and changed this whenever it is too speculative.

Please report all of the Nb sequences that were used in the study in the supplemental. This is essential for reproducibility. Why does the description of the method of how the Nbs were made differ so significantly between the Nat Comm manuscript and the Biorxiv?

All the Nb sequences used for cryo-EM are now part of the methods section (Expression constructs and Nanobody sequences) in the revised manuscript. We are aware that these sequences are relevant and of interest to other researchers for follow-up studies, reproducibility and new experiments, and must hence become available to other researchers. Regarding the difference between the NatComms submission and the bioRxiv preprint on the method section for the Nbs we were confronted with the situation that the Nbs were generated more than ten years ago. We did not yet have the detailed records from the method for immunization (initially only the information of the antigen used in the selection, the presence of LEV during the selection process and the ELISA positive selected clones) when we started to evaluate the Nbs for this project. The executing scientist for the immunization was already out of the institute for a long time, and we decided to submit a preprint because we had no aspects in our work where this information would be critically required to support our finding. Of course, we informed the author that this information will be required. Before the submission to Nature Communications, we needed to retrieve this information together with the performing scientist from the labnotes. For this, we looked up all the details of this immunization that are needed for a methods section in its final form.

Line 390 - SV2C does not bind LEV, please remove this speculation that it does. There are many reports showing that LEV binds only to SV2A.

We noted 100% identity between SV2A and C of the residues directly interacting with LEV and a predicted near-identical fold, which drove us to mention this observation. We agree though that our description is too strong/speculative and have changed this passage now following the reviewer's recommendation. Potentially, differences outside of the binding pocket could thus account for this difference in LEV binding but also a different preferred conformation of SV2C, which we now express in the respective paragraph.

Line 399-402 - the disulfide may be present also in the recombinant protein but not well-resolved. Since the LD in these structures is sandwiched by Nb and TeNT this may reduce the flexibility in this region which would allow the disulfide to be resolved. This possibility should not be excluded.

Indeed, a lower resolution in this region could account for the invisibility of the disulfide bond in the structures of Yamagata et al. (NatComms, 2024) and Mittal et al. (NatStructMolBiol, 2024) which both used recombinant protein. And we agree that this region is apparently very well resolved in our structures due to the stabilization of the LD4 by PMb5 and TeNT. However, in the structures of Yamagata et al. we see well resolved density of sidechains nearby the potential disulfide bridge in LD4 and the TM part which makes it somewhat difficult to argue that the missing disulfide bond is just not resolved. Instead, this supports the idea that the disulfide bond is not formed in these samples. The authors themselves expect a disulfide bond at this position. The cysteines are highly conserved, and Alphafold models are built with disulfide bond too, which we have now added in a new Supplementary Figure 7B). Their MS analysis could not reveal such a bond, and they concluded it is not present in their samples. On the other hand, we have very clear density for this disulfide bridge and given its position and conservation of the two cysteines we do not think it is artificially induced in our preparation. Yamagata and colleagues raise also the possibility that the redox potential in neurons could differ from the ER-lumen in their expression system (insect cells). For Mittal et al., the resolution at the LD4 is indeed too low to draw safe conclusions about the presence/absence of a disulfide bridge (they used HEK cells as expression host, different from Yamagata et al.). In the new version of our manuscript we do not rule out that this bond is labile or potentially dynamically forms and breaks as an element of regulation though. For the binding of LEV, it may not be required. The structures of Yamagata et al. and our structures align very well globally (see the new Supplementary Figure 13A), and thus impact on the structure of the TM part of SV2A are probably minor, at least in this conformational state. The MD simulation of Yamagata et al. shows very high motion in LD4, including even the loss of the most N-terminal beta-strand of the LD4. This could be different if the bond was present and requires more investigation.

The authors report that their structure is outward-open. As this protein is found in vesicles, this should be changed to luminal-open to reflect that SV2s are residents of the SV. Throughout the paper, 'out' should changed to 'lumen'. In addition, other groups have reported that this conformation is luminal-occluded due to the conformation of the ditryosine motif. Are there any conformational differences between this structure and other recent reports?

We think the terminology "outward-open vs. inward-open" is broadly applied in the structural description of transporters, also for transporters residing in intracellular membrane structures. Since the lumen of a synaptic vesicle is equivalent to the extracellular space our chosen description is not

misleading or incorrect. Further, synaptic vesicle transporters are facing the extracellular space during their exo-/endocytosis cycle and thus outward-open is more neutral and refers to both locations, the endocytosed synaptic vesicle and the extracellularly exposed SV2A after fusion at the PM before retrieval (this is also the location where TeNT binds SV2A). Thus, the topology of a protein residing permanently in intracellular membranes (e.g. ER) would be more precisely described with luminal instead of outside, but for an SV resident we have a cycle that includes both locations. Yamagata et al., Nat Comms, 2024, have chosen the same terminology for their SV2A structure and Mittal et al. have chosen to use luminally-open (or -occluded), we thus think this is further a decision of the individual authors rather than a strict convention.

Without insights into the full conformational space and the natural substrate, it is not always trivial - from our point of view- to discriminate an outward-occluded from an outward-open state. In related transporters (see e.g. URAT1 (Dai et al., CellResearch, 2024)), different states are described and intermediate sub-states could be defined. In URAT1 transporters, the outward-occluded state as compared to the outward-open state is characterized by the obstruction of the central cavity by movements of TM-helix 7 towards the center. The helix 7 movement in the outward-occluded state of URAT1 constricts the passage to the binding pocket much stronger than the phenylalanines (corresponding to the di-tyrosine motif) alone. Further, the overall architecture of SV2A in our structure and the location of the tyrosines resemble more the state that has been identified as outward-open in the related URAT1 (Dai et al., CellResearch 2024) as compared to (outward)-occluded. Whether such an additional occlusion through a bend in the homologous helix 7 in an outward-occluded state occurs in SV2A is not clear yet. In the revised manuscript we discuss now the potential occlusion through the di-tyrosine motif and why we think that the state is likely outward-open. Additionally, we have now included a figure (Suppl. Fig. 13) with structural alignments of recent SV2A structures, including ours, and URAT1 for which three states have been solved. In conclusion, the structures of Yamagata et al. and ours align very well with each other and the outward-open state of URAT1. In both cases, SV2A was solved as apo or LEV-bound. In contrast, the structure of Mittal et al. looks indeed as if it was occluded due to movements of Helix 1 towards the center. Interestingly, the occluded state of URAT1 is characterized by movements in Helix 7 towards the central cavity instead of helix 1. The structure of Mittal et al. has been solved with a derivative of Padsevonil, apparently this compound stabilizes a different conformational state or binds only to such a state. It is noteworthy that the occlusion in SV2A in the structure of Mittal et al. occurs through helix 1 instead of helix 7 because the first half (helix 1-6) is known to be a stator-like element with almost no changes in the helix positions during the transport cycle in this family of SLC transporters (SLC22, where SV2A is also known as SLC22B1). We further point out that a definite answer must await future investigations and the capture of more states of SV2A. We thank the reviewer for this comment as this improves the corresponding section in our manuscript.

The writing of the paper is rough and I would encourage the authors to polish the writing further as it reads like a draft. Many sentences are understandable but awkwardly written. This is important work so I would appreciate if the authors could improve this as it will make the manuscript difficult for non-experts to understand. I will highlight several sentences from the abstract/introduction where this is an issue, but the authors should go through the entire manuscript to fix these problems:

We followed the reviewer's recommendation to look through the manuscript and polish the writing to become better understandable for the readers.

Line 15: bona fide - please choose a more appropriate word here.

We exchanged the word 'bona fide synaptic vesicle (SV) constituent' with 'genuine synaptic vesicle (SV) constituent'.

Line 18: as receptor - as a receptor.

We changed the wording as suggested.

Line 20: we subjected native SV2A to cryo-EM - please define that native SV2A means that it has been purified from brain tissue.

We changed the respective sentence to: 'We subjected native SV2A purified from brain tissue for cryo-EM'.

Line 24: Further, a LEV-bound structure - Further, our LEV-bound...

We changed the expression as suggested.

Line 29-30: Synaptic vesicles (SVs) that store neurotransmitters for release into the synaptic cleft upon fusion with the presynaptic membrane are among the best studied organelles in the cell. - Synaptic vesicle store neurotransmitter...and are among...

We changed the respective sentence to the following as suggested by the reviewer: 'Synaptic vesicles (SVs) store neurotransmitters for release into the synaptic cleft upon fusion with the presynaptic membrane and are among the best studied organelles in the cell.'

Reviewer #2 (Remarks to the Author):

This manuscript describes a cryo-EM structural study of native SV2A in complex with TeNT HC and an antiepileptic drug, LEV. The authors developed Pro-Macrobodies (PMbs) recognizing SV2A and successfully purified the SV2A from sheep brains. Further, TeNT-HC binding to SV2A greatly improved the quality of the cryo-EM images and allowed the authors to determine the tripartite complex of SV2A-PMb-TeNT HC. The cryo-EM structure reveals that TeNT HC binds to the N-terminal β-strand of the SV2A LD4, which is the opposite side from the binding interface with BoNT/A. Thanks to the native SV2A, the authors first identified the endogenous gangliosides that allow TeNT (and BoNT) to target the neuronal cell membrane in the cryo-EM map. In addition, the LEV-bound SV2A structure was also determined. The LEV in this report was placed differently from the other group's structure.

Overall, the manuscript is well-written, and the experimental data appear to be of good quality. Although similar structural studies for the SV2 proteins have been reported recently (Yamagata et al. 2024; Mittal et al. 2024), the TeNT-bound structure is a great advance. In addition, the structures of the native SV2A provide several new findings, which were not identified in the other structures using the recombinantly expressed protein. However, I have some concerns, particularly about the bound LEV structure.

Major

1) The authors placed LEV differently from the other group's report. The authors proposed that Cys297 forms a hydrogen bond with the carbonyl of LEV, which may explain why SV2B does not bind to LEV. However, it is difficult for me to tell if the current modeling of LEV is reasonable or not from the cryo-EM densities shown in Fig. 5C. Please provide the cryo-EM densities in different orientations. I am concerned that the authors modeled LEV to satisfy that SV2C does not bind to LEV, rather than the experimental cryo-EM densities. Did the authors test the refinement with the same orientation of LEV as the other group's report? The authors improved the cryo-EM map with local refinement, followed by the density modification with Phenix. Although I agree that these techniques are already established and highly reliable, I am a little concerned that these modifications reduce the detail of the densities of the bound LEV and induce some artificial effects. Therefore, I ask the authors to show the cryo-EM maps of the global refinement, the local refinement, and the density modification so that the readers can understand how these modifications improved the cryo-EM densities of the bound LEV. Also, cryo-EM densities of the LEV-binding site in the apo state should be shown.

We have included now several views in different orientations from the substrate pocket with bound LEV that make it clear why we chose this orientation of LEV (see Suppl. Fig. 11A). With the chosen orientation, LEV fits better into our map than it would when the ethyl-group was facing toward Cys297. There is a slight bifurcation apparent of the arms of the butanamide moiety that is more in line with the amide. We have further included density maps with models of the region of interest with global NU refinement, local refinement, local refinement sharpened and the Phenix density modified map that document the improvement of the LEV density step by step (Suppl. Fig. 11B). We further include a view on the binding pocket in the Apo-form vs. the LEV-bound form in Suppl. Fig. 11C. A clear density is apparent in the LEV sample which is absent in the Apo-structure. Ultimately, higher resolution and probably the use of other racetam-derivatives will be required for a definite answer on the orientation of the LEV molecule (i.e. its butanamide moiety) in SV2A's binding pocket. But with the observed densities, chemical environment in the binding pocket and additional information from mutagenesis binding studies (Mittal et al.) we see the orientation of LEV as shown in our manuscript to be better supported (see also point 2 of reviewer 2). We thank the reviewer for his comments and think the suggested additional Supplementary figure 11 has improved our manuscript and justifies our interpretation better than in the first version.

2) The previous mutational studies indicated that the mutation of Cys297 to alanine showed only a limited effect on the binding affinity with the racetam derivative (Shi J., et al. 2011 Biochem. Soc. Trans. Doi:10.1042/BST0391341), which is controversial to the structure in this paper, in which Cys297 likely plays a critical role in binding. Please consider the additional biochemical assays with the associated mutations, which could strengthen the paper and alleviate these concerns.

The reviewer is right that the Cys297Ala substitution had little effect on the binding of a racetam derivative (Shi J., et al. Biochem. Soc. Trans., 2011. Doi:10.1042/BST0391341). This was not LEV though, but a compound (called compound1) with a large hydrophobic moiety at the same position as the propyl extension on the γ-lactam ring in Brivaracetam. One must bear in mind that this difference in the molecule can obscure the effect that substituting Cys297 with alanine could have on LEV, because other parts of the molecule compound 1 are involved in interactions and could also result in different positioning of the molecule. The alkylation of Cys297 showed an effect but the bulky addition may block the entire pocket, hence this modification provides only limited insight.

Additional biochemical experiments would require establishing recombinant (mutant) protein production for SV2A which currently is not part of our methods and thus this would need too much time for the review. However, Mittal et al., NatStructMolBiol 2024, have performed a similar experiment with LEV that strongly supports our notion on Cys297. When they mutated Gly240 in SV2B to cysteine (corresponding to Cys297 in SV2A), they observed almost the same K_i for LEV as in SV2A (SV2B WT: 1mM, SV2B G240C: 13 µM, SV2A WT: 4µM). Substituting Gly240 with Cysteine thus turned SV2B almost into an SV2A-protein with respect to its binding of LEV and an almost 100fold increase of K_i. According to these results and our observed density, Cys297 is apparently a highly critical residue for the binding of LEV different than the effect seen for binding of compound 1 in Shi J., et al. 2011. Akin to the effect seen for Cys297Ala and compound 1 in Shi et al, 2011, Mittal et al. did also see only minor effect of substituting Gly240 for cysteine for the binding of BRV. BRV binds to almost the same position in the substrate pocket of SV2A as LEV. Still, the propyl-moiety of BRV could dominate its binding and leaving only D670 as the relevant interaction for the amide but not the cysteine, maybe caused by a slightly different position in the pocket.

3) The authors performed a thermal shift assay to verify the binding of SV2A to LEV. However, 250 µM LEV used in this assay seems unusually high, considering a few micromolar levels of the binding affinity of LEV. Did the authors observe a similar thermal shift with the lower concentration of LEV (such as 50, 100 µM)?

Indeed, 250 µM is a high concentration when referring to the binding affinity of LEV. But in thermal shift assays the potential small molecule ligands are added in high concentrations, also for substrate searches. As a guideline for our experiments, we have used the protocols of Majd et al., eLife, 2018 (citation #76). Performing this type of assay close to the KD leads only to a minute thermal shift, and depending on the precision and uniformity of the thermal cycler this may not be sufficient to get a clearly different signal than in the control. The assay is intended here as a qualitative test for binding of the drug. We have also used this concentration to obtain the cryo-EM structure of LEV-bound SV2A, again it is common to use a ligand in large excess, both for cryo-EM or crystallization. LEV is very water soluble; it can thus be used at high concentrations, and if there is no impact on the control protein or strong effects on the buffer-only-sample, a good excess provides a clearer answer in the thermostabilization assay for a qualitative experiment.

We have performed a LEV titration experiment where we used lower and higher concentrations of LEV (as low as 2.5nM and up to 2.5mM), to demonstrate the impact of the ligand concentration on the thermostabilization of SV2A. We have included this experiment into suppl. figure 9C and D to address the request of the reviewer.

4) The native SV2A structure reveals the disulfide bond between Cys198–Cys583, which was not observed in the previous studies using the recombinantly expressed proteins. Consider examining the disulfide bond formation of the native SV2A protein using mass spectrometry. This would provide an important resource for future research.

As suggested by the reviewer we have tried to verify the disulfide bond through mass spectrometry in a VIB core facility of our institution (VIB proteomics core Ghent), and this series of experiments has required most of the time for our revised manuscript. Our sample contained (unavoidably) also the obvious disulfide bond in the nanobody (PMb5). Our sample should therefore in principle be well suited to identify a disulfide bond in the target and the equimolar internal Nb control. However, for

both proteins it was not possible to retrieve crosslinked peptides even with a series of proteases used, and with differential labelling of cysteines. Initially, using trypsin, the peptides where the disulfide bond would occur were not detectable in the unreduced sample, which we considered a good sign. Probably the peptides were too big when unreduced, and thus this region was only covered reasonably well under reducing conditions. However, when using elastase, peptides including the cysteines in question could be detected in unreduced sample but no disulfide containing peptides. Because the same was true for the Nb moiety of the PMb, where the disulfide bond is also clearly visible in our cryo-EM maps (and documented in numerous reported X-ray structures), we cannot exclude a technical reason. We thank the reviewer for the comment and fully agree that additional verification of the disulfide bond by mass spectrometry would have provided additional important information. However, either the bond is very labile, the region of SV2A too difficult to cover by MS, or a technical reason at the MS facility is causing this. Reasons why we consider the disulfide bond in our sample as natively occurring are mentioned already in a response to reviewer 1.

Others

1) The model of the full-length TeNT bound to SV2A in Supplementary Fig. 4E is interesting. The horizontal orientation of the translocation helices (colored in magenta in this figure) seems similar to those of the full-length BoNT/A model bound to SV2A. However, the distance of translocation helices from the membrane of TeNT might be longer than that of BoNT (~40Å). Could the authors discuss how these structural similarities and differences are related to the functional differences of these toxins?

We agree with the reviewer that the comparison of the full length toxins bound to SV2A would be a very interesting and a next logical step towards understanding the functioning of the CNTs. Our simple comparison however just served to illustrate that the binding of TeNT holotoxin would be possible without major clashes with SV2A as additional support for this newly identified binding site. Currently, neither a SV2A-TeNT-holotoxin, nor a SV2A-BoNT-holotoxin structure is available, and we thus think that this would involve too much speculation to be addressable. In addition, the holotoxin (at least for TeNT) was shown to undertake major conformational changes (large scale movements of the translocation domain and the light chain relative to the RBD) upon lowering of the pH (Masuyer et al., EMBO reports, 2017). We currently do not know if these changes occur also in e.g. BoNT/A. We think thus that the pH-dependent motions in these toxins and distances of the holotoxins towards the membrane are very complex and still not understood sufficiently to compare TeNT with BoNT/A on this aspect.

Minor

1) As two similar structural studies have already been published and their structures are already available from the PDB, the part of the figures should be updated accordingly. For example, in Fig. 4D, the BoNT/A2 HC–SV2A structure is recommended instead of the SV2C LD4–BoNT/A1 HC structure.

We have used now the new PDB entry from Yamagata et al. (BoNT/A2 on SV2A LD4) for the comparison of BoNT/A and TeNT binding in figure 3D.

2) Please cite Yamagata et al. 2024 in line 299.

We cannot see why the reviewer would like to see Yamagata cited in the context at this position. Maybe another line was meant? We have cited the Yamagata paper now relatively often in the revised form (BoNT/A2 binding to SV2A, disulfide bridge, outward-open vs. occluded, LEV placement), and we think, wherever it was necessary. We can only excuse if an additional citation of the study is wished for, but we cannot find which point in the manuscript was referred to by the reviewer, certainly not line 299.

3) I am a bit confused with the right panel of the Supplementary Fig.8. Is it necessary?

Since the description of the angle of TeNT vs. BoNT on the respective ends of the LD4 is probably not so easy to follow for the non-specialist, we considered an illustration useful to grasp this idea on a first glance. Our structure shows for the first time the simultaneous binding of protein receptor and gangliosides by CNTs and the different angle of the binding loop for the N- or C-terminal end is a remarkable adaptation and fully in line with the requirement of both receptors. Flipping the two toxins on the ends of the LD4 in a graphic illustrates this in the best way from our point of view, and makes it immediately understandable. It is further a supplementary figure, not impacting space requirements, and we would very much prefer that it remains available for readers.

REVIEWERS' COMMENTS

We thank both reviewers for their invested time and additional input and address all comments point by point below in italics.

Reviewer #1 (Remarks to the Author):

I am satisfied with the revised experiments/figures in this manuscript. However, there are two major issues that need to be addressed in a revised manuscript.

I thank the authors for providing the sequences of the Nbs. However I would point out that the sequence of Nb2 is the same sequence as Nb-8783 reported in Mittal et al, 2024 and the other Nbs appear to be from the same Nb discovery campaign. My understanding is that these Nbs are the IP of UCB Pharma, as this is how Mittal et al have obtained them. I understand that the Nbs were raised likely before many of the authors began studying these proteins. However, the authors should cite Mittal et al for Nb2 and possibly clarify how these Nbs were generated and their ownership.

The nanobodies that we used in our study were raised under a service contract over ten years ago between UCB Pharma and the Vlaams Instituut voor Biotechnologie (VIB, Flemish Institute for Biotechnology). We are part of the VIB. There are items/materials that remained property of UCB which are purified proteins that have been used in the selection process or chemical compounds that they provided. The cDNAs of the nanobodies are however excluded from this contract and are also property of VIB. We have clarified this ahead of our research (and would otherwise have contacted UCB Pharma). VIB has confirmed this for us. As we are part of VIB, we are thus allowed to use these nanobodies for research purposes and to publish the results. The reviewer is right, Nb2 is identical to Nb-8783. Mittal et al. have used one of the nanobodies from the same nanobody discovery campaign in the mentioned service contract and they are equally eligible to use them as UCB gave them the right to do so (UCB is also part of the study). This explains why both groups have access to these nanobodies.
We have provided the corresponding legal document to the editorial team of Nature Communications to give all necessary insight.

The other problem is that the writing is still quite rough and the authors have not done an adequate job to fix these problems. I have now gone through the manuscript and have listed each line where I feel could be improved. As I have said in my earlier review, the authors could do more than this to improve how the manuscript is communicated:

Line 15: genuine < change to 'resident'
changed accordingly

Line 19: potentially tetanus neurotoxin < this has been shown in many studies, it is not 'potentially' the biochemical studies that have been cited are definitive.
We have changed "potentially" to "presumably". The binding of Tetanus toxin to SV2A was questioned because BoNT/A and TeNT did not compete for entry in experiments by Blum et al., which are also experts in the field of neurotoxins, and we express with "presumably" that this question is debated by some scientists (e.g. Thy1 is also suggested as a receptor on central neurons). The word reflects only that this debate might not be settled for everyone in the field.

Line 23: central neurons < neurons in the brain or central nervous system
The term "central neurons" instead of CNS neurons is frequently used in the field. For example the term 'central neurons' can be found in the paper by Yeh et al. from the Chapman lab in the title: "SV2

*mediates entry of tetanus neurotoxin into **central neurons**.", Plos Pathogens, 2010. We keep this expression in the manuscript.*

Line 35: early proposed < In early studies it was proposed..
changed accordingly

Line 36: further considered as a regulator of presynaptic Ca2+ levels < this is unspecific. Some studies proposed it was a Ca2+ transporter but it does have a clear function in Syt regulation which involves Ca2+.
We are referring here only to the proposed regulation of presynaptic Ca2+ levels through a suggested transporter function in SV2 (uptake of Ca2+ into SVs leading to a (faster) lowering of Ca2+ levels at terminals) We are now pointing out this idea more clearly, even though the reference is already given.

Lines 37-38: However, a substrate, potentially even a neurotransmitter or modulator, could not be assigned to SV2A 37 (but see7), and a Ca2+ uptake activity could not be supported. < this is awkwardly written.
The passage has been rephrased

Line 40: On electrophysiological level < Electrophysiological studies show..
changed accordingly

Line 45-46: but no clear molecular explanation could be suggested derived from these experiments < I don't think this is accurate. Syt trafficking is normal in these experiments so this is suggests another function.
Yes, Syt trafficking is normal in these mutants, but still no molecular reason why Trp300 is important could be derived from the data except that the data rules out Syt in this context . The authors carefully suggest (as we do) that this supports the idea of SV2A working as a transporter, but in the absence of knowledge about its substrates one cannot state that Trp300Ala is critical because SV2A is a transporter. This must be first shown. We point out clearly in the next sentence that the data is in support of a transport function. The sentence in line 45-46 was already criticized by reviewer 1 in the first review round but reviewer1 is now referring again to the first version, not to the updated version.

Line 48: at the core of the protein in the center of the membrane < at the primary binding site.
We rephrase here but we refrain from calling this a primary binding site. This would imply that we know where an unknown substrate is binding and also that there is even a secondary binding site.

Line 48: SV2 proteins are interacting with < SV2 proteins interact with
changed accordingly

Line 53: SVs through attached keratan sulfates < this has been debunked and did not make sense to begin with. Please see Bradberry et al 2023, this is discussed later on in the manuscript.
We changed this paragraph to rule out confusion.

Line 59-60: of which the latter two were previously identified as essential in rescue experiments of the SV2A KO < of which the Trps were shown to be essential for SV2A function in neurons.
changed accordingly

Line 64: To settle questions < To address the binding site of LEV
changed accordingly

Line 67: together with < and
rephrased

Line 70: for the largest part of < for the majority of
We think this is just a matter of taste how to express here, we can't see what could be wrong in our choice

Line 75: the peculiar way of TeNT to enter neurons < the peculiar pathway of entry of TeNT in neurons
We have refined the sentence including the term pathway

Line 75-77: The toxin enters first motoneurons, to reach the postsynaptic endings thereof where signal input from central neurons is received, by retrograde trafficking inside endosomal structures. < TeNT first enters into motor neurons which receive inputs from the CNS, reaching the postsynaptic terminals by retrograde trafficking via endosomal pathways.
We have refined the sentence once more. Although both terms are used in scientific literature we changed motoneurons to motor neurons.

Line 77: TeNT is then released into the synaptic cleft and enters the presynaptic central neurons, but different from the entry, now by endocytosis into SVs < TeNT is released into synapses and enters CNS neurons by endocytosis into SVs.
We consider synaptic cleft more precise because the synapse includes pre- and postsynaptic terminals and the cleft in between.

Line 107: valid < conserved
changed accordingly

Line 115: thus not < thus could not
changed accordingly

Line 117: that the LD4 is by itself a flexible element, too < LD4 is also inherently flexible. *changed accordingly*

Line 140: roofed < capped? One could also say canopied.
We think the readers can perfectly understand what is meant with "roofed" and that this is only a minor detail in the choice of words. Capped implies also that the entry is closed by the LD4, whereas we want to say that the LD4 is just lying above this entry. We do not use "canopied" because it sounds too much like vegetation providing shade.

Line 154-155: as judged from the clear density in our map < subjective, please remove and its also not necessary. The density is present and I agree with the modeling of the disulfide.
Yes, but in other published structures there is no such density and the reader could wonder if the density we have is weak or distinct. This is therefore not subjective. Since MS data could not reveal this bond for us, the map is even more of importance and emphasizing this serves to support this finding.

Line 157: transmembrane part < transmembrane domain
changed accordingly
Line 164-165: but whether the LD4 would serve the perception of such molecules is currently difficult to state. < whether LD4 is a binding site for hydrophobic molecules is not known.
changed accordingly

Line 166: We noticed that SV2A is not residing in the center of the micelle < We observed that SV2A does not..
changed accordingly

Line 168-169: cause the asymmetric micelle < result in asymmetry in the position of SV2A in the detergent micelle
changed accordingly

Line 169: but a loose association with SV2A might cause this. < this is speculation and I would remove this.
changed accordingly

Line 177: We do not see indications < We do not see any densities for
changed accordingly

Line 197: TeNT is also binding to SV2B (but not SV2C) < TeNT binds SV2B but not SV2C. *changed accordingly*

Line 213: is apparently only possible with full-length protein due to the requirement of all windings < this does not make sense, please rephrase.
rephrased

Line 220: TeNT binds to gangliosides as nearly all CNTs < TeNT like nearly all CNTs binds to gangliosides.
rephrased

Line 224: and could, fit < and could fit
comma deleted

Line 235-236: A possible explanation for this is that in the previously reported of the X-ray structure only the oligosaccharide moiety has been applied while in our study we report the interactions with native gangliosides which are further embedded in a detergent micelle. < Only the isolated oligosaccharide was added to the protein during crystallization?
Yes. Otherwise detergent micelles would have been a necessity to keep the gangliosides in solution

Line 259: results in precise levelling of the height < Rephrase, this is not understandable.
Rephrased and placed into discussion instead of results
Line 263-273: This idea of exchanging the binding sites of TeNT and BoNTA I do not understand. It seems like the main point is that TeNT needs to bind where it does because it also needs to bind the gangliosides. It would be simpler to say that.
That is actually not what we want to express because gangliosides must also be present (in neuronal membranes) at the C-terminal side of the LD4 where BoNT/A binds. There is just no structure yet that would visualize this. What we mean is that the LD4-binding hairpin of the toxins is adapted to ensure binding simultaneously to the gangliosides and the LD4.
We rephrased this passage and shortened it. We think because it is the first structure of a CNT simultaneously bound to lipids and SV2 it is an important observation that deserves room.

Line 293: For a molecule with the size of Padsevonil < For larger molecules like padsevonil..
changed accordingly.

Line 300: In SV2B that is only very weakly targeted by LEV (Ki ~1mM) < LEV binds very weakly to SV2B, Ki ~1 mM.
changed accordingly.

Line 312: This circumstance speaks also for the chosen < This mutagenesis result explains the pose of.
changed accordingly.

I have noted a few more lines in the discussion but this list is not exhaustive:

Lines 326-327: Their apparently indispensable involvement in the neurotransmission of vertebrates even exacerbates this state of knowledge. < Their indispensable involvement in neurotransmission suggests that their function is essential.
We have deleted this sentence

Lines 359-360: Rephrase.
changed accordingly.

Lines 364: motor neurons
changed accordingly.

Lines 381-385: These sentences are awkward, please rephrase.
changed accordingly.

Lines 378-379: Rephrase.
changed accordingly.

Lines 381-385: Rephrase.
changed accordingly.

Lines 397-427: I would consider rewriting this to leave a bit more possibility that the other pose reported by Yamagata is the correct pose. While I understand the authors reasoning, the densities are not definitive. Moreover, it is possible that the fitting of Yamagata is correct and that the result of Mittal et al mutating G240C in SV2B which facilitated LEV binding could have occurred through a water at the binding site that cannot be observed at this resolution.
We refer already to the paper of Yamagata et al.. If we argue that the result of Yamagata et al. is equally likely to be correct as ours, we do not need to substantiate what we see, that would be somewhat awkward. Further, we do not criticize the placement of LEV in Yamagata et al.. The observations and argumentations are based on two different densities/maps obtained in two different labs. Therefore, based on our deviant observation, we conclude that this question is probably not settled yet and will require future investigations, i.e. higher resolution or other derivatives.

Reviewer #2 (Remarks to the Author):

It is somewhat disappointing that the authors have not provided the biochemical data for LEV-binding and the experimental evidence for the disulfide bond formation in their sample. However, I agree that they have made faithful efforts to detect the disulfide bond with MS spectrometry analysis despite the potential technical difficulties. I also understand the technical challenges of mutational analysis in their experimental system using the native protein. Overall, I have no major concerns. I think it might be necessary for the authors to read through the manuscript and polish it

up. The followings are additional suggestions that the authors should consider and discuss with the editor.

Line124: Apparently, TeNT-Hc… substantially. -> TeNT-Hc appears to stabilize … substantially.
We have changed it accordingly

Line152: The LD4 is connected to the TM parts through the N-terminal helix 7 and on the C-terminal end by TM8. -> The LD4 is connected to the TM parts through the C-terminus of helix 7 and the N-terminus of TM 8.
Changed accordingly. Indeed this expression is much simpler and better to understand.

Line191: ..side chain interactions are strengthening… -> … side chain interactions strengthen…

Line194: …with Asn1280TeNT outside of LD4. ("out side of LD4" should be removed)
We have changed it accordingly

Line197: TeNT is also binding to… -> TeNT also binds to…
We have changed it accordingly

Line212: Consequently, revealing the binding of … -> Consequently, the binding of CNT to the N-terminal end of LD4 is only possible with the full-length protein.
We have changed it accordingly

Line237-239: The carbohydrate moiety is complexed mainly… -> I recommend re-editing for clarification.
We re-edited this sentence to make it clear which carbohydrate unit is hydrogen-bonded.

Line308: the lack of LEV-binding in SV2B -> very weak binding to LEV by SV2B
We have changed it accordingly

Line432: Higher resolution structures… -> High-resolution structures, possibly better than 2 A resolution, will be needed… (please note that the previous SV2A structures' resolutions are higher than those in this manuscript.)
We have rephrased the passage to make it more clear.

Reply to the author's comment (for Minor comments #2 in the previous revision);
The authors questioned my comments in the revision (Minor comments #2). I intended to cite the reference about the contribution of Y461 and I663 on binding to BRV. It has been cited in the revised version (line 318 in the revised manuscript).
Ok