pH-Dependent Membrane Binding Specificity of Synaptogyrins 1-3 with Distinct Isoelectric Points (pI) Identified by Structural Bioinformatics and Molecular Dynamics

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Abstract

Synaptogyrins are integral membrane proteins involved in an essential process for neuronal communication: neurotransmitter release from synaptic vesicles. The synaptic vesicles maintain an acidic environment due to the action of proton pumps (V-ATPases), which actively transport H□ into the vesicles. This creates a gradient of protons, which enables the transport of neurotransmitters into the vesicles. In contrast, the pH of synaptic vesicles increases upon neuronal activation. To evaluate the effect of varying pH environments on synaptic vesicle proteins, synaptogyrin-1 and synaptogyrin-3 were selected for this study because of their distinct isoelectric points (pI), but highly similar structures (RMSD=1.273Å). We conducted molecular dynamics simulations to examine the membrane binding dynamics of AlphaFold3 predicted structures at the active pH of 7.25 and the resting pH of 5.5. Our results reveal that the electrostatic properties of synaptogyrins affect their membrane binding properties, such as gyration radii and root mean square fluctuations. Their conformational characteristics were analyzed in correlation with variant analysis and evolutionary profiling, which uncovered several key residues contributing to their diversification. The study identifies the potential roles of synaptic vesicle pH in modulating synaptogyrin conformational and variational space, offering new perspectives on their contributions to synaptic function.

Key Words: Neuromodulation, Neuronal activation, Membrane protein design, Protein 3D structural predictions

Introduction

Neuronal communication is a well-regulated process that requires the precise release of neurotransmitters from synaptic vesicles (Teleanu et al. 2022). This release is mediated by molecular interactions such as those between proteins and lipids within the neuron (Rizo 2022). Among the key proteins in the release of neurotransmitters are the synaptogyrins (SYNGRs), a family of integral membrane proteins that are known to be involved in synaptic vesicle trafficking (Janz et al. 1999). Synaptic vesicles are vesicles that store neurotransmitters and are responsible for their rapid release into the synaptic cleft upon neuronal activation (Gowrisankaran and Milosovic 2020; Farsi et al. 2017). The pH changes upon neuronal activation, typically due to the vesicle's proton release during fusion and neurotransmitter unloading, are essential for the synaptic vesicle cycle (Gowrisankaran and Milosovic 2020; Farsi et al. 2017). Despite their importance, the specific mechanisms by which synaptogyrins contribute to or are affected by these processes remain incompletely understood (Gowrisankaran and Milosovic 2020).

Synaptogyrins are integral membrane proteins localized to synaptic vesicles that modulate synaptic vesicle trafficking and may influence synaptic strength and plasticity by affecting vesicle availability and fusion dynamics (Janz et al. 1999). Despite their highly conserved features and fundamental roles, some studies show that their knockout does not significantly affect health (Park et al. 2024; Stevens 2012). High isoform counts with overlapping functions or compensatory mechanisms may be responsible for this (Park et al. 2024). Synaptogyrins also have essential functions for synaptic plasticity (Janz et al. 1999). In the brain of an Alzheimer's disease patient, the binding interaction of Tau to synaptic vesicles is mediated by synaptogyrin-3 (SYNGR3) (Largo-Barrientos et al. 2021). Interestingly, Alzheimer's disease and aging often involve pH changes (Decker et al. 2021).

This study intends to explore the membrane binding behaviour of synaptogyrin-1 and synaptogyrin-3. Our main reason for focusing on these proteins was their distinct biochemical diversification. Synaptogyrin-1 and synaptogyrin-3 are two isoforms that, although similar in structure, exhibit distinct isoelectric points (pI). This difference in pI suggests that they may interact differently with the lipid environments that have different pH levels, such as the synaptic vesicles. The synaptic vesicles maintain an acidic environment (~pH 5.5) due to the action of proton pumps (V-ATPases) that actively transport protons (H^+) into the vesicles (Forgac 2007; Farsi et al. 2017). This creates a proton gradient, which is essential for neurotransmitter storage, as the acidic pH facilitates the proton-coupled transport of neurotransmitters into the vesicles (Gowrisankaran and Milosovic 2020; Farsi et al. 2017). It has been shown that the lumen of synaptic vesicles achieves a pH of approximately 5.5 (Miesenböck 1998; Ahdut-Hacohen et al. 2004). Upon neuronal activation, the fusion of synaptic vesicles with the plasma membrane releases their neurotransmitter cargo into the synaptic cleft (Gowrisankaran and Milosovic 2020). This process is accompanied by a shift in vesicle pH, which not only affects the vesicle's ability to participate in neurotransmitter release but also can affect the reacidification of the vesicle post-release, thus ensuring a cycle of vesicle reuse and neurotransmitter recycling (Farsi et al. 2017). This pH change is likely to impact the binding affinity of proteins whose function is pH dependent. Understanding these interactions is crucial, as the pI of a protein can significantly influence its electrostatic interactions with membrane lipids, thereby affecting its binding affinity and specificity. The pH-dependent properties are also crucial in targeted drug release because they enable drugs to be released at specific locations in the body where the pH is distinct from other areas (Abdella et al. 2023).

To investigate these interactions, we employed all-atom molecular dynamics simulations in lipid bilayers, allowing for the detailed study of protein interactions in a dynamic environment. Lipid bilayers were constructed using a realistic model based on previously established lipid maps (Binotti et al. 2021), according to the quantitative lipidomic analysis of mammalian synaptic vesicles (Takamori et al 2006). By simulating the interactions of synaptogyrin-1 and synaptogyrin-3 with synaptic vesicles under varying pH conditions, we revealed how pI and membrane composition influence the binding specificity and functional roles of these proteins. The evolutionary basis of diversification and mutational clustering of specific amino acids was inspected through a statistical analysis of homologous sequences. Statistical frameworks were enhanced by taking into account of confounding effects that complicates underlying variational dynamics. Accordingly, we utilized nonparametric correlation analysis and multivariate statistics to distinguish amino acid changes that result the diversification we see. Furthermore, we employed variant analysis and phenotypical mapping to

establish supporting clinical evidence and uncover a potential epistasis between variants of these proteins. Understanding these interactions will provide valuable insights into the molecular mechanisms that regulate synaptic proteins, potentially revealing new aspects of synaptic function and neurosecretion.

Results and Discussions

Comparative structural and evolutionary analysis of synaptogyrins 1 and 3

The two proteins in question exhibit remarkable structural similarity, reflected by a minimal root-mean-square deviation (RMSD) value of 1.273Å, indicating near-identical 3D conformations (Figure 1). The similar protein structures across sequences imply that the core structural elements and functional domains are preserved. Although significant changes were observed between the two sequences, the amino acid alterations may occur in less critical regions that do not dramatically impact the overall protein conformation. In support of this, notable non-homogeneity and clustering were observed in the regions of variation. Certain C-terminal residues showed chemically distinct variations, which could be indicative of diversification of membrane head interactions at the intracellular side (Figure 1).

The high degree of structural similarity was further reinforced by significant sequence homology. The amino acid variations in homologous sequences of the two proteins were significantly correlated, indicating a shared evolutionary origin and functional constraints that prevent significant deviations in their sequences. The strong correlation coefficient between aligned residue-wise conservation scores (rho = 0.8819, $p<0.001$) suggests that although there are some minor differences, they maintain a high degree of similarity in evolutionary pathways, likely due to shared structural requirements. The observed covariation could be indicative of potential compensatory mutations, where changes at one position might be balanced by alterations at another to preserve overall protein functionality. These covarying residues are likely located in structurally or functionally significant regions, such as binding sites or transmembrane domains, where precise residue interactions are critical.

Interestingly, mong the 20 standard amino acids, histidine showed least correlation in homologous sequences (rho=0.5633). Histidine is unique among amino acids because its imidazole side chain can exist in both protonated and unprotonated states at physiological pH close to 7. This allows it to act as a proton acceptor or donor, making it highly versatile for catalytic activity in enzymes (Liao et al. 2013). However, this versatility also means that histidine is more frequently substituted according to specific functional needs, depending on the protein's environment and function (Table 1). Similarly, the amino acids M, C, N, P, R, and W have weaker correlations in homologous sequences (rho < 0.66), suggesting they are less constrained and can vary more independently. The less constrained positions may provide opportunities for adaptive sequence diversification without compromising core structural integrity. In contrast, highly conserved amino acids (rho > 0.80) like A, F, G, and S are likely critical for maintaining the core structural fold or key functional interfaces.

Analysis of surface charges and relative solvent accessibilities

The similar solvent accessibility profiles may imply that they interact with their surrounding environment in comparable ways, potentially engaging with similar substrates or ligands. Accordingly, a strong correlation was found in aligned relative solvent accessibilities, rho = 0.7481(95% CI = 0.6357-0.8364). SYNGR1 shows a slightly more hydrophobic character overall compared to SYNGR3. The molecular lipophilicity potential (MLP) scores for the two structurally similar proteins reveal subtle differences in surface properties. For SYNGR3, the MLP values range from a minimum of -28.01 to a maximum of 23.17, with a mean of -1.837. In contrast, SYNGR1 shows a slightly broader range, with a minimum of -31.67, a maximum of 23.98, and a mean of -1.247. These results indicate that while both proteins have comparable hydrophobic and hydrophilic surface regions, SYNGR1 is slightly less hydrophilic (mean MLP closer to zero) and exhibits a slightly higher maximum lipophilicity than SYNGR3.

Conversely, the isoelectric points (pI) of these proteins emerge as the primary distinguishing feature between them, despite their otherwise high similarity (Figure 2). This pattern indicates that while the core structural architecture remains conserved, the proteins' surface charges undergo significant alterations. The

divergence in pI between the two proteins could reflect differences in their amino acid compositions, particularly the presence of positively and negatively charged residues (e.g., lysine (K), arginine (R), glutamate (E), and aspartate (D)). For example, an increase in basic residues like K and R would shift the pI higher, making the protein more positively charged under physiological pH conditions, whereas an increase in acidic residues like E and D would lower the pI, resulting in a more negatively charged protein. On the other hand, the underlying variational dynamics are complex, since the correlations of homologous amino acid profiles showed that K, R, E, and D were varied, but they were not as independent as H and N (rho = 0.5633 and 0.6251, respectively. If direct changes of charged residues are driving the pI divergence, we would expect them to show relatively weak correlations. A multivariate analysis was conducted using amino acids that showed lower correlations (Table 2). Interestingly, it appears that the dynamics among the amino acid varieties in the homologous sequences of SYNGR1 and SYNGR3 are asymmetrically related to H and N. For K, R, and E, removing H from SYNGR3 tends to result in the greatest decrease in correlation values, suggesting a positive interaction between H and these amino acids. On the other hand, the relationship for D appears to be enhanced by N variations in SYNGR1 homologs. D is least affected by the confounding amino acids H and N. Meanwhile, the correlation of K in homologous aligned sequences significantly decreased when controlled by H (rho = 0.6655).

Membranes are typically negatively charged due to the presence of charged phospholipids like phosphatidylserine. Our model system of synaptic vesicles contains cholesterol (CHOL), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SM), and phosphatidylinositol (PIns), with the following molar percentages: 40 mol%, 17 mol%, 20 mol%, 6 mol%, 4 mol%, and 1 mol%, respectively. Among them, phosphatidylcholine and phosphatidylethanolamine are zwitterionic phospholipids, meaning they have both positive and negative charges on different parts, but their net charge is neutral. Meanwhile, phosphatidylserine and phosphatidylinositol are negatively charged. Accordingly, an increase in the positively charged areas on the protein surface could enhance electrostatic interactions with the negatively charged membrane, potentially increasing the protein's membrane-binding affinity (Dalbey 1990). Positive charges on the protein surface could help stabilize its positioning within the lipid bilayer or increase the likelihood of membrane association, especially if these regions are near the transmembrane domain (Dalbey 1990). In this context, the Coulombic potential of a protein surface indicates the distribution of its electrostatic charges. The Coulombic values for the SYNGR1 surface are as follows: minimum -12.18, mean -0.86, maximum 12.17. Meanwhile, the Coulombic values for the SYNGR3 surface are minimum -11.70, mean 1.24, maximum 12.27. For SYNGR1, the negative mean potential combined with its minimum value suggests the presence of negatively charged patches that might engage favourably with positively charged lipid headgroups. Conversely, SYNGR3's more positive mean potential might indicate a higher prevalence of positively charged surface regions, potentially interacting with negatively charged lipid regions, though it might rely more on local negative patches for stable membrane anchoring. Variations at these sites may facilitate adaptive changes to the protein's surface properties, potentially modulating pI, binding interactions, or environmental responses, all while preserving the conserved structural core.

Dynamic behaviours of the synaptogyrin-1 in membrane systems

The distinct isoelectric points (pI) of SYNGR1 (4.5) and SYNGR3 (8.42) provide an important context to their structural dynamics. The significant difference in pI suggests these proteins have fundamentally different electrical charges at physiological pH, which makes their structural similarity even more intriguing. Molecular dynamics (MD) simulations were conducted to evaluate the structural stability of SYNGR1 under different pH conditions (Figure 3). At a physiological pH of 7.2, SYNGR1 exhibited greater conformational changes through the 50ns equilibrated trajectory, as evidenced by an average root-mean-square deviation (RMSD) plateau of approximately 2 nm relative to its initial structure (Supplementary Figure S1). Residue-wise root-mean-square fluctuations (RMSF) demonstrate a remarkable variance. This indicates that SYNGR1 undergoes a conformational change in a neutral environment. However, at a more acidic pH of 5.5, the protein exhibited extended instability, suggesting a slower transition. On the other hand, the deviations remained at 1.5 nm, which is lower than the active pH of 7.5. These results highlight the sensitivity of SYNGR1's stability to pH changes, which may impact its functional role in cellular processes under varying environmental conditions.

The observed alterations in the radius of gyration profiles between the two pH conditions further indicate that the structure of the molecule or system is sensitive to pH changes (Supplementary Figure S1). As

the simulation progresses, the lower pH environment (resting state) shows a smaller radius of gyration than the active state pH. The gyration axes also vary with pH changes. This is expected, as charged residues are not uniform (Figure 2, Figure 3). At the acidic pH of 5.5, protonation of acidic side chains likely increases intramolecular repulsion, promoting a more expanded structure initially. However, as the simulation proceeds, local structural rearrangements may reduce these repulsive forces, resulting in a gradual decrease in the radius of gyration. In contrast, at pH 7.2, deprotonation of these residues stabilizes intermolecular interactions, maintaining a more compact configuration from the onset. Notably, the differences in gyration axes indicate that the conformational response to pH is anisotropic, likely driven by changes in specific regions of the protein structure. These findings imply that pH-induced structural rearrangements are not uniformly distributed but instead localized to regions where charged residues play key roles in maintaining structural integrity. Consequently, the system's sensitivity to pH could influence its functional states, particularly if the conformational changes impact binding sites or allosteric regions critical for activity.

Dynamic behaviours of the synaptogyrin-3 in membrane systems

For SYNGR3, with a pI of 8.42, at pH 5.5, the protein will be positively charged overall because the pH is below the pI. This net positive charge at pH 5.5 might help the protein engage with negatively charged surfaces (such as membranes), promoting stability through electrostatic interactions. Concurrently, SYNGR3 is more stable at pH 5.5 compered to SYNGR1, likely as a consequence of its net positive charge at this pH, which could facilitate electrostatic interactions with negatively charged entities like membranes. This stability could also be influenced by favourable protonation states, a more stable protein conformation, and the environmental conditions at this pH. At active pH (7.25), SYNGR3 reaches a plateau earlier, 20ns instead of 35ns. This could be due to better electrostatic interactions, a more favourable protonation state, or a more stable protein conformation at this pH, leading to quicker stabilization in simulations. On the other hand, SYNGR3 shows a greater conformational change during simulations.

SYNGR1 demonstrates lower stability in a neutral environment, while SYNGR3 shows higher stability both at acidic pH and in neutral environment due to its positive charge and electrostatic interactions. The distinct behaviours of these proteins under different pH conditions may have implications for their functional roles in cellular processes in varying environmental contexts. The distinct RMSF differences observed in residues 200–229 (C-terminus) of SYNGR3 at different pH conditions suggest a pH-dependent conformational flexibility that is not present in SYNGR1 (Supplementary Figure S2). Despite their structural similarity, SYNGR3 exhibits significant fluctuations in this region, particularly at pH 5.5, whereas C-terminal region of SYNGR1 maintains similarities across both pH conditions. This behaviour may be attributed to differences in protonation states, leading to alterations in electrostatic interactions, hydrogen bonding networks, and solvent accessibility. At lower pH, C-terminal region in SYNGR3 might experience protonation, disrupting stabilizing intramolecular interactions and increasing local flexibility.

Residue-wise deviations and pH dependent effects

When comparing the simulations of SYNGR1, we observe that the core transmembrane helices remain predominantly unaltered after a 50ns equilibrated simulation at active and resting pHs (RMSD below 1.5 Å). This indicates that SYNGR1 demonstrates dynamical similarities in both active and resting pH (Figure 4). Minimal fluctuations are visible, likely due to the pI of SYNGR1 (4.5), which suggests it is negatively charged at both pH values and stabilized by interactions in its environment. SYNGR1 exhibits high atomic mobility but maintains a relatively stable overall structural configuration during active pH conditions. This suggests that while specific regions of the protein are highly flexible, its core structure remains largely consistent. Conversely, SYNGR3 undergoes more significant changes over 50 ns, with red regions (RMSD over 1.5 Å), dominating the loop regions and termini, suggesting higher movement. Some areas of the transmembrane helices also show moderate flexibility in the core region. The increased flexibility in SYNGR3 is likely due to its pI (8.42), making it less negatively charged and more adaptable to environmental changes at neutral pH compared to its behaviour at the resting pH of 5.5.

The comparison between proteins after 50ns equilibrated simulation suggests that resting SYNGR1 is more similar to the active SYNGR3 state than to its resting state. Surprisingly, the resting SYNGR1 showed more structural similarity to the active SYNGR3 than to its own resting configuration. This observation suggests that conserved structural elements critical for vesicle recycling may exist, indicating that these

synaptic proteins might share key conformational features during different functional states. This similarity between resting SYNGR1 and active SYNGR3 suggests that structural elements critical for vesicle recycling (SYNGR1's role) may be either conserved or transiently adopted by SYNGR3 during activation. This further suggests functional convergence, where active SYNGR3 mimics SYNGR1's conformation to facilitate vesicle recycling after neurotransmitter release. Thus, our findings reinforce a more fluid, adaptable model of protein behaviour where structural elements can be transiently shared or borrowed across different protein states.

Variant analysis

Since we identified a differential response to pH changes, altered synaptic vesicle cycling and membrane instability caused by neurological conditions might disproportionately affect proteins like SYNGR1 and SYNGR3. SYNGR1, retaining its conformation during pH differences, might be sensitive to these pathological perturbations. Accordingly, we identified that most ClinVar-reported pathological variants are located at sites that have major differences between SYNGR1 and SYNGR3 (Figure 5). SYNGR1 has more damaging variants reported compared to SYNGR3, which could be a result of differences in their evolutionary conservation or functional roles. Variants reported for Gly3, Ser36, Asn45, Ile43, Gly143, Asp145, Ala146, Arg101 are predicted to be damaging (PolyPhen score > 0.85). Most of these damaging variants are located in regions with low structural mimicry. Among them, p.Arg101Cys introduces a Cys residue capable of forming covalent disulfide bonds. The transition from a charged to a nucleophilic residue indicates an ionic interaction networks while creating the potential for aberrant cross-linking.

Conversely, the same phenomenon could not be said for SYNGR3 variants. In this case, the damaging Ser156, Ala148, and Pro55 variants occur near less flexible regions, potentially affecting transmembrane stability. SYNGR3 variants are located in regions of high structural mimicry, as evidenced by RMSD maps. However, these variants demonstrate polarity changes: serine (polar, hydroxyl group) to phenylalanine (non-polar, aromatic), alanine (non-polar) to threonine (polar, hydroxyl group), and proline (non-polar, cyclic) to arginine (positively charged). This suggests that these changes may affect protein function by altering local chemical environments. Variants in SYNGR1, which predominantly affect regions of low structural mimicry, induce significant disruptions to the resting-state architecture. In contrast, SYNGR3 variants, localized to regions of high structural mimicry, potentially disrupt functional dynamics. Therefore, we suggest that active SYNGR3 adopting a more SYNGR1-like conformation could reflect a compensatory mechanism to preserve vesicle recycling while mitigating neurotransmitter release disruptions, such as in early synaptic failure in AD.

Future scopes and the potential applications

Molecular dynamics simulations reveal that protein functionality is not solely determined by static structural characteristics but involves complex, dynamic interactions. While the 50 ns simulation timeframe might be considered relatively short, it provided valuable insights into the initial stability of interactions, consistent with observations from our previous studies (Karagöl et al. 2024a). These results may be useful for identifying the importance of dynamic modelling in understanding protein behaviour, particularly under varying pH conditions, which were shown to influence structural dynamics through alterations in radius of gyration and conformational stability. Extending the simulations could further refine these insights.

The structural resemblance between SYNGR1's resting state and SYNGR3's active state implies a potential evolutionary conservation of functional protein modules. These findings challenge traditional views of protein specificity and suggest a more fluid, adaptable model of protein behaviour where structural elements can be transiently shared or borrowed across different protein states. This supports the hypothesis that structural and functional modularity in proteins is an evolutionarily conserved strategy for optimizing adaptability and functionality across different environmental and cellular contexts. The observed dynamic interchangeability of structural features between SYNGR1 and SYNGR3 supports the role of evolutionary plasticity in shaping protein functionality. Future studies could investigate whether similar adaptations are observed in orthologous proteins across species that inhabit environments with varying pH levels, such as extremophiles.

Further research could explore how pH-induced structural changes impact the functional properties of proteins, including ligand binding, allosteric regulation, or enzymatic activity. This could have direct implications for understanding diseases where pH dysregulation plays a role, such as cancer (White et al. 2017) or heart failure (Lyu et al. 2023), where the extracellular environment is altered. While this study

focused on pH values of 5.5 and 7.25, extending the investigation across a broader spectrum could offer a more comprehensive understanding of the molecule's behaviour under extreme acidic or alkaline conditions. This would be particularly relevant for systems that operate in varied biological environments, such as the gastrointestinal tract or lysosomes.

Conclusion

The findings from this study deliver significant insights into how pH variations influence the structural dynamics of synaptogyrins, particularly through alterations in their radius of gyration and conformational stability. This study further demonstrates how pI influences the diversification of amino acid variance in homologous sequences. The differential stability observed under varying pH conditions might provide insights into how proteins evolved to function optimally in specific cellular compartments with distinct pH levels. The findings of this study could have a far-reaching implication on fields ranging from molecular neuroscience and drug development to synthetic biology and evolutionary research.

Methods

Protein sequence alignments and related characteristics

Protein sequences for SYNGR1 and SYNGR3 were retrieved from UniProt (accession numbers O43759 and O43761) (https://www.uniprot.org) (Uniprot consortium 2023). Molecular weights, amino acid compositions, and isoelectric points were calculated using the Expasy tools (https://web.expasy.org/) (Gasteiger et al. 2005; Bjellqvist et al. 1994; Bjellqvist et al 1993).

AlphaFold3 (https://alphafoldserver.com) was used to predict structures according to default settings (Abramson 2024). The structures were then superposed using PyMOL Molecular Graphics System version 2 (https://pymol.org/2/) (Shrödinger LLC 2024). The similarities between structures were benchmarked quantitatively by calculating root-mean-square deviation (RMSD) values for all-atom RMSDs (cycles=0), ensuring the accuracy of the comparisons. Residue-wise SASA calculations and secondary structure deductions were analyzed with Stride (https://webclu.bio.wzw.tum.de/cgi-bin/stride/stridecgi.py) (Frishman and Argos 1995).

Analysis of Evolutionary Homology and Residue-wise conservations

ConSurf server (https://consurf.tau.ac.il/) was utilized for generating residue-wise evolutionary conservation profiles (Yariv et al. 2023; Ashkenazy et al. 2016; Celniker et al. 2013; Landau et al. 2005). The server was run with native structures predicted by AlphaFold that were also used for RMSD calculations. The homolog search algorithm HMMER was utilized, which is a hidden Markov model-based approach for detecting remote protein homologues (E-value set to 0.0001). The sequences were then aligned using the MAFFT-L-INS-i method to generate a Multiple Sequence Alignment (MSA). The conservation scores were calculated using the Bayesian method, selecting the amino acid substitution model based on the best fit (default). The resulting output provides normalized conservation scores, with values ranging from 1 (representing variable positions) to 9 (indicating highly conserved positions).

By comparing the percentage variations of the same amino acid residues in the aligned sequences, we statistically measured how consistently the evolutionary trends in SYNGR1 correlated with those in SYNGR3, reflecting potential co-evolutionary patterns or shared evolutionary constraints between these two proteins. The Shapiro-Wilk normality test was performed and demonstrated that the distribution of the conservation scores departed significantly from normality (Shapiro and Wilk 1965). Due to the lack of adherence to the assumptions of linearity and normality, nonparametric methods were applied, and bootstrapping was used for confidence interval calculation (Hervé 2020). Spearman's rank correlation coefficient (Spearman's ρ or rho) was utilized to evaluate the monotonic connection between two variables (Hervé 2020). This nonparametric method is particularly well-suited for analysing ranked data, as it does not assume a normal distribution of the variables (Kim et al. 2015), making it ideal for assessing evolutionary amino acid profiles where distributions may deviate from normality. The confounding effects of H and N on K, R, E, D frequencies were analyzed using partial Spearman coefficients. Statistical calculations were performed and visualized using R (https://www.r-project.org/) (The R Foundation for Statistical Computing, Vienna, Austria), version 4.3.1 (R Core Team 2016)

Comparative molecular dynamics simulations

Molecular dynamics simulations were performed on the AlphaFold-predicted structures to ensure a consistent comparison. All MD simulations were performed using GROMACS 2024.3 (Abraham et al. 2015), which were executed on Google Colab, utilizing L4 GPUs, 106 GB RAM, and 43GB VRAM. To optimize computational efficiency, the simulations were parallelized across multiple cores within VM, and the source code was recompiled using all available cores with CUDA GPU support (DGMX_GPU=CUDA) and OpenMP enabled (GMX_OPENMP_MAX_THREADS = 128) for L4 GPUs according to our recent benchmarking analysis (Karagöl et al. 2024b). Configuration files and codes for the simulations are publicly available with step-by-step instructions (Karagöl et al. 2024b).

The membrane-protein systems were constructed utilizing the CHARMM-GUI web server (Wu et al. 2014; Jo et al. 2008; Jo et al. 2009), similar to our previous study on glutamate transporters (Karagöl et al. 2024a). Spatial positions of proteins in the lipid bilayer are refined through the PPM 2.0 method, which incorporates the hydrogen-bonding profiles and anisotropic water-lipid environment (Lomize et al. 2012). Protonation states assigned based on the local pH. The lipid composition is critical for the function of synaptic vesicle proteins (Binotti et al. 2021). For our study, we used previously reviewed lipid composition profiles (Binotti et al. 2021) according to the quantitative lipidomic analysis of mammalian synaptic vesicles conducted by Takamori et al 2006. Our system of synaptic vesicles contains cholesterol (CHOL), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SM), and phosphatidylinositol (PIns), with the following molar percentages: 40 mol%, 17 mol%, 20 mol%, 6 mol%, 4 mol%, and 1 mol%, respectively.

The systems were built with neutralizing K^+, Cl^- ions for the resting state (concentration = 0.15M), calcium levels physiologically increase in an active state and Ca^{+2} ions (concentration = 0.1M) were further added to active state systems. The ions in membrane systems were determined through 2000 step Monte Carlo (MC) simulations using a primitive model. The systems were solvated in TIP3P water. CHARMM36m force field (all-atom) was used (Huang et al. 2017). The relaxation of both protein-membrane systems was conducted via multi-step minimization and equilibration, similar to our previous study (Karagöl et al. 2024a). The system energies were minimized using the steepest descent method, and until the maximum forces converged to values under 1000 kJ/mol/nm. For both systems, 125-ps equilibration simulations were then performed using the standard CHARMM-GUI protocol (Wu et al. 2014; Jo et al. 2008; Jo et al. 2009). After the NVT and NPT equilibration, a 50-ns production MD simulation was run with timestamps for every 0.5ns, in line with our previous publication which produced insights on the initial interactions of macromolecules (Karagöl et al. 2024a). The Particle Mesh Ewald (PME) method was employed for electrostatics, with a cutoff distance of 1.2 nm for both Coulomb and van der Waals interactions. The temperature was held at 303.15 K and pressure was held at 1 bar. The Parrinello-Rahman barostat with semi-isotropic coupling and the Nose-Hoover thermostat were utilized.

Comparative trajectory analysis was conducted for analysing the dynamical changes of both systems. The equilibrated trajectories were combined with the GROMACS gmx traj tool. The gyration radii were measured using the gmx gyrate tool. As a measure of the flexibility of residues, the residue root mean square fluctuation (RMSF) was also calculated for the Cα atoms of proteins for the full 50ns equilibrated simulation trajectory. The solvent accessible surface area (SASA) of the side chains of the protein residues was calculated according to a solvent probe radius set to 1.4Å (Eisenhaber et al. 1995). Plots that were generated through analyses were visualized utilizing Grace (https://plasma-gate.weizmann.ac.il/Grace/). The structures and 50ns trajectories were visualized using UCSF ChimeraX version 1.8. Residue-wise root-mean-square deviation (RMSD) values were analyzed and visualized using UCSF ChimeraX version 1.8 (Meng et al. 2023).

Variant analysis

Variants for *SYNGR1* and *SYNGR3* genes were obtained from large-scale sequencing projects in the Genome Aggregation Database (gnomAD v4.1.0, http://gnomad.broadinstitute.org/) (Chen et al. 2024). The Ensembl canonical transcripts for SYNGR1 and SYNGR3 were ENST00000328933.10 and ENST00000248121.7, respectively. Disease-related variants were obtained from the ClinVar database (Landrum et al. 2016) (https://www.ncbi.nlm.nih.gov/clinvar/) and *in silico* assessments of variant impact obtained via PolyPhen

(http://genetics.bwh.harvard.edu/pph2/), which evaluates the likelihood of a variant affecting protein structure or function based on sequence, phylogenetic, and structural information (Adzhubei et al. 2010). PolyPhen score > 0.85 is considered likely to be damaging. Sampled variants were visualized on the residue-wise RMSD mapping of SYNGR1 and SYNGR3 using UCSF ChimeraX version 1.8 (Meng et al. 2023).

Supplementary information (SI)
Supplementary Information.pdf
Supplementary data file featuring graphical results from the molecular dynamics simulations.

Ethics approval
Ethics approval was not required for this computational study as it did not involve animal subjects, human participants, and identifiable data.

Consent to participate
Not applicable. This computational study did not involve human participants.

Consent for publication
Not applicable. This computational study did not involve human participants.

Availability of data and materials
Each statistical and computational analysis of this study, included with step-by-step instructions where possible, are publicly available to ensure repeatability. To access detailed information on the statistical analyses, input files, and detailed results, please refer to the website: https://github.com/karagol-taner/pH-dependent-membrane-binding-synaptogyrins.

Competing financial interests
Both authors of this study have an equal 50% share in a pending patent application for inhibition of SNARE complex formation by a specific truncated isoform, targeting synaptic modulation. Patent application No. 2024/018735. The patent has been published. Both authors of this study also have an equal 50% share in a pending patent application for a method of designing pH-specific proteins (Patent Application No. 2024/018998). However, the research presented in this manuscript does not involve the utilization of any artificially designed proteins or truncated isoforms. This study focuses on the pH-dependent membrane binding specificity of synaptogyrins 1 and 3 through molecular dynamics simulations. This study's methodology is independent of the patented approaches.

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Table 1. Residue-wise evolutionary correlations of SYNGR1 and SYNGR3.

Variable[1]	Spearman's rho[2]	Confidence intervals[3]
A	0.8173	0.7428, 0.8795
C	0.6566	0.5020, 0.7695
D	0.7220	0.6247, 0.8120
E	0.6903	0.5891, 0.7794
F	0.8233	0.7455, 0.8860
G	0.8193	0.7488, 0.8761
H	0.5633	0.4251, 0.6779
I	0.7093	0.6108, 0.7887
K	0.7089	0.5832, 0.8058
L	0.7161	0.6137, 0.7954
M	0.6006	0.4698, 0.7021
N	0.6251	0.5141, 0.7244
P	0.6057	0.4862, 0.7070
Q	0.7530	0.6639, 0.8242
R	0.6508	0.5290, 0.7572
S	0.7660	0.6892, 0.8378
T	0.7082	0.6205, 0.7895
V	0.7267	0.6375, 0.8001
W	0.6521	0.4473, 0.8105
Y	0.7286	0.6035, 0.8254
Residue-wise Conservation	0.8819	0.8345, 0.9208

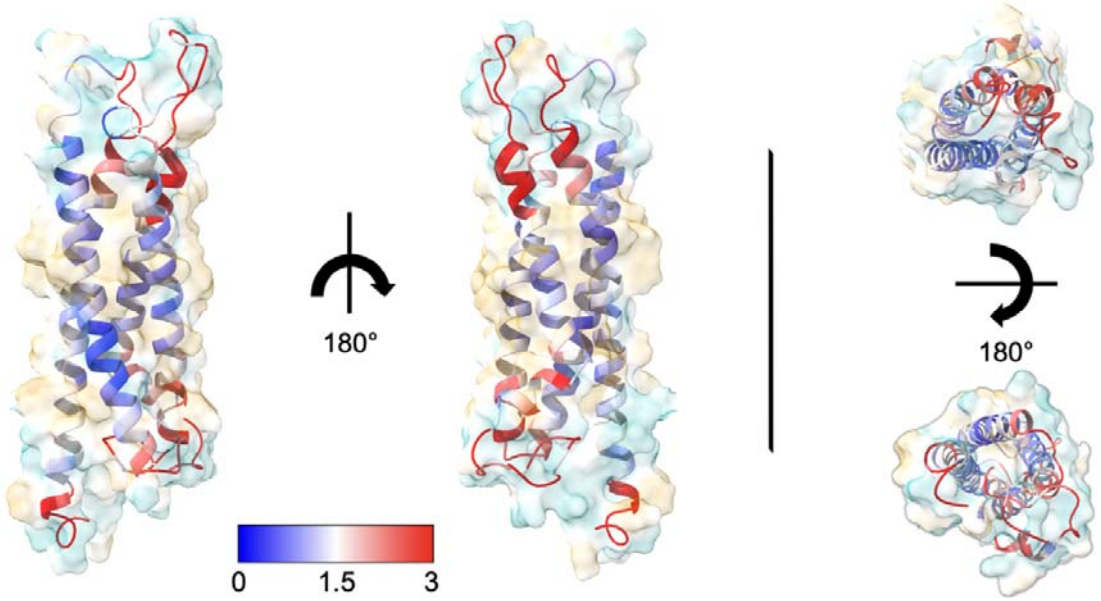
[1]Percentage distribution of amino acids at each residue position in homologous sequence alignments of SYNGR1 and SYNGR3. Residue-wise conservation is ConSurf conservation grade for each residue (1=variable 9=conserved).
[2]Spearman's ranked correlation ($p<0.001$).
[3]95% confidence intervals.

Table 2. Cofounding effects of H and N on charged amino acid (K, R, E, D) varieties in SYNGR1 and SYNGR3 homologous alignments.

AA	Control Variable[1]	Partial Spearman's rho[2]	Confidence intervals[3]
K	H (SYNGR1)	0.6741	0.5469, 0.7708
	H (SYNGR3)	0.6655	0.5356, 0.7646
	N (SYNGR1)	0.7059	0.5847, 0.7963
	N (SYNGR3)	0.7071	0.5855, 0.7976
R	H (SYNGR1)	0.6358	0.5118, 0.7338
	H (SYNGR3)	0.6187	0.4926, 0.7194
	N (SYNGR1)	0.6503	0.5278, 0.7463
	N (SYNGR3)	0.6508	0.5283, 0.7467
E	H (SYNGR1)	0.6880	0.5777, 0.7736
	H (SYNGR3)	0.6773	0.5674, 0.7635
	N (SYNGR1)	0.6883	0.5799, 0.7729
	N (SYNGR3)	0.6913	0.5833, 0.7753
D	H (SYNGR1)	0.7206	0.6128, 0.8021
	H (SYNGR3)	0.7207	0.6129, 0.8021
	N (SYNGR1)	0.7026	0.5885, 0.7893
	N (SYNGR3)	0.7100	0.5976, 0.7951

[1]Confounding variable as specific amino acids varieties in homologous sequences. For instance, N (SYNGR3) indicates N amino acid varieties in the SYNGR3 homologous sequences (aligned).
[2]Spearman's ranked correlation ($p<0.001$).
[3]95% confidence intervals.

Figures and Figure Legends



Graphical Abstract. Surface profile of SYNGR1 and residue-wise RMSD between SYNGR1 (resting) and SYNGR3 (active) after 50ns of equilibrated trajectory. SYNGR1 is shown in molecular surface representation, coloured by hydrophobicity (cyan: hydrophilic, gold: hydrophobic). The underlying colour map represents the residue-wise RMSD between SYNGR1 (resting pH) and SYNGR3 (active pH). RMSD values are colour-coded from blue (low deviation) to red (high deviation), as indicated by the scale bar (0–3Å).

a

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SYNGR1/1-233   1 MEGGAYGAGKAGGAFDPYTLVRQPHTILRVVSWLFSIVVFGSIVNEGYLNSASEGEEFCI  60
SYNGR3/1-229   1 MEGASFGAGRAGAALDPVSFARRPQTLLRVASWVFSIAVFGPIVNEGYVNTDSGPELRCV  60

SYNGR1/1-233  61 YNRNPNACSYGVAVGVLAFLTCLLYLALDVYFPQISSVKDRKKAVLSDIGVSAFWAFLWF 120
SYNGR3/1-229  61 FNGNAGACRFGVALGLGAFLACAAFLLLDVRFQQISSVRDRRRAVLLDLGFSGLWSFLWF 120

SYNGR1/1-233 121 VGFCYLANQWQVSKPKDNPLN-EGTDAARAAIAFSFFSIFTWAGQAVLAFQRYQIGADSA 179
SYNGR3/1-229 121 VGFCFLTNQWQRTAP--GPATTQAGDAARAAIAFSFFSILSWVALTVKALQRFRLGTDMS 178

SYNGR1/1-233 180 LFSQDYMDPSQDSSMPYAPYVEPTGPDPAGMGGTYQQP--ANTFDTEPQGYQSQGY     233
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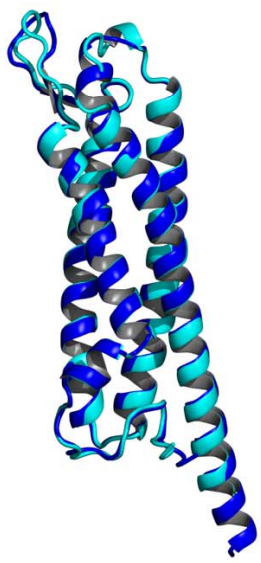
b



c

Name	pI	Mw	RMSD
SYNGR1	4.50	25455.61	1.273 Å
SYNGR3	8.42	24554.96	

Figure 1. Sequence alignment and structural comparison of SYNGR1 and SYNGR3. (a) Sequence alignment of SYNGR1and SYNGR3, highlighting conserved and variable regions. Residues with high variability are coloured red, with darker red indicating greater differences. Gaps in the alignment are represented by dashes. (b) Structural superposition of SYNGR1 and SYNGR3. The SYNGR1 structure is shown in cyan and the SYNGR3 structure is in blue. (c) Summary of the isoelectric point (pI), molecular weight (Mw), and root-mean-square deviation (RMSD) between the two structures. SYNGR1 has a pI of 4.50 and Mw of 25455.61, while SYNGR3 has a pI of 8.42 and Mw of 24554.96, with an RMSD of 1.273 Å between the two proteins.

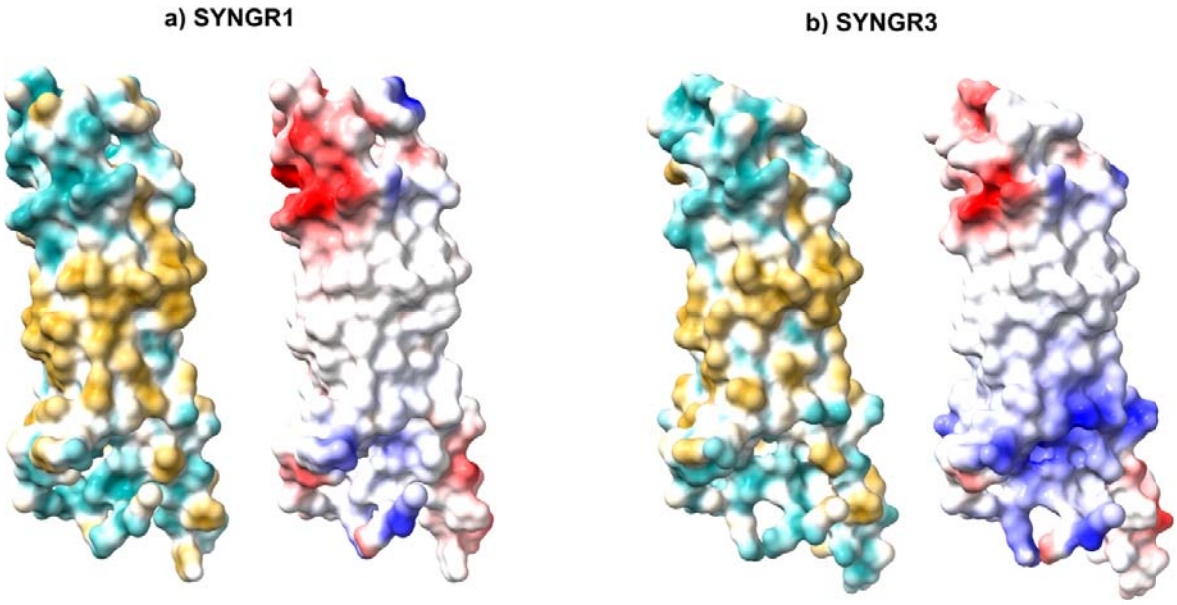


Figure 2. Surface profiles of SYNGR1 and SYNGR3. (a) SYNGR1 and (b) SYNGR3 are shown in molecular surface representations. The left panels depict hydrophobicity using a colour gradient (cyan indicates hydrophilic regions, and gold indicates hydrophobic regions). The right panels represent the electrostatic potential mapped onto the molecular surfaces, with red indicating negative charge, blue indicating positive charge, and white representing neutral regions. While hydrophobic surfaces show similarities, SYNGR3 has larger regions with positive charge (blue). Differences in charge distribution highlight the distinct pI values between the two isoforms.

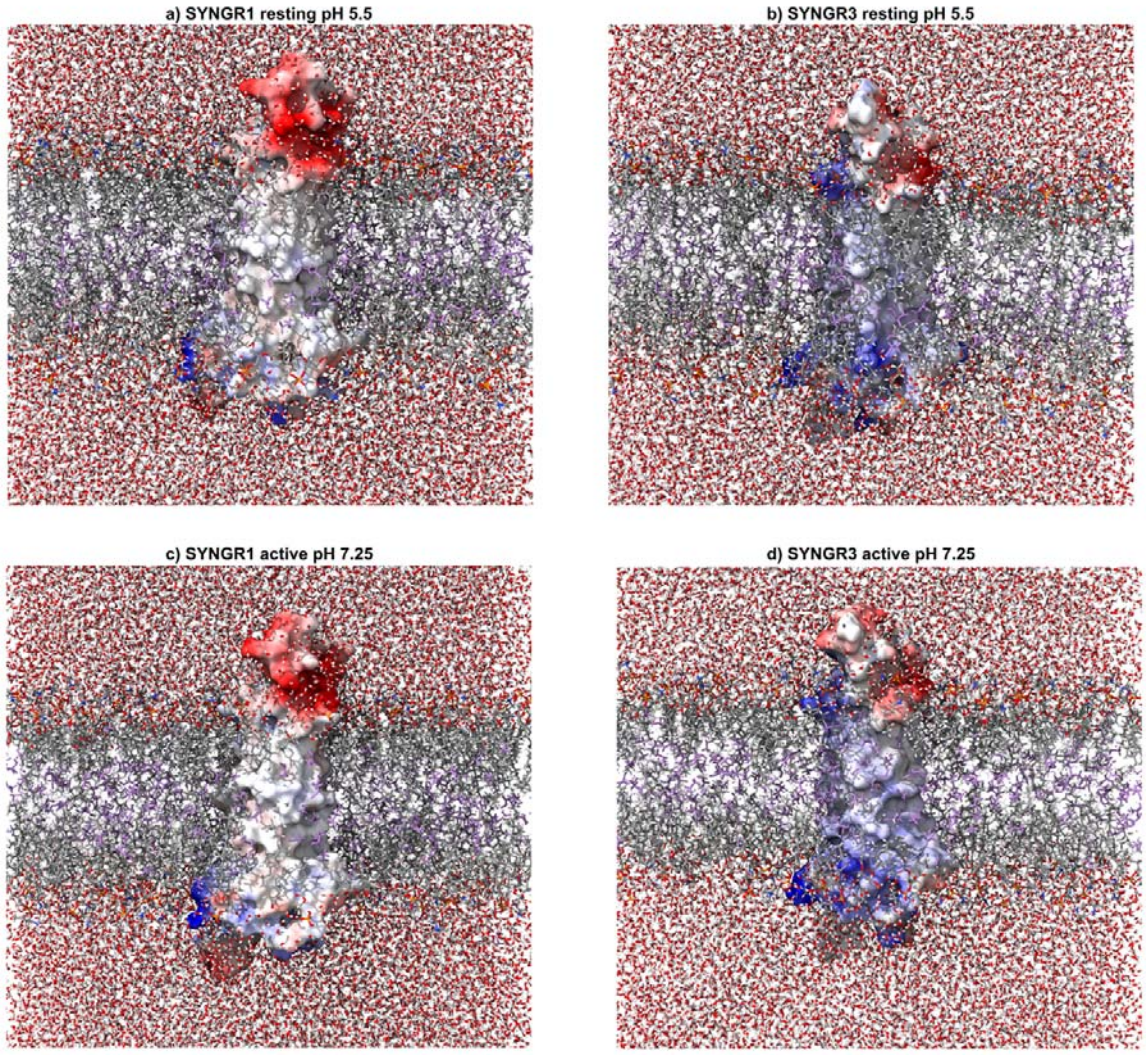


Figure 3. Equilibrated membrane systems of SYNGR1 (a and c) and SYNGR3 (b and d) in active and resting pH. The electrostatic potential mapped onto the molecular surfaces, with red indicating negative charge, blue indicating positive charge, and white representing neutral regions. Regions with negative charges usually located outside of the lipid bilayer. Differences in charge distribution highlight the distinct pI values between the two isoforms. Neutralizing K^+, Cl^- ions (concentration = 0.15M) for resting state, calcium physiologically increases in an active state and Ca^{+2} ions (concentration = 0.1M) further added to active state systems (see Methods).

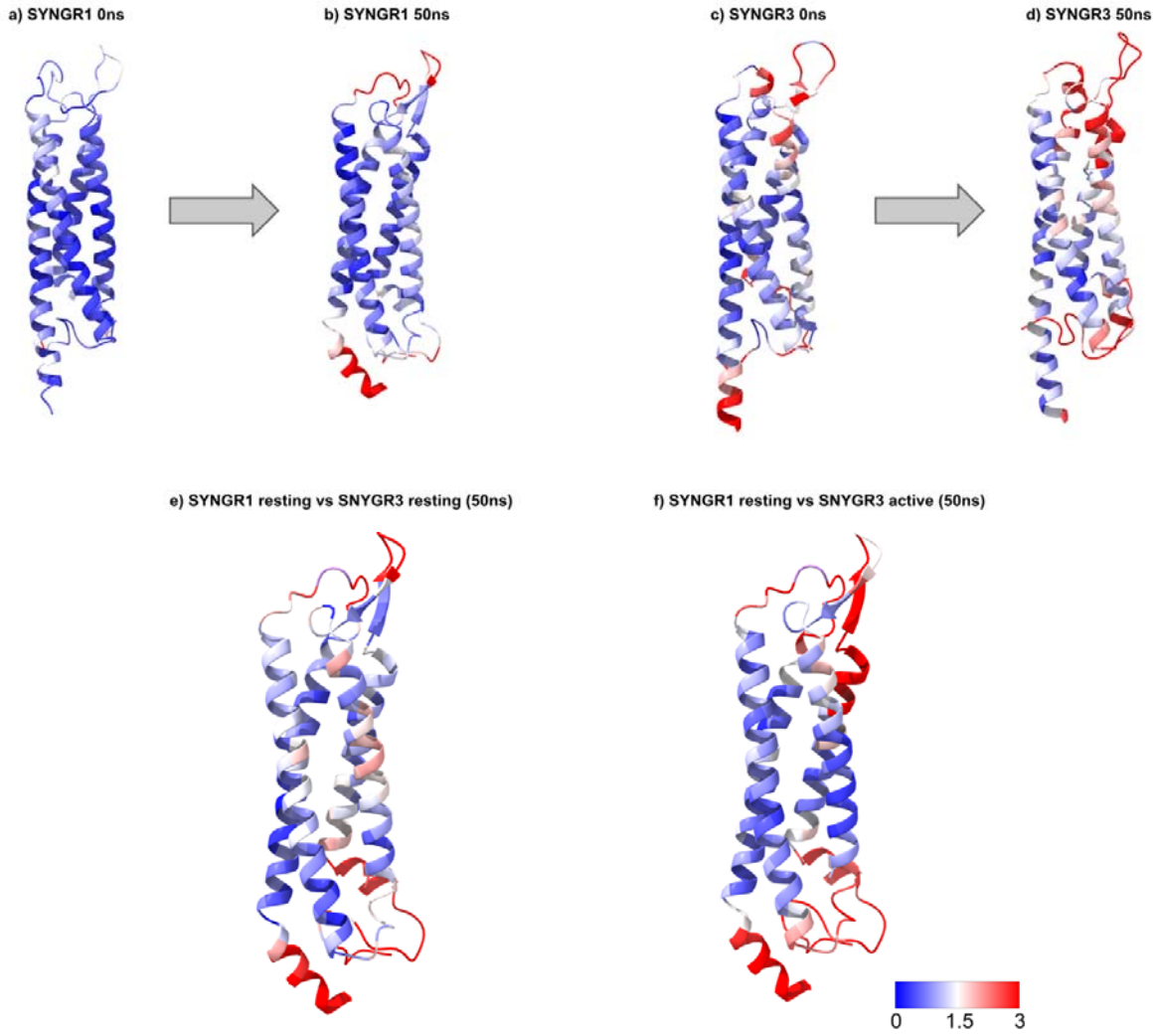


Figure 4. Residue-wise RMSD of SYNGR1 and SYNGR3 structures between initial and 50ns equilibrated trajectories. Panels (a) and (b) display the residue-wise deviations of SYNGR1 from its initial conformation at 0ns (a) to its equilibrated structure at 50ns (b). Similarly, panels (c) and (d) illustrate the structural progression of SYNGR3 from 0ns (c) to 50ns (d). Panels (e) and (f) compare the residue-wise RMSD between different states: (e) SYNGR1 resting pH versus SYNGR3 resting pH at 50ns, and (f) SYNGR1 resting versus SYNGR3 active pH at 50ns. The RMSD values are colour-coded, with blue indicating low deviations and red representing higher deviations, as depicted by the scale bar (0–3Å).

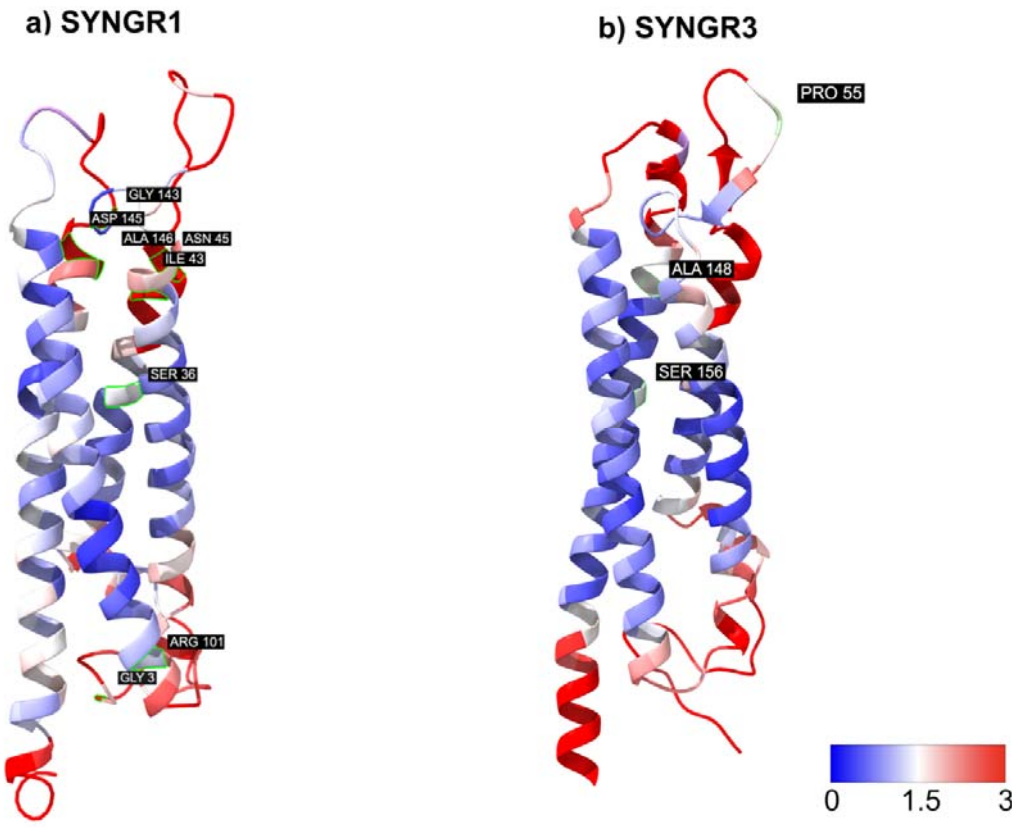
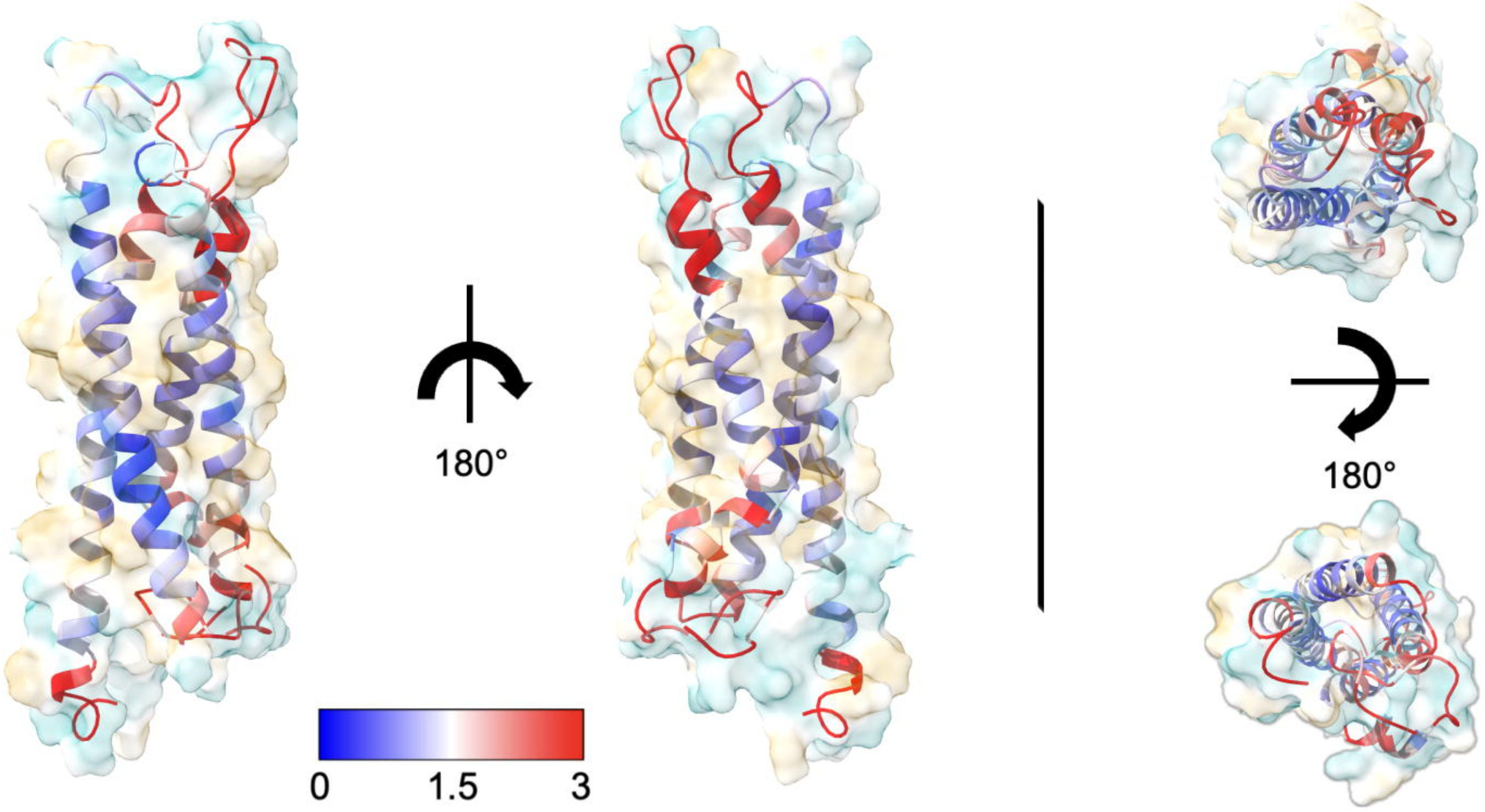


Figure 5. ClinVar reported variants with PolyPhen scores predicted to be damaging (0.85-1.0). Underlying colour maps compare the residue-wise RMSD between SYNGR1 resting versus SYNGR3 active pH at 50ns. The RMSD values are colour-coded, with blue indicating low deviations and red representing higher deviations, as depicted by the scale bar (0–3Å). Reported ClinVar variants for SYNGR1 were p.Gly3Arg, p.Asp145Tyr, p.Ala146Thr, p.Arg101Cys, p.Glu142Lys, p.Ser36Phe, p.Ile43Met, and p.Asn45Asp. Most of these damaging variants are located in regions with low structural mimicry. Conversely, same phenomenon could not be said for SYNGR3 variants. Reported ClinVar variants for SYNGR3 were p.Ser156Phe, p.Ala148Thr, and p.Pro55Arg.


180°
180°
0
1.5
3

a

```
SYNGR1/1-233   1 MEGGAYGAGKAGGAFDPYTLVRQPHTILRVVSWLFSIVVFGSIVNEGYLNSASEGEEFCI  60
SYNGR3/1-229   1 MEGASFGAGRAGAALDPVSFARRPQTLLRVASWVFSIAVFGPIVNEGYVNTDSGPELRCV  60

SYNGR1/1-233  61 YNRNPNACSYGVAVGVLAFLTCLLYLALDVYFPQISSVKDRKKAVLSDIGVSAFWAFLWF 120
SYNGR3/1-229  61 FNGNAGACRFGVALGLGAFLACAAFLLLDVRFQQISSVRDRRRAVLLDLGFSGLWSFLWF 120

SYNGR1/1-233 121 VGFCYLANQWQVSKPKDNPLN-EGTDAARAAIAFSFFSIFTWAGQAVLAFQRYQIGADSA 179
SYNGR3/1-229 121 VGFCFLTNQWQRTAP--GPATTQAGDAARAAIAFSFFSILSWVALTVKALQRFRLGTDMS 178

SYNGR1/1-233 180 LFSQDYMDPSQDSSMPYAPYVEPTGPDPAGMGGTYQQP--ANTFDTEPQGYQSQGY     233
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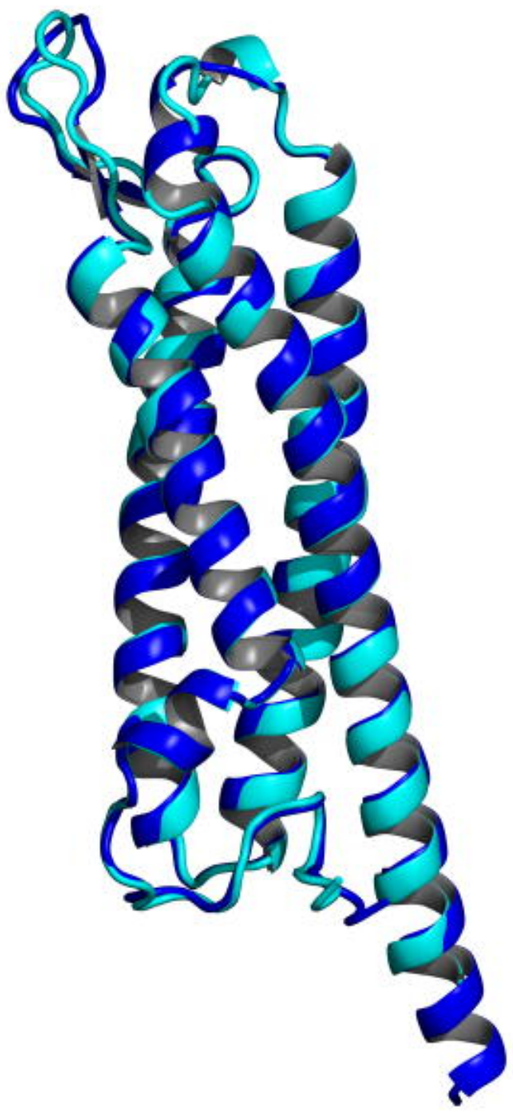
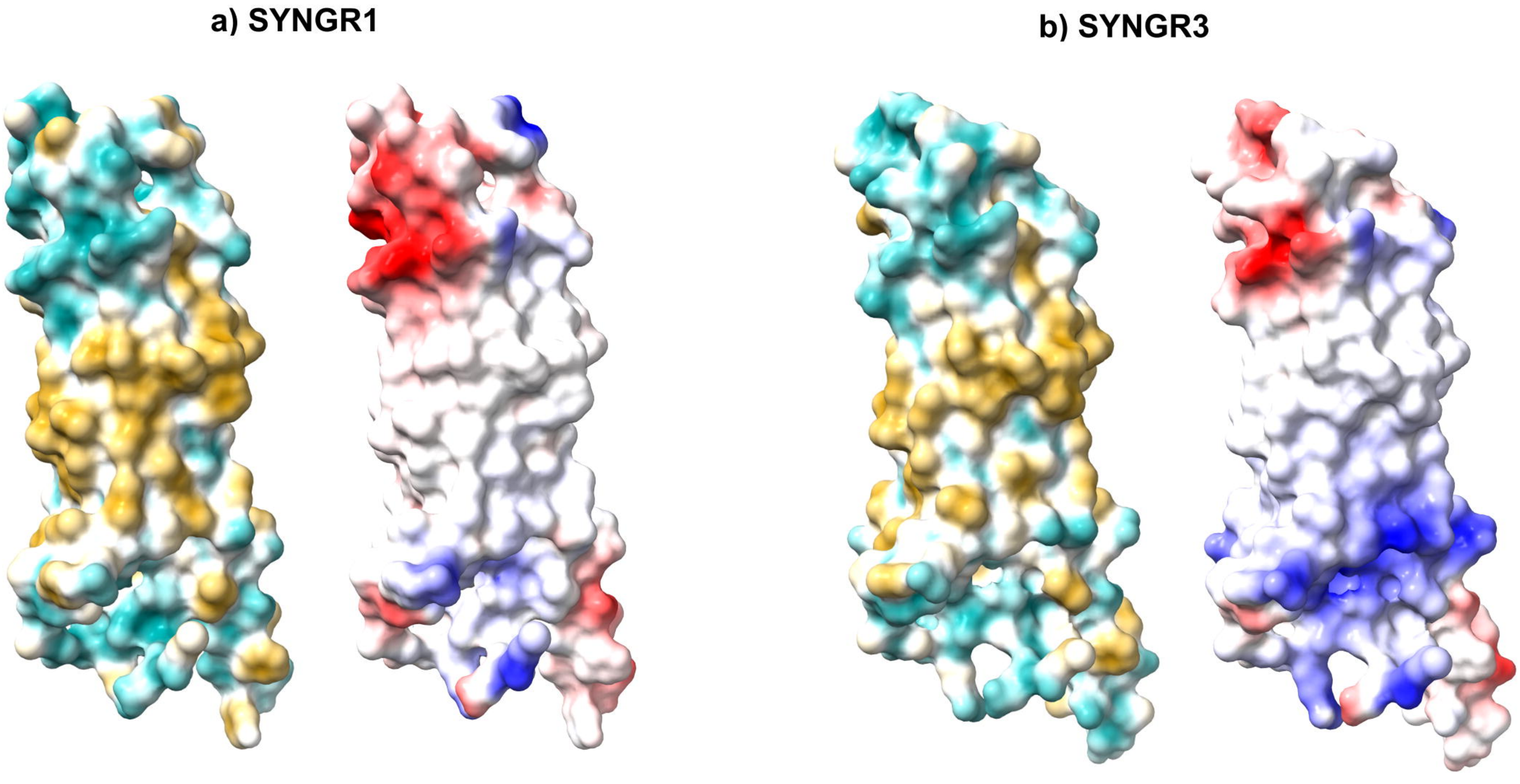
b

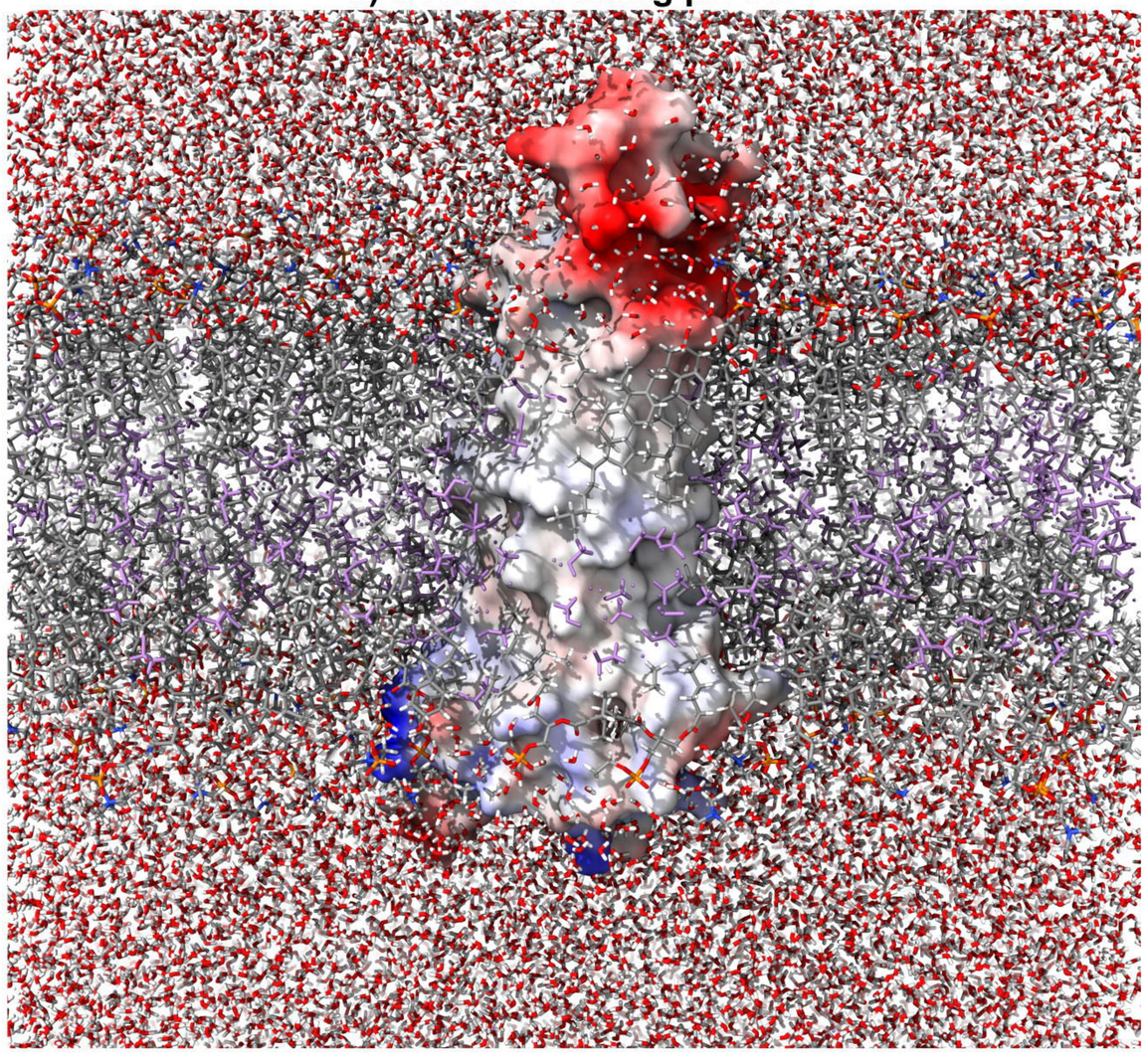


c

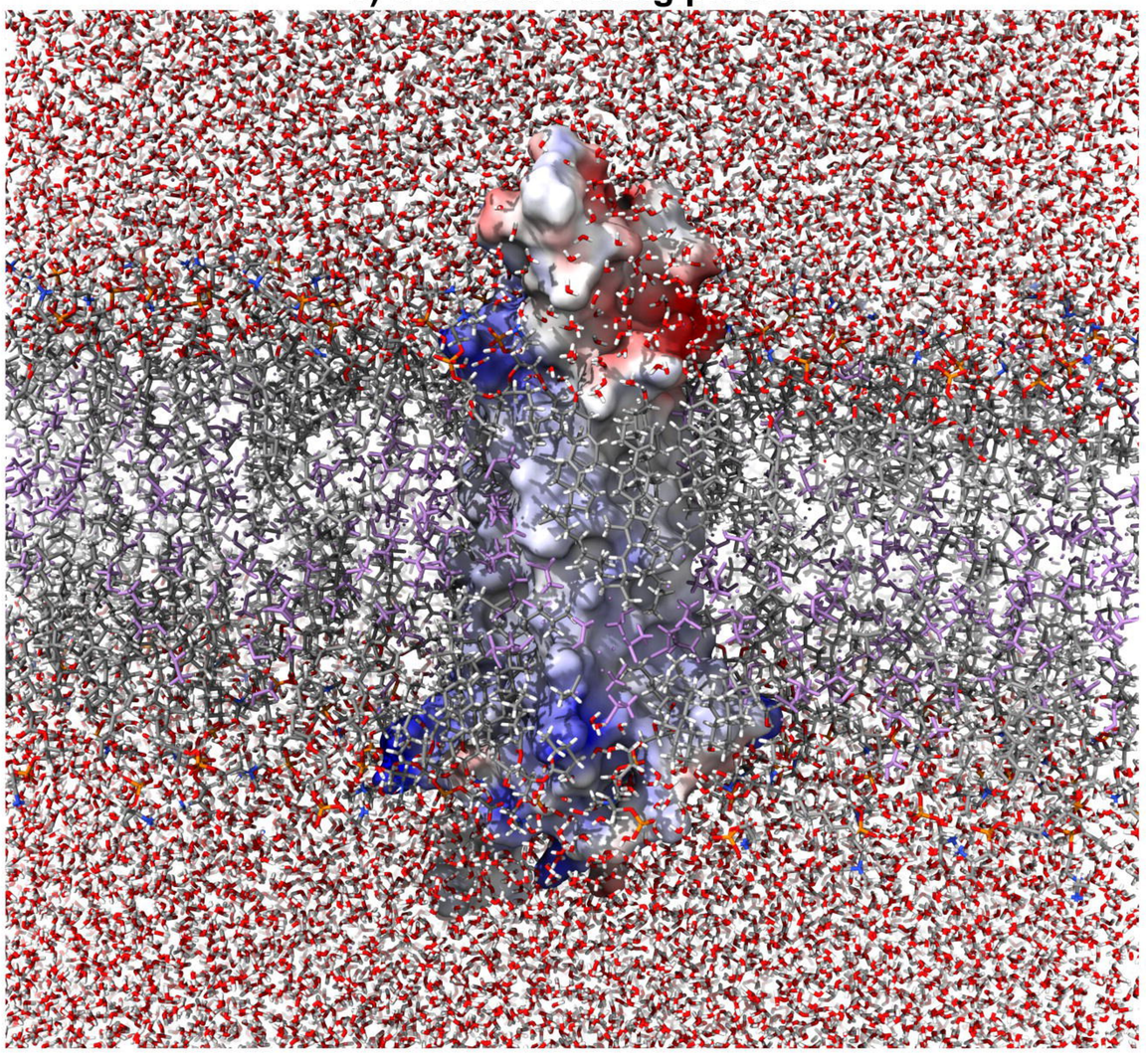
Name	pI	Mw	RMSD
SYNGR1	4.50	25455.61	1.273 Å
SYNGR3	8.42	24554.96	


a) SYNGR1
b) SYNGR3

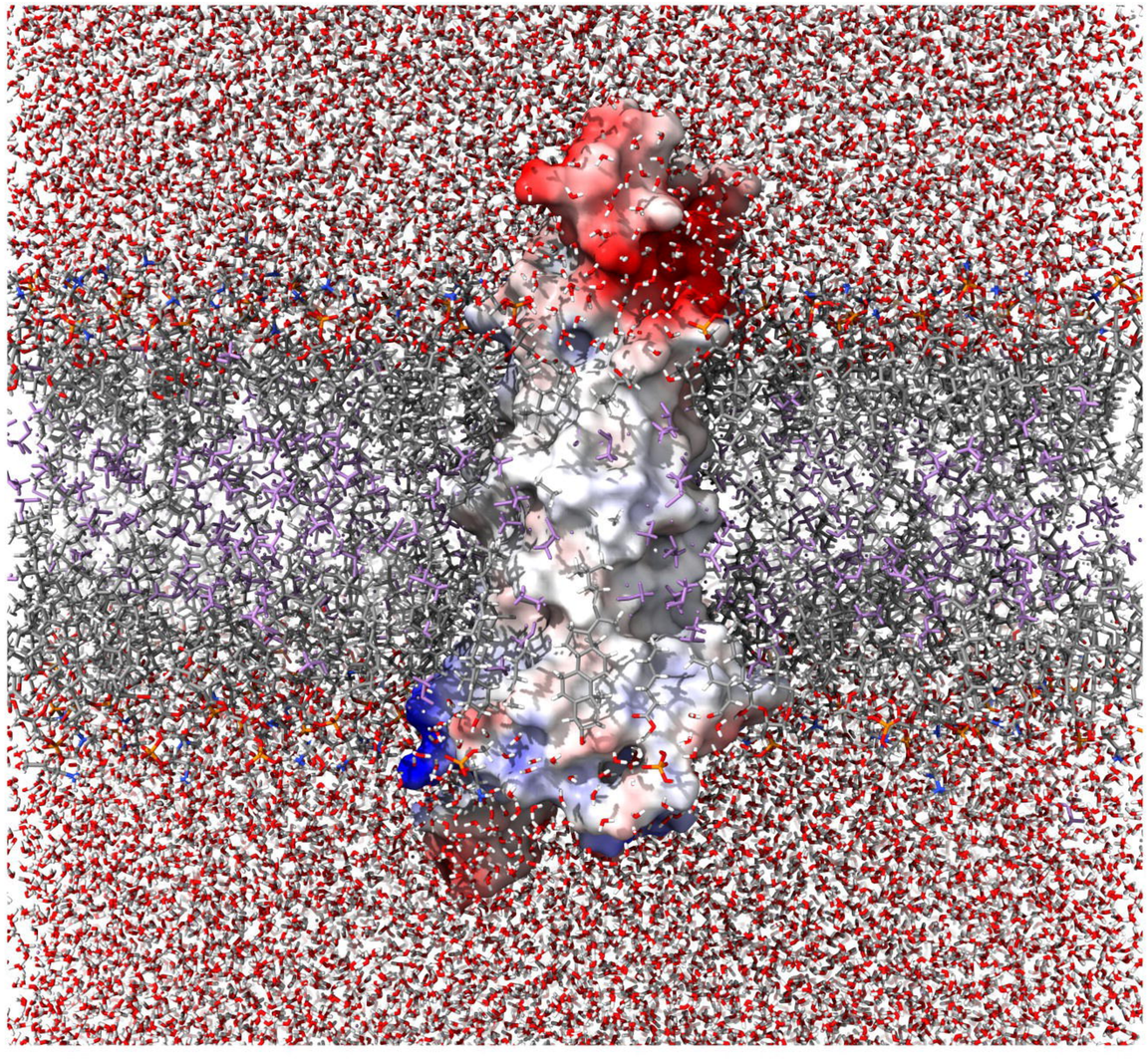
a) SYNGR1 resting pH 5.5



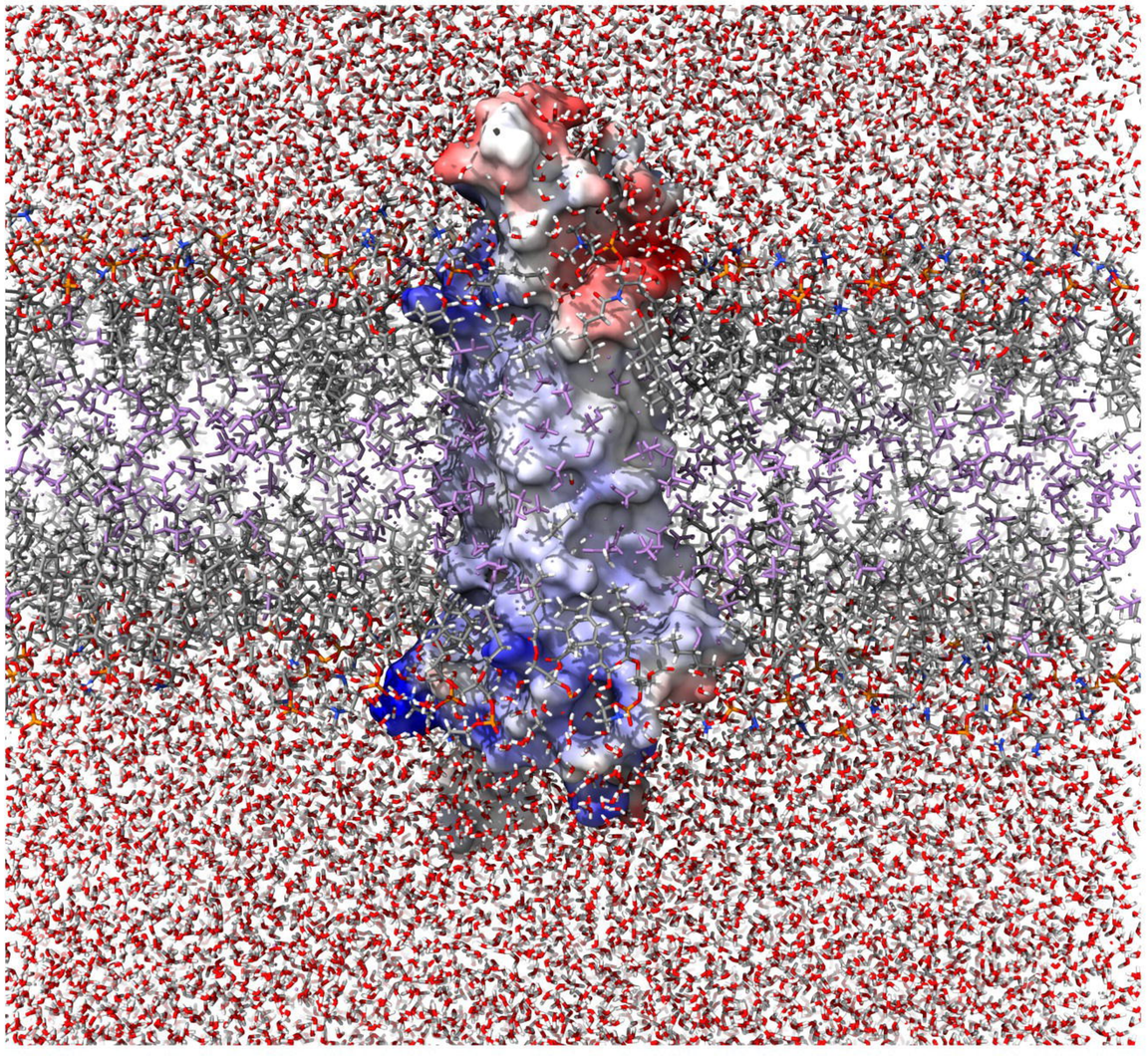
b) SYNGR3 resting pH 5.5

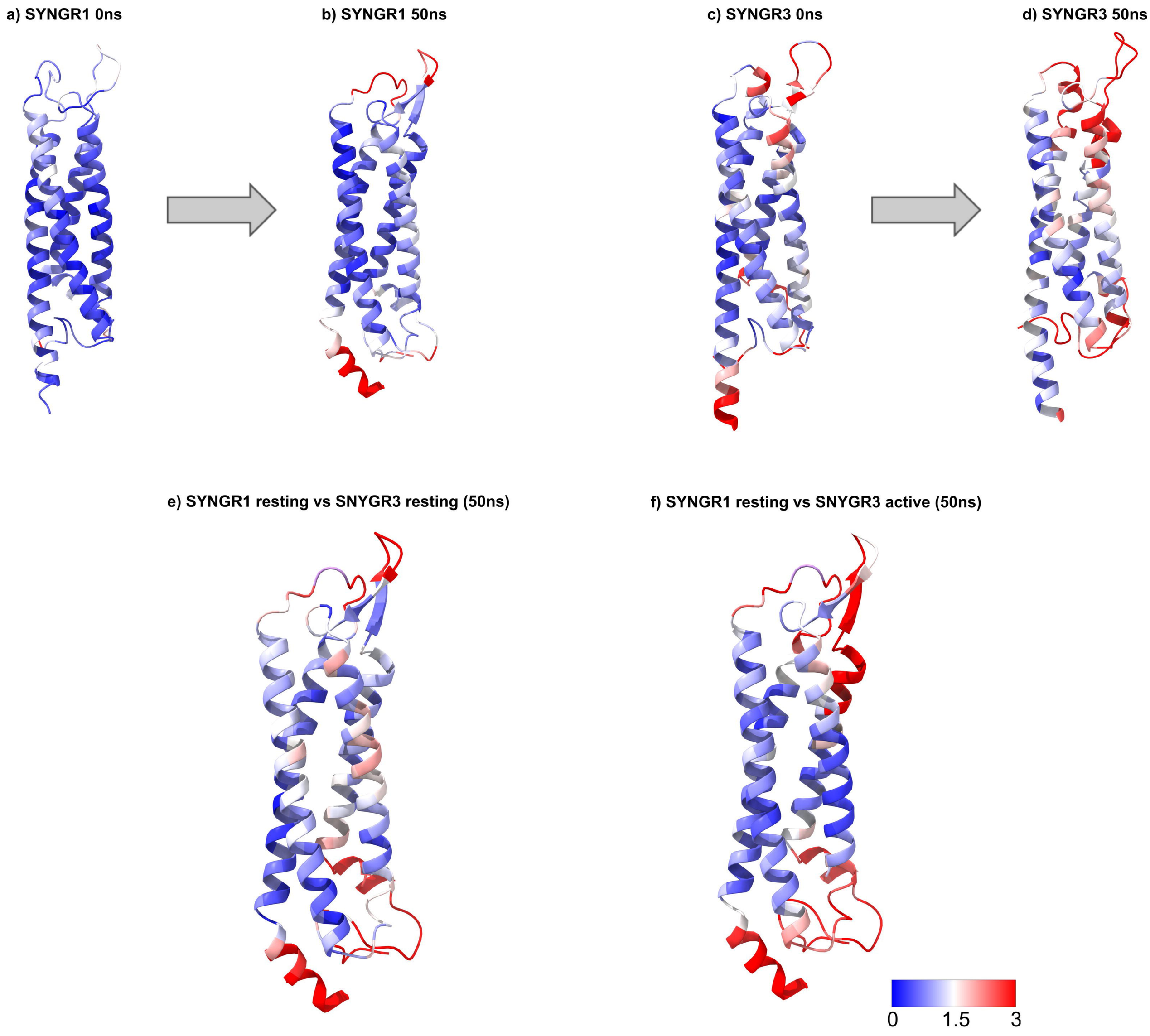


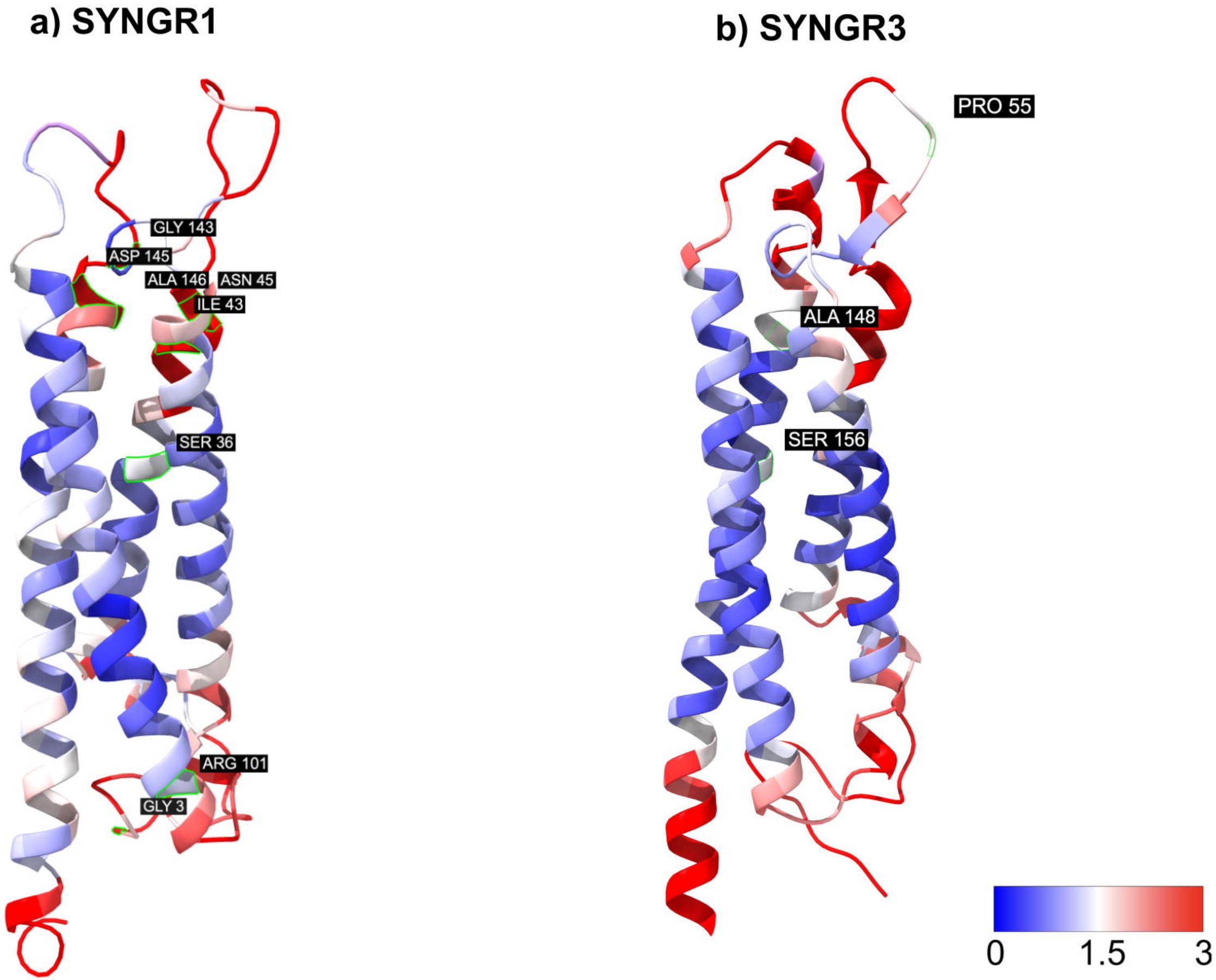
c) SYNGR1 active pH 7.25



d) SYNGR3 active pH 7.25




a) SYNGR1 0ns
b) SYNGR1 50ns
c) SYNGR3 0ns
d) SYNGR3 50ns
e) SYNGR1 resting vs SNYGR3 resting (50ns)
f) SYNGR1 resting vs SNYGR3 active (50ns)
0
1.5
3


a) SYNGR1
GLY 143
ASP 145
ALA 146
ASN 45
ILE 43
SER 36
ARG 101
GLY 3
b) SYNGR3
PRO 55
ALA 148
SER 156
0
1.5
3