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# NMDA Receptors' Structural Asymmetry

Farzad Jalali-Yazdi[1], Eric Gouaux[1,2*]

[1.] Vollum Institute, Oregon Health and Science University, Portland, OR, USA.
[2.] Howard Hughes Medical Institute, Oregon Health and Science University, Portland, OR, USA.
* Corresponding author: gouauxe@ohsu.edu

*N*-methyl-D-aspartate receptors (NMDARs) are members of the ionotropic glutamate receptor family (iGluRs). The structure of the full-length homotetrameric AMPA receptor, first of any iGluR family members, revealed a 2-fold symmetric receptor with a ~4-fold symmetric gate, exhibiting symmetry mismatch between the transmembrane domain (TMD) and the extracellular domain (ED) [1]. The iGluR receptors have similar architectures with the ED comprised of the more distal amino terminal domain (ATD) and the membrane proximal ligand binding domain (LBD), and both adopting bi-lobed clamshell like structures. NMDARs are obligate heterotetramers, predominantly consisting of two GluN1 and two GluN2 subunits. The first structures of the full length NMDA receptor utilized a disulfide bond in the ATD, locking the receptor in an inactive state to improve crystal diffraction [2, 3]. This disulfide-bridged inactive conformation was 2-fold symmetric. Interestingly, Lee et al., noted that if the disulfide bond was removed, two halves of the receptor were observed in the asymmetrical unit with different ATD conformations. The next set of diheteromeric NMDAR structures were solved using cryo-electron microscopy (cryoEM), with constructs lacking the disulfide-locked ATDs [4, 5]. Zhu et al., obtained a pseudo C2-symmetric structure for the agonist bound conformation, with one ATD heterodimer bent at a steeper angle towards the pseudo symmetry axis [4]. Several other diheteromeric NMDAR cryoEM structures, however, were solved with applied C2 symmetry [5-7]. Here, we analyze receptor symmetry, and pinpoint the challenges in identifying the correct symmetric state.

Our recent work, elucidating the molecular mechanism of high-affinity zinc and proton inhibition of the GluN1/GluN2A diheteromeric NMDARs, resulted in 18 structures in 5 structural classes [8]. We noticed that none of the classes exhibited symmetry. The structural class associated with the receptor in the presence of agonists and absence of antagonist was comprised of 6 independent structures, allowing us to measure the degree of asymmetry with statistical accuracy. The most distinct sign of broken C2-symmetry was the bending of one ATD heterodimer towards the pseudo symmetry axis, as previously seen in the GluN1/GluN2B receptor [4]. To quantitate the receptor asymmetry, we measured the ATD-LBD angle, the angle between the two ATD lobes and the two LBD lobes. We found a ~12° difference between the two GluN2A subunit's conformations (Figure 1A). This asymmetry is propagated towards the TMDs, with the distance between the lower LBD lobe and the receptor gate being 1.1 ± 0.2 Å smaller in one GluN2A subunit compared to the other (Figure 1B).

One of the main challenges with regards to assigning correct symmetry for the receptor is limitations in the current software for cryoEM reconstructions. Two of the most commonly used software in the field, Relion [9] and cisTEM [10], use "local searches" to solve the issue of computational needs during finer sampling of particle orientation. Once sampling has shifted from a "global search" of all possible orientations to a local search, the sampling is performed only within a narrow angular range. While this method greatly reduces the computational resources needed for finer sampling, it can "lock" particles in the wrong orientation, at the time when only low-resolution information is being used to align the particles. We noticed this issue with the GluN1/GluN2A diheteromeric receptor. By classifying the

particles after our initial refinement and allowing only local angular searches for classification, we obtained two main classes which showed high-resolution features. When we overlaid and fitted these two densities into one another, there were marked differences between them (Figure 1C), however, when we rotated one class by 180° and re-fitted the maps into each other, the maps were superimposable (Figure 1D). The particles' distribution between the two classes was 29% to 18%. To improve the overall resolution of our map, we manually rotated the particles in the less populated class, recombined the two classes, and refined the structures while allowing only local searches, leading to a 0.37 Å resolution improvement (Figure 1D-E). The fact that we were able to classify these two orientations apart, further confirmed the asymmetry of the GluN1/GluN2A receptor. Another pitfall of symmetry misassignment is that application of C2 symmetry can generally improve the resolution of the structure, as measured by gold-standard FSC, specially compared to the global refinement value (Figure 1E). Software upgrades, allowing for pseudo symmetry during local refinement, can greatly help overcome the current challenges of solving nearly symmetric receptors, and lead to correct assignment of symmetry.

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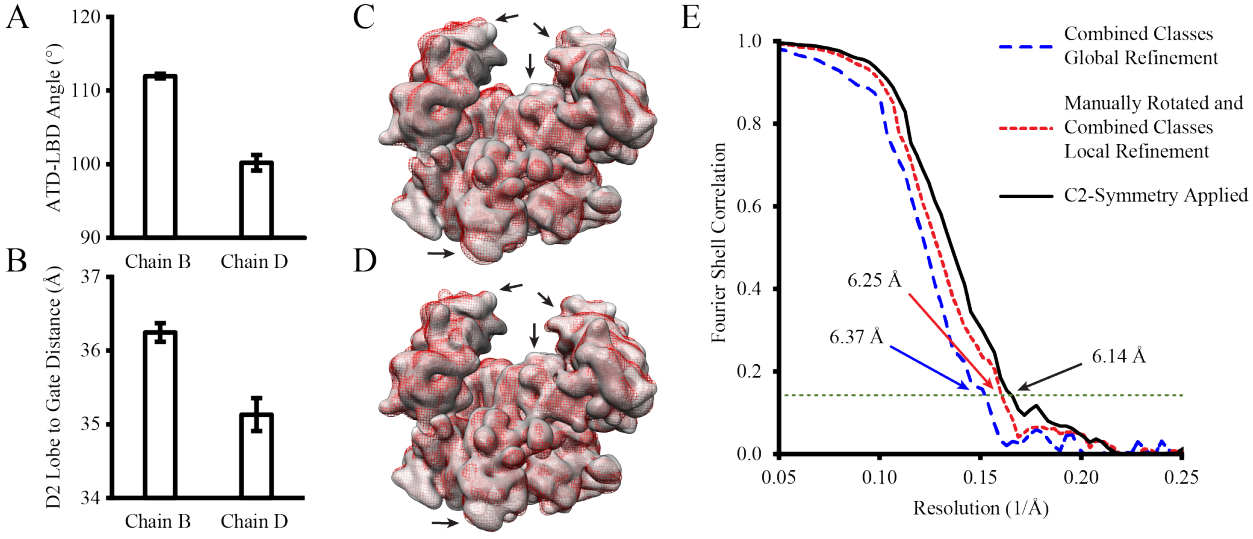


**Figure 1.** Asymmetry in GluN1/GluN2A NMDAR. (A-B) The ATD-LBD angle (A) and the distance between the lower LBD lobe to the receptor gate (B) for the two GluN2A subunits. Error bars represent SEM of 6 independent structures. (C-D) Overlay of the two classes obtained after classification with local searches (C), vs. the overlay after one class was rotated by 180° prior to alignment (D). Arrows indicate large differences in structures in (C). (E) Fourier Shell Correlation of the globally refined vs. manually aligned and locally refined vs. C2-symmetry applied structures.

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