DOI: 10.11134/btp.2.2026.4

UDC 577.112.5 : 543.456 : 577.322 Original Article

DESIGN, EXPRESSION AND PURIFICATION OF VIAAT PROTEIN CONSTRUCT FOR CRYO-EM STRUCTURAL ANALYSIS

Lukashenko N.[1,†], Yantsevich A.[2,†], Patapovich M.[1*]

[1] *Faculty of Biology, Belarusian State University, Nezavisimosti ave., 4, Minsk, 220030, Republic of Belarus*
[2] *Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, Academician Kuprevich str., 5/2, Minsk, 220084, Republic of Belarus*
† These authors contributed equally to this work.
** Corresponding author: potapovich@bsu.by*

ABSTRACT

In GABAergic neurons GABA is being transported into the synaptic vesicles by unique vesicular inhibitory amino acid transporter (VIAAT). Dysfunction of GABAergic system has been implicated into various diseases of neurological spectrum, making VIAAT a reasonable target for treatment. In order to develop specific modulators of VIAAT functioning, a well resolved three-dimensional structure of the transporter is needed. Such data is absent for the moment due to protein behavior in course of cryo-electron microscopy. In this work, interspecies VIAAT hybrid protein was designed in order to improve overall stability and introduce compatibility to the Fab fiducial marker, thus facilitating structure determination. Each variant consisted of the main part of drosophila VIAAT, in which the C-terminal part was replaced with the corresponding Fab binding region of human VIAAT. Interspecies parts of the hybrid protein were linked using a helical linker. Total of 9 hybrid protein variants were expressed in HEK293F cell line. Variants able to bind Fab were selected for further purification and applied to cryo-EM for structure determination. Three-dimensional data can contribute to the understanding of VIAAT structural organization which, in turn, facilitate the search for specific functional modulators as the perspective therapeutic agents for a wide variety of diseases.

Key words: vesicular inhibitory amino acid transporter (VIAAT), γ-Aminobutyric acid (GABA), hybrid protein construct, neurotransmitters, fragment antigen binding (Fab), protein purification, cryo-EM

INTRODUCTION

In the mature brain γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter. GABA, when released into the synaptic cleft, can exert its regulatory potential on GABA receptors, that are classified into 3 types: $GABA_AR$, $GABA_BR$ and $GABA_CR$ receptors. [1] Among them, $GABA_ARs$ are considered the most abundant inhibitory neurotransmitter receptors in the central nervous system. [2, 3] In contrast, during the early development of the brain, the GABAergic neuronal system provides the formation of the main excitatory impulses. [4]. The impairment of GABARs functioning leads to the loss of excitation/inhibition balance in nervous system and associated with a wide spectrum of neurological diseases, such as epilepsy, major depressive disorders, Alzheimer's disease etc. [5, 6, 7] Being promising targets for functional modulation, many components of GABAergic system (predominantly GABARs) have undergone extensive research from the point of their structure and mechanism of functioning. It allowed to discover some pharmacological properties, including the development of specific agonists, such as benzodiazepines, neurosteroids and the new generation of anesthetics. [8]

The release of neurotransmitters in the nerve terminals requires their previous accumulation in secretory vesicles in presynaptic terminal. In GABAergic neurones GABA is being transported into the synaptic vesicles by vesicular inhibitory amino acid transporter (VIAAT). [9] It consists of 525 amino acid residues and has the molecular weight of 57 kDa. VIAAT was first identified through studies of unc-47 deficient C. elegans, where it was shown to be essential for packaging GABA into synaptic vesicles. In the same experiment, the membrane topology of VIAAT was first predicted, comprising 10 transmembrane domains with N-terminus facing cytosolic side of the vesicle and C-terminus facing luminal side. [10] Despite its well-established physiological role, the three-dimensional structure of VIAAT is still absent, making it the only neurotransmitter transporter with unresolved structure. In the studies of other neurotransmitter transporters, the method of cryo-EM structure determination was applied. Transporters' molecular weight is close to the lower limit for resolution via cryo-EM, which usually requires structure modification. In the works dedicated to vesicular monoamine transporter (VMAT2) and vesicular acetylcholine transporter (VAChT), a maltose-binding protein (MBP) tag was added to the N-terminus of VMAT2 using a helical linker and ankyrin repeat protein (DARPin), specific to MBP, was fused to the C-terminal region. DARPin and MBP form a clamp-like structure, greatly stabilizing the transporter and adding molecular weight. [11, 12] It is important to note, that the ability of hybrid transporters to specifically transport the corresponding substrates wasn't greatly disrupted. For VIAAT structure determination such fusion strategy has initially failed, making it necessary to develop a protein construct, that would stabilize the VIAAT behavior in course of expression, purification and cryo-EM sample preparation and finally allow for successful image alignment and electron density maps construction during structure determination workflow.

MATERIALS AND METHODS

VIAAT hybrid protein construct design and cloning

In order to obtain VIAAT protein, suitable for cryo-EM analysis, fusion strategy was applied. The main part of the protein was drosophila VIAAT. Previous structure determination attempts showed that dVIAAT possesses better behavior and is more suitable for image alignment. As a fiducial marker, fragment antigen binding (Fab) region of antibody, specific to human VIAAT C-terminus, was used. As far as

DOI: 10.11134/btp.2.2026.4

dVIAAT doesn't possess the sequence for Fab recognition, we attempted introducing hVIAAT C-terminal sequence (EGLIEAYRTNAED) into dVIAAT through a helical linker extended with 3 GS repeats. We attempted to incorporate such construct as the extension of different transmembrane helices of dVIAAT. Total of 9 variants of VIAAT hybrid protein were designed. In the process of designing, we payed attention to whether the linker-hVGAT C-terminus is fused to the alpha-helix facing cytosolic or luminal part of VIAAT (both strategies were attempted) and that the engineered part of the protein and native alpha-helix form a united long and straight helix without turns and excessive bends. It's needed in order to ensure the same rigid positioning of Fab relative to the protein. For each variant 2 or 3 pairs of primers were designed, containing the sequences of C-terminus and linker. The purpose of the design was to amplify and assemble the fragments of newly engineered protein in the right order using Gibson assembly and also to incorporate the rigid linker sequence using overlapping pair of primers. Using the designed primers along with cDNA template of dVGAT gene 12 PCR reactions were done in order to synthesize the desired fragments. The PCR reaction consisted of 25 rounds with denaturation temperature of 95 °C, 54 °C annealing and 72 °C extension. FastPfu Fly DNA polymerase was used. After the PCR, 2 or 3 DNA fragments (depending on the design) were assembled together via Gibson assembly to obtain a specific construct. pEASY®-Basic Seamless Cloning and Assembly Kit was used. As the vector, GFP-Ctag plasmid, containing eGFP coding gene, was used. (Figure 1)

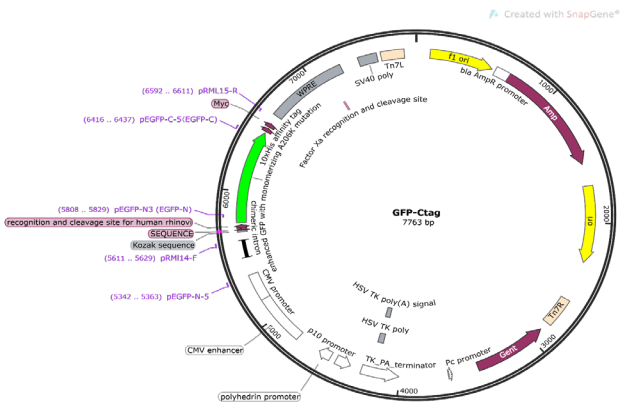

Figure 1 - Map of GFP-Ctag plasmid used for VIAAT expression in HEK293F cells

The final construct was later verified by DNA sequencing. After Gibson assembly, the *E. coli* DH5α cells (Trans1-T1) were transformed with the constructs and incubated on agar ampicillin containing plates overnight prior to positive clones' selection. Transformation was done with heat-shock method (50 °C, 90 seconds). Then liquid LB medium was added and the culture was incubated during 1 hour in shaker incubator. Afterwards, the cell suspension was centrifuged and the pellet resuspended in little volume of medium. The suspension was plated on a solid medium containing ampicillin. After the confirmation of positive clones, the construct was obtained from cell colony using plasmid extraction kit.

Expression and purification

HEK293F cells were transfected with VIAAT-GFP construct in order to obtain the target protein. Prior transfection, cells were cultured until the concentration meets 2.5-2.8 million cells/mL and diluted to 1.5 million cells/mL for transfection. For 1 liter cell culture 1 mg of plasmid was used. Polyethylenimine (PEI) was used as the transfection reagent. The PEI:plasmid ratio was 2.5 to 1. After the transfection cell culture was incubated for 6 hours in CO2 incubator under. In order to promote expression of VIAAT in HEK293F cell culture sodium butyrate was added to meet the concentration of 10 mM. Cell culture was incubated during 48-72 hours. After expression the cell culture was collected and centrifuged. Cell pellet was resuspended in lysis buffer (cocktail of detergents: 1 % LMNG, 0.3 % GDN, 0.1 % CHS, 1 mg/mL DNase, 300 times diluted protease inhibitor cocktail, 300 mM NaCl, 50 mM HEPES, 15 % glycerol, 500 times diluted βME). All further procedures were done on ice or at 4 °C to avoid protein aggregation or any structure disturbances. After that, the mixture was homogenized with mechanical homogenizer and incubated for 2 hours in cold room. The lysate was centrifuged 2 rounds for 10 and 5 minutes at 18000 g and mixed with the beads, containing GFP-specific nanobodies, then transferred to the gravity column. ATP and MgCl2 were added to the mixture beforehand, with final concentration of 2 mM and 10 mM respectively. The beads were previously washed with water (3 c.v.) and balanced with washing buffer (3 c.v.). Washing buffer contained 150 mM NaCl, 25 mM HEPES, 0.02 % detergent (DDM:CHS ratio was 10:1). The pH was adjusted with NaOH to reach 7.5. The buffer was filtered through 0.22 µm filter. After mixing protein suspension with beads in column, it was washed with 40 c.v. washing buffer. 100 µl of PreScission Protease (PPX - is a fusion protein of glutathione S-transferase (GST) and human rhinovirus (HRV) type 14 3C protease), specific for the link between GFP and VIAAT, was added to the column. The mixture was incubated overnight and after that eluted with 5 c.v. through another gravity column containing beads with GST, that bind PPX. Elution through GST beads was performed twice in order to get rid of PPX. GFP stays attached to the nanobody beads, being then eluted with guanidine HCl in order to recover the nanobody beads. After that the eluate was concentrated using Amicon Ultra-15 (50 kDa) to 1 ml volume at low speed (3900 g). Concentrated sample was transferred to the centrifuge tube and centrifuged at maximum speed for 10 minutes.

Protein-containing supernatant was collected and applied for size-exclusion chromatography to obtain a high-purity VIAAT sample. For VIAAT purification AKTA pure liquid chromatograph with Superose 6 Increase 10/300 GL column (Cytiva) was used. Washing buffer was used as a running buffer. The flow rate was set to 0.4 ml/min.1 ml of sample was injected into the loop. The fractions with expected VIAAT were collected with the volume of 0.4 ml per tube. Finally peak fractions were mixed and the concentration was measured. After chromatography purification step VIAAT sample was mixed with Fab in 1 to 1.4 molar ratio. Subsequently, the sample was applied to the second round of chromatography purification in order to separate the formed VIAAT-Fab complexes from the excess of Fab.

Antibody binding assay

In order to select the suitable protein construct, containing C-terminal Fab-binding sequence, the ability to bind Fab was

DOI: 10.11134/btp.2.2026.4

accessed. VIAAT-GFP sample was applied to antibody containing sorbent specific to GFP and washed similarly to the VIAAT purification protocol. Subsequently, Fab was added and also washed with 3 column volumes of wash buffer. After all the possible interactions between VIAAT and Fab were established, loading buffer was added directly to the sorbent and applied to SDS-electrophoresis. The successful samples should be represented on the electrophoresis lanes with separate bands for VIAAT and Fab.

Cryo-EM structure determination

After the second round of size-exclusion chromatography that allows for separation of VIAAT-Fab complex from unbound Fab molecules, the sample is considered pure enough and ready to undergo structural assay under cryo-EM workflow. Cryo-EM grids were glow discharged by ionizing air under low vacuum to ensure even distribution of the sample. A droplet of sample was directly applied in the carbon side of the grid. The grid was blotted to remove the excess of sample and plunge frozen in liquid ethane. For the process of vitrification, the Thermo Fisher Scientific Vitrobot was used. After vitrification, the grid was screened on 200 kV Cryo-TEM Talos Arctica with a K2 camera module to confirm, whether the sample is successfully applied on the grid and there's no ice contamination. Images were collected on 300 kV Cryo-TEM Titan Krios G3 equipped with a BioQuantum K3 camera. Up to 10,000 two-dimensional images (depending on the sample and grid quality) were collected. 2D dataset was processed with CryoSPARC software. 3D density map of VIAAT was obtained at the end of the workflow, which can be assessed in order to improve the design of protein for the next round of structure determination.

RESULTS AND DISCUSSION

VIAAT modeling

In course of previous experiments aimed on VIAAT structure determination via cryo-EM it was noted that the drosophila homolog of human VIAAT (dVIAAT) shows higher stability and better behaviour during expression and purification. dVIAAT is presumably less prone to oligomerization, which leads to protein loss, and able to give cryo-EM raw data with higher quality. The evaluation of human and drosophila VIAAT protein sequences has shown similarities between hVIAAT and dVIAAT (Table 1). Both proteins have similar pI and relatively close aliphatic index. Their difference in total length and weight is predominantly due to the varied N-terminal disordered region. Drosophila VIAAT has less cysteine residues, which can be one of the reasons for its structural stability, promoting less spontaneous disulfide bridges. Alignment of proteins has shown only 42.7 % amino acid sequence homology, however AlphaFold 3 modelling shows common three-dimensional organization and conservative disordered N-terminus, which make dVIAAT a suitable candidate for structural elucidation (Figure 2).

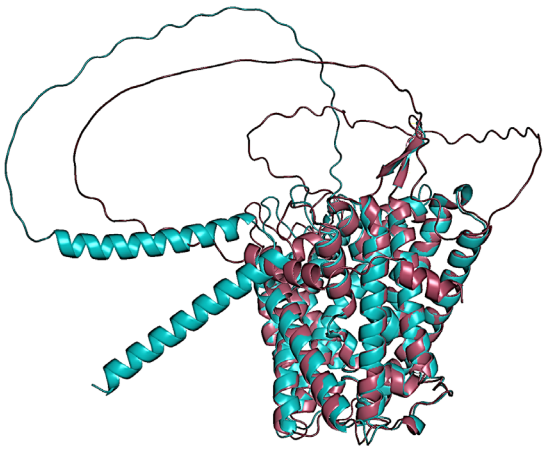

Figure 2 - Aligned 3D structures of dVIAAT (shown in purple) and hVIAAT (shown in blue)

High predicted three-dimensional structure similarity and close values of basic protein characteristics between hVIAAT and dVIAAT make drosophila-originated protein a suitable candidate for determining VIAAT structure in general.

VIAAT hybrid protein construct design and cloning

The hybrid protein construct, with dVIAAT as the main part and hVIAAT Fab-binding sequence allows to combine the stability of drosophila protein with hVIAAT ability to interact with antibodies, serving as the fiducial marker. In order to find the most suitable region of dVIAAT for fusion of hVIAAT sequence, the linker, carrying hVIAAT C-terminus, was introduced between dVIAAT residues 311, 310 or 309 and 312; 433, 432 or 431 and 434; and after 540, 539 or 538 (3 variants for each position). The final protein construct consisted of the following domains (from N- to C-terminus): 1-311 (310, 309) AA dVIAAT sequence as the «main body» (1-433 (432, 431) AA or 1-540 (539, 538) AA); alpha-helical linker (AEEEKRK), that extends one of the dVIAAT

Table 1 - Comparison of drosophila and human VIAAT basic protein characteristics

Parameters	hVIAAT	dVIAAT
Protein length	525	549
Molecular weight	57.42 kDa	60.84 kDa
Estimated pI	6.19	6.18
Aliphatic index	105.33	91.82
Length of N-terminal disordered region (residues)	117	142
Cysteine residues number	17	13

DOI: 10.11134/btp.2.2026.4

transmembrane helices to form a rigid attachment place for Fab-binding sequence; C-terminal sequence of hVIAAT forming Fab-binding site; 3 GS-repeats, separating Fab-binding site from the transporter body to avoid steric obstacles; the remaining C-terminal sequence of dVIAAT (312-549 AA (434-549 AA or none)).

Antibody binding assay

The antibody binding assay allowed to select the hybrid VIAAT variants, capable of actually binding Fab protein. The resulting SDS-PAGE profiles show two bands near 25 kDa (Fab molecular weight) corresponding to several protein variants. It makes three constructs suitable for cryo-EM analysis (lane 8,9 on the figure 3a and lane 5 on the figure 3b).

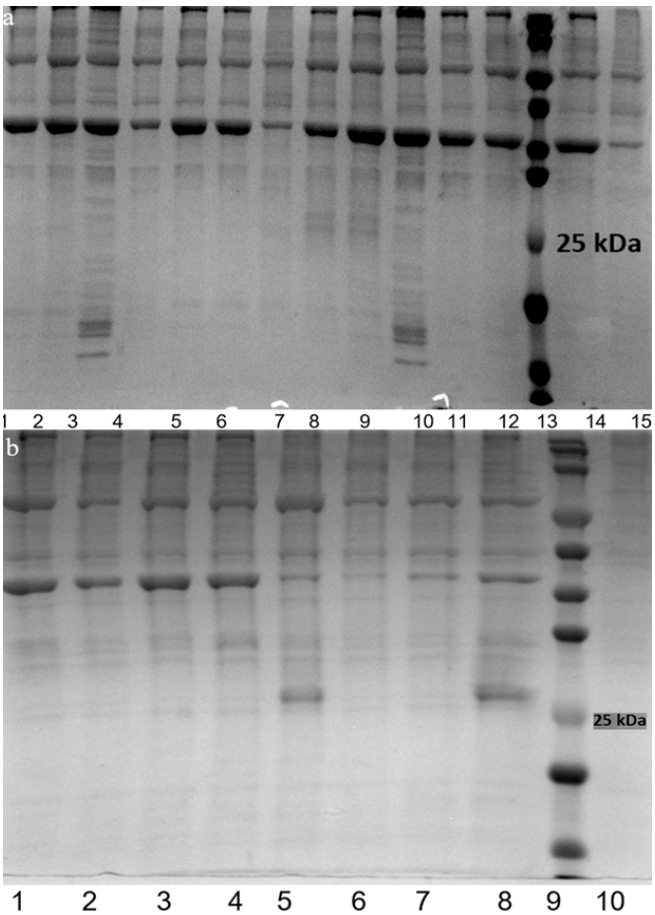

Figure 3 - SDS-PAGE profiles of VIAAT hybrid proteins, showing their ability to bind Fab.

a: 1-5 – constructs 1, 3, 4, 7, 9 without Fab; 6 – positive control without Fab; 7 – negative control without Fab; 8-12 – constructs 1, 3, 4, 7, 9 with Fab; 13 – ladder; 14 – positive control with Fab; 15 – negative control with Fab. 25 kDa bands (corresponding Fab) are visible in case of variant 1 and 3 (lanes 8, 9). b: 1-3 – constructs 2, 5, 6 without Fab; 4 – positive control without Fab; 5-7 – constructs 2, 5, 6 with Fab; 8 – positive control with Fab; 9 – ladder; 10 – negative control with Fab. Construct 2 showed interaction with Fab (lane 5)

Expression and purification

VIAAT is initially expressed fused to GFP on its C-terminus. The expression vector carries the eGFP gene directly after the cloned target sequence. GFP is used in course of affinity chromatography, where VIAAT-GFP is attached to GFP-specific nanobodies. After the sample is washed, VIAAT is cleaved away from GFP with human rhinovirus 3C protease, which recognition and cleavage site is located between VIAAT and GFP.

Hybrid VIAAT protein was successfully expressed and purified with chromatography methods. During the first round of size-exclusion chromatography, monomeric VIAAT was separated from the oligomer fraction. (Figure 4) The concentration of VIAAT in the collected sample made up 3.4 mg/ml. Despite relatively high sample concentration, some protein was lost during size-exclusion chromatography due to its tendency to aggregate. The final content of VIAAT from 1 ml of sample was 0.8 mg comparing to 3 mg before chromatography.

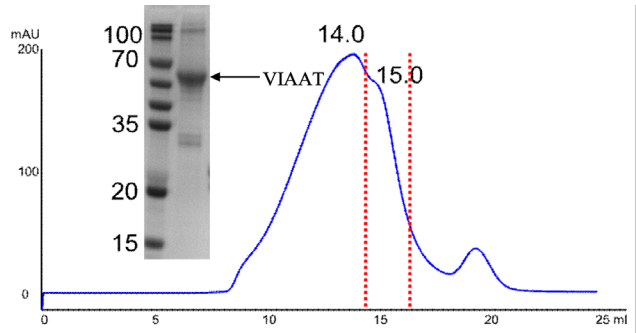

Figure 4 - Size-exclusion chromatography profile of purified VIAAT.

A major oligomer peak is eluted around 14 ml. Fraction collection window is indicated with red dashed lines and corresponds to monomer VIAAT. Inset, SDS-PAGE of the collected fractions demonstrating protein homogeneity and correspondence to the expected molecular weight of 55 kDa.

Second round of size-exclusion chromatography was performed after VIAAT mixing with Fab (Figure 5). As a result, VIAAT-Fab complex was separated from the remaining VIAAT oligomers and the excess of Fab. The SDS-PAGE profile showed the presence of formed complex in the collected fractions, as well as partially degraded VIAAT while containing low level of oligomers and Fab.

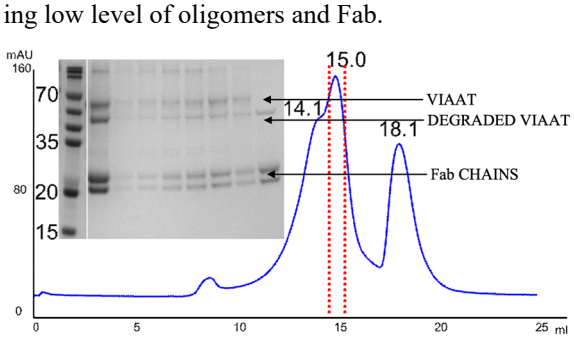

Figure 5 - Size-exclusion chromatography profile of purified VIAAT-Fab complex.

An oligomer peak is eluted around 14.1 ml. Fraction collection window is indicated with red dashed lines around 15 ml and corresponds to VIAAT-Fab complex. The excess of Fab is eluted around 18.1 ml. Inset, SDS-PAGE of the collected fractions from 13 to 18 ml. Fab is around 20 kDa, while VIAAT-Fab complex is around 70 kDa.

Cryo-EM structure determination

In the result of VIAAT cryo-EM 2D classification from

DOI: 10.11134/btp.2.2026.4

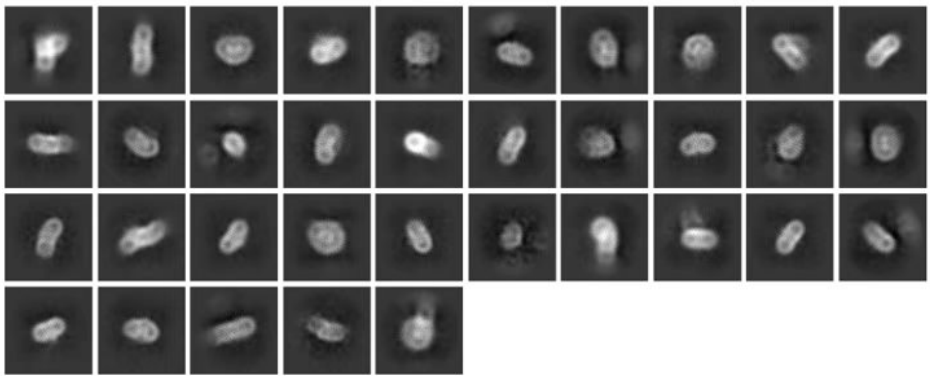

Figure 6 - 2 D Cryo-EM dataset of VIAAT

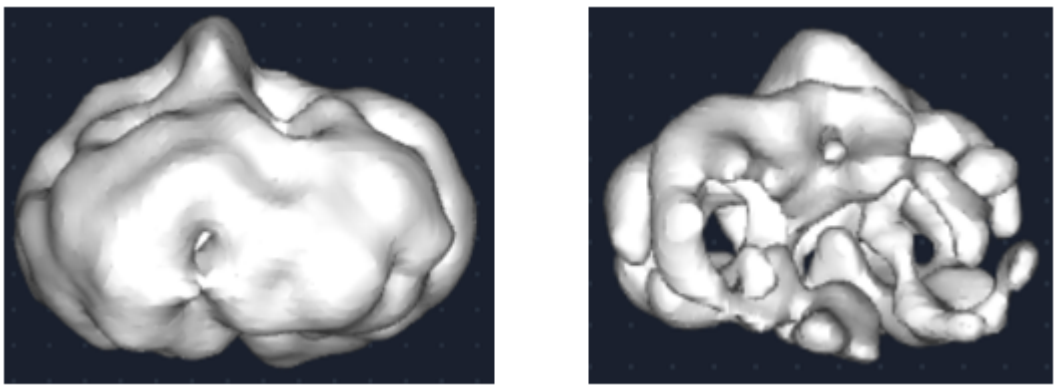

Figure 7 - 3 D density map of VIAAT

raw photographs was obtained. Typical classes are represented in the figure 6.

Further processing of 2D dataset resulted in 3D density maps of VIAAT (Figure 7).

However, the obtained density maps don't give sufficient information to construct the complete 3D structure of VIAAT, the resolution level represents major structure elements. In course of the following experiments, the protein construct should further be optimized in order to achieve more effective positioning of fiducial marker. In order to stabilize the structure, it is suggested to attempt rigid substrate binding within the transporter pore, in order to lock it in the inward or outward-facing state.

Conclusion

In current work, a hybrid protein construct of vesicular inhibitory amino acid transporter was obtained in order to facilitate structure determination. As native membrane transporters are rarely applicable to cryo-EM method, the construct was designed to bind additional elements. As the backbone, drosophila VIAAT was picked because of its better performance in course of expression, purification and cryo-EM sample preparation. In the structure of dVIAAT, C-terminal Fab-binding sequence of human VIAAT was incorporated. It allows to specifically bind Fab, serving as the fiducial marker in cryo-EM image alignment. The hybrid VIAAT expression was successfully achieved in HEK293F system. In process of VIAAT purification, size exclusion chromatography showed aggregate fraction of VIAAT, which means, that some amount of protein expressed is inaccessible for Cryo-EM structure analysis. In order to check the ability of hybrid VIAAT constructs to bind Fab, they were screened with antibody binding assay. Purified VIAAT-Fab complexes were prepared for cryo-EM analysis. In the result, 2D photographs dataset was collected and its interpretation resulted in obtaining the density maps of VIAAT. The following models are representative, however additional optimization of hybrid protein is required in order to get electron density maps with higher resolution for exact determination of protein structure. VIAAT cryo-EM data can contribute to the understanding of structural organization of the whole transporter which, in turn, facilitate the search for specific functional modulators as the perspective therapeutic agents for a wide variety of diseases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, P.M.; methodology, L.N., Y.A.; statistical analysis, L.N., Y.A.; study design, P.M., L.N., Y.A..; data interpretation, P.M., L.N., Y.A..; writing-original draft preparation, L.N., Y.A.; writing-review and editing, P.M.; supervision, P.M.

REFERENCES

1. Olsen R. W. GABAA receptor: Positive and negative allosteric modulators // Neuropharmacology. – 2018. – Vol. 136. – P. 10–22. – DOI: https://doi.org/10.1016/j.neuropharm.2018.01.036

2. Zhang W., Xiong B., Zhang L., Huang X., Yuan X., Tian Y., Tian X. The Role of the GABAergic System in Diseases of the Central Nervous System // Neuroscience. – 2021. – Vol. 470. – P. 88–99. – DOI: https://doi.org/10.1016/j.neu-

DOI: 10.11134/btp.2.2026.4

roscience.2021.06.037

3. Kim J. J., Hibbs R. E. Direct Structural Insights into GABAA Receptor Pharmacology // Trends in Biochemical Sciences. – 2021. – Vol. 46, No. 6. – P. 502–517. – DOI: https://doi.org/10.1016/j.tibs.2021.01.011

4. Deidda G., Bozarth I. F., Cancedda L. Modulation of GABAergic transmission in development and neurodevelopmental disorders: investigating physiology and pathology to gain therapeutic perspectives // Frontiers in Cellular Neuroscience. – 2014. – Vol. 8. – P. 119. – DOI: https://doi.org/10.3389/fncel.2014.00119

5. Wang Y., Wang Y., Chen Z. Double-edged GABAergic synaptic transmission in seizures: The importance of chloride plasticity // Brain Research. – 2018. – Vol. 1701. – P. 126–136. – DOI: https://doi.org/10.1016/j.brainres.2018.09.008

6. Luscher B., Fuchs T. GABAergic Control of Depression-Related Brain States // Advances in Pharmacology. – 2015. – Vol. 73. – P. 97–144. – DOI: https://doi.org/10.1016/bs.apha.2014.11.003

7. Rivera J., Sharma B., Torres M. M., Kumar S. Factors affecting the GABAergic synapse function in Alzheimer's disease: Focus on microRNAs // Ageing Research Reviews. – 2023. – Vol. 92. – P. 102123. – DOI: https://doi.org/10.1016/j.arr.2023.102123

8. Evenseth L. S. M., Gabrielsen M., Sylte I. The GABAB Receptor—Structure, Ligand Binding and Drug Development // Molecules. – 2020. – Vol. 25, No. 13. – P. 3093. – DOI: https://doi.org/10.3390/molecules25133093

9. Aubrey K. R. Presynaptic control of inhibitory neurotransmitter content in VIAAT containing synaptic vesicles // Neurochemistry International. – 2016. – Vol. 98. – P. 94–102. – DOI: https://doi.org/10.1016/j.neuint.2016.06.002

10. McIntire S. L., Reimer R. J., Schuske K., Edwards R. H., Jorgensen E. M. Identification and characterization of the vesicular GABA transporter // Nature. – 1997. – Vol. 389, No. 6653. – P. 870–876. – DOI: https://doi.org/10.1038/39908

11. Pidathala S., Liao S., Dai Y., Li X., Long C., Chang C., Zhang Z., Lee C. Mechanisms of neurotransmitter transport and drug inhibition in human VMAT2 // Nature. – 2023. – Vol. 623, No. 7989. – P. 1086–1092. – DOI: https://doi.org/10.1038/s41586-023-06727-9

12. Zhang Y., Dai F., Chen N., Zhou D., Lee C., Song C., Zhang Y., Zhang Z. Structural insights into VAChT neurotransmitter recognition and inhibition // Cell Research. – 2024. – Vol. 34, No. 9. – P. 665–668. – DOI: https://doi.org/10.1038/s41422-024-00986-5