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Dissecting diverse functions of NMDA receptors by structural biology

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10% highlighted references (94 total), * of special interest, ** of outstanding interest

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Tajima N, Karakas E, Grant T, Simorowski N, Diaz-Avalos R, Grigorieff N, Furukawa H: Activation of NMDA receptors and the mechanism of inhibition by ifenprodil. <i>Nature</i> 2016, 10.1038/nature17679	** Cryo-EM structures of three major different agonist-bound classes suggest concerted movements of the ATD and the LBD are required to activate the NMDAR ion channel. The structure-based functional experiments validated the structural observation.
Lu W, Du J, Goehring A, Gouaux E: Cryo-EM structures of the triheteromeric NMDA receptor and its allosteric modulation. <i>Science</i> 2017, 355 :eaal3729	** First structure of the triheteromeric NMDAR using antibody fragments to distinguish highly similar subunits.
Regan MC, Grant T, McDaniel MJ, Karakas E, Zhang J, Traynelis SF, Grigorieff N, Furukawa H: Structural Mechanism of Functional Modulation by Gene Splicing in NMDA Receptors. <i>Neuron</i> 2018, 98 :521–529 e523.	** Cryo-EM structure of GluN1b-GluN2B shows the structural organization of the exon 5- encoded motif at the ATD-LBD interface and a channel blocker MK-801 in the TMD.
Karakas E, Simorowski N, Furukawa H: Subunit arrangement and phenylethanolamine binding in GluN1/GluN2B NMDA receptors. <i>Nature</i> 2011, 475 :249–253.	* Crystal structure of <i>Xenopus</i> GluN1 ATD and rat GluN2B ATD heterodimer shows that the ifenprodil binding site resides at the GluN1- GluN2B subunit interface. This study shows that the pattern of inter-GluN1-GluN2B interactions at ATD is distinct from those of non-NMDARs.
Gielen M, Retchless BS, Mony L, Johnson JW, Paoletti P: Mechanism of differential control of NMDA receptor activity by NR2 subunits. <i>Nature</i> 2009, 459 :703–707 Yuan H, Hansen KB, Vance KM, Ogden KK, Traynelis SF: Control of NMDA receptor function by the NR2 subunit amino-terminal domain. <i>J Neurosci</i> 2009, 29 :1204512058.	* The subunit identity of ATD and the linker between ATD and LBD (ATD-LBD linker) influences channel open probability of the NMDARs, as shown by swapping the ATD and the ATD-LBD linker between GluN2A, GluN2B and GluN2D subunits. Similarly, authors use GluN2A-GluN2D chimeras to show that the ATD not only influences channel open probability, but also mean open duration and deactivation time courses by extensive patch-clamp experiments.
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Declarations of interest: none

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Abstract

N-methyl-D-aspartate receptors (NMDARs) are glutamate-gated ion channels, which are critically involved in brain development, learning and memory, cognition, as well as a number of neurological diseases and disorders. Structural biology of NMDARs has been challenging due to technical difficulties associated with assembling a number of different membrane protein subunits. Here, we review historical X-ray crystallographic studies on isolated extracellular domains, which are still the most effective mean to delineate compound binding modes, as well as the most recent studies using electron cryo-microscopy (cryo-EM). A number of NMDAR structures accumulated over the past 15 years provide insights into the hetero-tetrameric assembly pattern, pharmacological specificities elicited by subtypes and alternative splicing, and potential patterns of conformational dynamics, however, many more important unanswered questions remain.

Keywords

N-methyl-D-aspartate receptors; multi-heteromeric membrane proteins; X-ray crystallography; cryo-EM; neuropharmacology

Introduction

The *N*-methyl-D-aspartate receptors (NMDARs) belong to the ionotropic glutamate receptor (iGluR) family and play critical roles in excitatory neurotransmission and synaptic plasticity. They are crucially involved in brain development as well as learning and memory, and their malfunctioning has been associated with a broad range of neurological diseases and disorders including depression, stroke, seizure, schizophrenia, and Alzheimer's disease [1–3]. NMDARs primarily co-localize with other non-NMDAR iGluRs, including α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) and kainate receptors, at the post-synapse and contribute to excitatory postsynaptic potentials (EPSPs) upon binding of the neurotransmitter, glutamate. While the early phase and the amplitude of EPSPs are mainly dominated by the activities of non-NMDARs, the late decay phase is governed by the slow deactivation kinetics of NMDARs [4,5]. Opening of NMDARs results in a high calcium flux and requires both binding of neurotransmitters and a relief of a magnesium block by depolarization, thereby coincidentally detecting information from the pre-synapse (glutamate release) and the post-synapse (depolarization) – the necessary functional features that initiate synaptic modifications for neuroplasticity according to the Hebbian theory [6].

Unlike non-NMDARs which can exist as homotetramers, NMDARs only function as heterotetramers composed of GluN1 subunits containing eight splice variants (1a-4a and 1b-4b) and GluN2A/B/C/D and/or GluN3A/B subunits. The GluN1 and GluN3 subunits bind glycine or D-serine, whereas the GluN2 subunits bind glutamate. Hence, for the most prevalent GluN1/GluN2 subtypes in the brain, binding of both glycine (or D-serine) and glutamate is required for channel opening whereas the GluN1/GluN3 subtype requires only glycine for the channel activation [7,8]. Like non-NMDAR subunits, every NMDAR subunit is composed of an amino-terminal domain (ATD), a ligand-binding domain (LBD), a

transmembrane domain (TMD), and a carboxy-terminal domain (CTD). However, there is little or no primary sequence similarity in the ATDs and CTDs between NMDARs and non-NMDARs. Consistently, the ATDs and CTDs of NMDARs are involved in functions that are distinct from non-NMDARs. For example, the NMDAR ATDs allosterically regulate open probability and deactivation speed, and some also bind allosteric compounds that inhibit or potentiate the channel activities [9–14] while such role of the non-NMDARs ATDs has not been observed, thus far. The CTDs of the GluN2 NMDARs are substantially long (ranging from 396 to 626 residues for rat GluN2) compared to those of non-NMDARs (~80 residues). The GluN2 CTDs have been proposed to work as ‘hubs’ for scaffolding postsynaptic density proteins to mediate cellular signaling for neuronal plasticity [15]. The GluN1 CTDs (~50–100 residues) contain one to two calmodulin binding sites to govern a mode of inhibition called calcium-dependent inactivation, the cytoplasmic mechanism to prevent overshoot of calcium influx [16–18]. Although outside the scope of this review, it is worth mentioning that a complex structure between the peptide from a part of the C1 cassette of the GluN1 CTD and calmodulin has been reported previously [19]. This review will focus on recent structures, including intact NMDARs as well as isolated NMDAR extracellular domains, as others have extensively reviewed pharmacological aspects of NMDARs [9,10,20–22].

High-resolution crystallography of NMDAR extracellular domains

Historically, structural studies of NMDARs lagged behind those of non-NMDARs due to technical difficulties associated with their hetero-oligomeric nature as described above. That is, sample preparation involving recombinant expression and protein purification required optimization to allow and maintain proper assembly of hetero-oligomers. Prior to 2014, all available structures on the NMDAR were limited to the isolated extracellular domains solved by X-ray crystallography, i.e. the GluN1/2/3 LBDs [23–30] and GluN1/GluN2B ATDs [11,31,32]. Despite the availability of an intact homotetrameric structure of another iGluR, the AMPA receptor [33], it became clear from studies on the GluN1-GluN2B ATD heterodimers that the overall architecture of the intact NMDAR would be rather distinct [11]. Specifically, the ‘twisted’ bi-lobe architecture of the NMDAR ATDs compared to non-NMDAR ATDs [11,31] would make it sterically impossible for NMDARs to follow the arrangement of the homomeric AMPAR ATDs [34–36] and homo- or heteromeric kainate receptor ATDs [37–39]. The later structural studies on the intact NMDARs (Fig. 1a) [40,41] took full advantage of these NMDAR domain structures and also showed that the architectures and the patterns of subunit arrangement in these isolated domains are preserved in the intact receptors, therefore demonstrating the physiological relevance of the isolated domain structures (Fig. 1b, 1c). The key advantage for conducting studies on the isolated extracellular domains is their high-resolution capability (up to ~1.5 Å for the GluN1-GluN2A LBD heterodimers and ~2.5 Å for GluN1-GluN2B ATD heterodimers), which has been unattainable by single particle electron cryo-microscopy (cryo-EM) or X-ray crystallography of intact NMDARs. The high-resolution crystallographic studies on the isolated domains have been instrumental especially in unraveling precise chemical natures of bindings of agonists, antagonists, and some allosteric modulators [12,27,42–48]. The limitation of this fragmented approach, however, is the difficulty in studying compounds potentially binding at the inter-ATD-LBD or inter-LBD-TMD interfaces [49–53].

Structural studies of intact NMDARs

The major shortcoming in the studies of isolated ATDs and LBDs is the lack of insights into inter-domain and inter-subunit interactions in the context of the heterotetrameric assembly. This is especially important in NMDARs because the ATDs regulate the ion channel activities [13,14] by interacting with the LBDs, a functional characteristic not present in non-NMDARs. However, how ATDs from different GluN2 subunits result in different channel open probabilities and how binding of allosteric modulators such as ifenprodil induce conformational changes in the ATD and result in allosteric inhibition of the GluN1-GluN2B ion channel at the TMD remained elusive. These fundamental questions drove structural biologists to obtain the first crystal structures of the intact GluN1-GluN2B NMDARs (from either rat or *Xenopus* frog), which were technically challenging [40,41]. For example, proper hetero-multimeric assembly of GluN1-GluN2B NMDARs required the usage of baculovirus-mediated gene transduction of mammalian cells (BacMam) [41,54] or baculovirus-mediated expression in insect cells using the *Drosophila* Hsp70 promoter instead of the conventional polyhedron promoter [40]. Availability of the high-resolution ATD [11] and LBD heterodimeric structures [24] facilitated obtaining sufficient phase information to result in electron density for the TMD from the diffraction data at around 4 Å. Improved resolution was achieved in both independent studies by introducing a disulfide crosslink between the two GluN2B subunits at the ATD, and by adding an allosteric inhibitor that binds at the ATD inter-subunit interface (ifenprodil or Ro25–6981), possibly suggesting a high degree of flexibility in the extracellular region. These intact tetrameric structures of the allosterically inhibited, closed channel with full or partial agonists bound have shown the overall assembly of alternating GluN1-GluN2B-GluN1-GluN2B subunits, the arrangement of “dimer of heterodimers” in the ATD and LBD layer, and a pseudo-fourfold symmetry in the TMD. Similar to AMPARs [33], domain swapping is observed in the extracellular region of the intact NMDARs, where the ATDs and LBDs change their respective heterodimer partners. Interestingly, structures of the desensitized GluK2 kainate receptor show a pinwheel arrangement for the LBD layer [55,56], different from any of the NMDAR or AMPAR structure to date. Considering the similarity between the GluK2 kainate receptors and the GluA2 AMPARs, a similar domain-swapping may also take place in non-desensitized states of the GluK2. The most pronounced difference between non-NMDARs and NMDARs is the inter-domain arrangement between the ATD and the LBD layers: the ATDs and LBDs in NMDARs have extensive inter-domain and inter-subunit interactions whereas the ATD-LBD interactions are minimal in non-NMDARs. Consequently, the NMDARs have a more compact ‘*hot-air balloon*’ architecture compared to the more extended ‘*Y-shaped*’ structure of AMPARs and kainate receptors.

Technological breakthroughs in cryo-EM in recent years have allowed many large protein complexes to be solved at a rapid speed [57–59], including more NMDAR structures in distinct functional states. While the resolutions of cryo-EM structures are typically lower (~4.5–15 Å) than those of crystal structures, they offer insights into large domain movements, which can be gleaned by extensive 3D classification in single particle analysis. Cryo-EM studies by Zhu *et al* and Tajima *et al* captured conformations distinct from the crystal structures in the agonist/allosteric modulator-bound condition [40,41], thereby providing the

first dynamic views of NMDARs and yielding structural insights into receptor activation, allosteric modulation, and antagonism [60,61]. The structure of the GluN1-GluN2B NMDAR in the presence of glycine and glutamate but in the absence of ATD-targeting allosteric inhibitors showed unique conformations in the extracellular domains that increases the distance between the GluN1-GluN2B ATD dimers, which is caused mainly by the opening of the GluN2B ATD bi-lobes compared to the ifenprodil-bound form. The majority of the captured 3D classes showed the ‘open’ GluN2B ATD bi-lobes and ‘closed’ LBD bi-lobes as observed in the previous agonist-bound crystal structures [11,24], and the TMD channel with the ‘closed’ gates, which could represent the ‘non-active’ pre-open or desensitized state [60,61]. One 3D class derived from ~16% of the NMDAR particles in the cryo-EM study by Tajima *et al* showed a conformation likely representing the ‘active’ state, but unfortunately, the TMDs are not well resolved in this particular 3D class and thus, limit mechanistic insights into gating [61] (Fig. 2a). Nevertheless, in this 3D class, in addition to the ‘opening’ of the GluN2B ATD bi-lobe, the inter-GluN1-GluN2B orientation is rotated by ~12°, which becomes translated to a rotation of the GluN1-GluN2B LBD dimers (Fig. 2a). This concerted movement between the ATD and the LBD increases the distances between the gating-ring residues that are proximal to the TMD channel by as much as ~20 Å compared to the other ‘non-active’ 3D classes, which may generate sufficient tensions in the LBD–TMD linker for ion channel gating [62,63]. The validity of naming this conformation ‘active’ was supported initially by the observation that trapping the ‘active’ GluN1-GluN2B subunit conformation at the ATD by chemical cross-linkers resulted in potentiation of agonist-induced currents as measured by electrophysiology [61]. Further support comes from recent work that trapped the ‘active’ conformation at the LBD layer by a disulfide bond, which also resulted in augmented activity reflected by a higher opening probability and mean open time [64]. Despite the significant advances represented by the above findings, the ‘active state’ structure with open gate and pore at the TMD is still required to obtain the complete mechanism of NMDAR activation.

Cryo-EM structures at 9–15 Å resolution of the GluN1-GluN2B NMDAR prepared in the presence of competitive antagonists 5,7-dichlorokynurenic acid (DCKA) and D-2-amino-5-phosphonovalerate (D-AP5) showed 3D classes with robust separation of the extracellular region compared to the agonist-bound form [60]. Based on the cryo-EM density and rigid-body model fitting, the study concluded that the gating-ring in those antagonist-bound NMDAR structures is ruptured in a way that the “dimer of dimers” arrangement is no longer maintained to mediate signal transduction from the LBDs to the TMD. Similar to the ‘active’ state [61], the TMDs were not resolved in those structures, thus, limiting the precise insights into how competitive antagonism works at the level of the ion channel. Furthermore, whether the structures of the antagonist-bound state resemble that of the resting state (i.e. no ligands) remains to be investigated. The observation in the cryo-EM study was further validated by double-electron-electron-resonance (DEER) experiments, which measures the distance between spin labelled residues to estimate inter-domain and inter-lobe distances [60]. Generally, it is important to carefully validate observed conformational changes in cryo-EM structures through a number of ways including functional assays, biochemical cross-linking, and DEER experiments, since a plethora of experimental factors in protein sample preparation as well as the vitrification process of cryo-EM sample preparation (e.g.,

shearing forces in vitreous ice, detergents, types of grids) can often lead to dissociation and deformation of protein assembly. For all of the observed conformational alterations associated with activation, competitive antagonism, and allosteric inhibition, it would be interesting to examine them by alternative methods such as luminescence resonance energy transfer (LRET) [65–68] and single-molecule fluorescence resonance energy transfer (smFRET) [69–72]. The key advantage of those methods is that the experiments can be conducted at room temperature, whereas DEER experiments are typically performed at cryogenic temperatures (~80 K) [73] and hence might not validate physiological relevance convincingly. Furthermore, probing conformational alteration using computational approaches [74,75] may provide an insightful path to understand the mechanistic details. The major challenge in this area of research is to correlate a number of modal patterns of activation observed by electrophysiology to the protein conformational landscape [76–80].

Addressing more specific questions

With the completion of *de novo* structures of the intact NMDARs and observation of fundamental conformational movement, the structural biology of NMDARs is at the stage where more specific questions can be pursued. Examples are structures of tri-heterotetrameric NMDARs composed of GluN1, GluN2A, and GluN2B [81], splice variants [82], and channel blocker binding [82,83].

In addition to di-heteromeric NMDARs, tri-heteromeric NMDARs containing three distinct subunits are present in the adult brain [84]. The first identified tri-heteromeric NMDARs was the GluN1-GluN2A-GluN2B combination [85]. Later studies demonstrated that the tri-heteromeric NMDARs are present in the synapses to mediate synaptic transmission and neuroplasticity [86–88]. Curiously, the GluN1-GluN2A-GluN2B tri-heteromeric NMDAR has functional properties, which are characteristic of both GluN1-GluN2A and GluN1-GluN2B di-heteromeric NMDARs but are more highly weighted toward the GluN1-GluN2A NMDAR [86,89,90]. The recent cryo-EM structure of the GluN1-GluN2A-GluN2B NMDARs from *Xenopus laevis* provided a possible answer to this phenomenon [81]. Technical difficulties included assembly of the GluN1-GluN2AGluN2B tri-heteromeric NMDAR proteins in a recombinant expression system and distinction of GluN2A and GluN2B during the single particle analysis due to the high structural similarity between the GluN2A and GluN2B subunits, which is reflected by their high sequence identity/similarity (71%/83% for *Xenopus* GluN2A/B without the CTD). By labeling the GluN2B subunit with an anti-GluN2B Fab fragment, Lü *et al* could however elegantly distinguish the subunits in the intact tri-heteromer [81] (Fig. 2c). The structure showed more extensive interactions between the GluN2A ATD and the LBD than between the GluN2B ATD and the LBD, possibly explaining why the pharmacological properties of these asymmetric tri-heterotetramers lean towards the di-heterotetrameric GluN1-GluN2A such as nano-molar zinc inhibition potency and a faster glutamate deactivation time course [86,90,91]. The most recent cryo-EM structures of the di-heteromeric GluN1-GluN2A NMDAR [92] now allow the field to make more detailed comparisons with the tri-heteromeric NMDARs and will help provide further explanation to the asymmetric pharmacological properties described above.

Another important feature of NMDARs is their differential pH-sensitivities controlled by alternative splicing [93]. It has been known that inclusion of the 21 amino acids encoded by exon 5 in GluN1 (in GluN1-1b to GluN1-4b splice variants) reduces potency of proton inhibition. A recent cryo-EM structure of the GluN1b-GluN2B receptor at 4.6 Å showed that the exon 5-encoded motif changes the local structure and protrudes from the GluN1 ATD core to form unique interactions with the GluN1 and GluN2B LBDs, thereby strengthening the heterodimeric GluN1-GluN2B LBD interaction [82] (Fig. 2d). This cryo-EM structure along with the structure-based experiments linked the exon 5 paradigm with the previous finding that the pH-sensor may lie at the GluN1-GluN2 LBD interface [94].

Improved cryo-EM density maps or electron density maps from X-ray crystallography for the TMD have now even enabled the field to locate the binding sites of small molecule channel blockers, such as MK-801 [82,83]. The cryo-EM study showed clear cryo-EM density of MK-801, which interacts closely with the residue Asn615 of the pore-lining M3 helices of rat GluN2B [81,82]. These findings were corroborated by X-ray crystallography as it generally remains the method of choice when probing binding of small molecules. In this case, removing the ATD helped in obtaining crystals with improved resolution and showed electron densities for the channel blocker MK-801 (Fig. 1d) [83]. While the LBD arrangement in these crystals seems different from the intact NMDARs containing the ATD, the TMD channel is assembled correctly, thus, the crystal structure serves as a valid tool to assess binding of channel blockers.

Conclusions

With the general structural framework of the NMDAR set and steadily improving resolution in cryo-EM studies, more detailed questions surrounding NMDAR subtypes as well as functional states and some compound binding patterns can be probed. However, at this point, X-ray crystallography remains to be the superior method for obtaining detailed compound binding mechanisms that are useful for therapeutic interventions. Combining all those structures with spectroscopic and computational experiments and correlating the outcomes with functional readouts from electrophysiology is the desired approach to complete the sophisticated mechanistic scheme of NMDARs.

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Highlights

- The X-ray crystallographic structures of the intact NMDARs showed unique domain and subunit assembly patterns explaining the distinct functions from the non-NMDARs.
- Implementation of single particle cryo-electron microscopy revealed multiple conformations of the intact NMDARs that are linked to functions.
- X-ray crystallography on isolated domains of NMDARs remains the best method to observe compound binding modes at high-resolution.
- Despite significant advances over the last decade, sophisticated questions such as modal gating of NMDARs have not been well explained by the structural biology at this point.

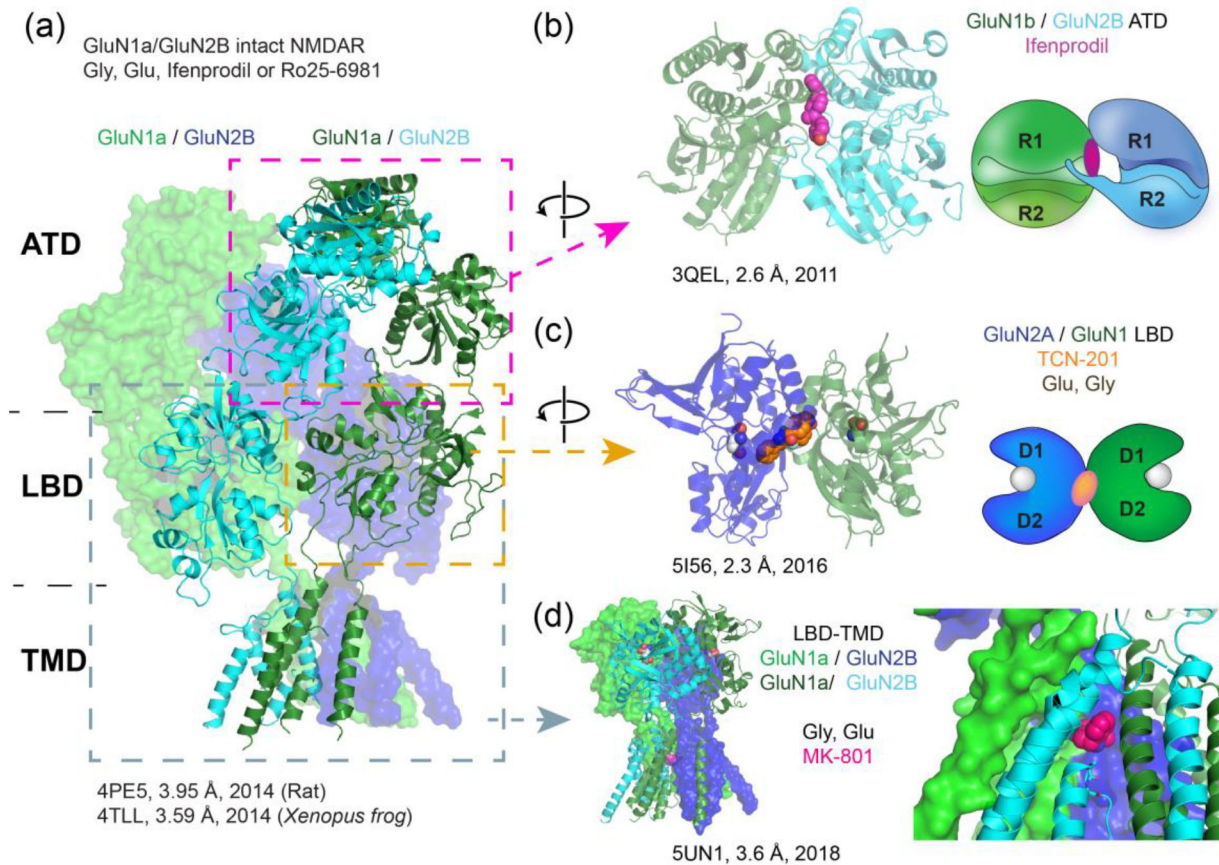


Figure 1. Crystallographic studies on the NMDAR.

(a) Crystal structures of the intact GluN1-GluN2B di-heteromeric NMDARs (without CTD) from rat and *Xenopus* frog showing a GluN1-GluN2B-GluN1-GluN2B arrangement (GluN1: green/light green, GluN2B: cyan/dark blue) and a domain-swap between the ATD layer and the LBD layer. **(b-d)** Fragmented approach to effectively monitor ligand binding sites at high resolution. **(b)** The crystal structure of the GluN1b-GluN2B ATD heterodimers shows the ifenprodil (pink) binding site at the GluN1b-GluN2B subunit interface. Binding of ifenprodil promotes the closure of the bi-lobed structure defined by the upper (R1) and lower (R2) lobes for the GluN2B ATD. **(c)** A number of crystal structures of NMDAR LBDs, including the ones of GluN1, GluN2A, and GluN3A, have been solved over the years with agonists, competitive antagonists, and allosteric modulators. Shown here is the crystal structure of the GluN1-GluN2A LBDs complexed to glycine (light gray), glutamate (light gray), and TCN-201 (orange). Glutamate and glycine bind at the cleft between the upper (D1) and lower (D2) lobes, whereas TCN-201 binds at the LBD dimer interface. **(d)** The crystal structure of the GluN1 ATD – GluN2B ATD NMDAR shows the binding site of the channel blocker MK-801 (red). Removing the ATDs changes the subunit arrangement in the LBD layer, which highlights the importance of ATDs for proper heterotetrameric subunit assembly. Resolutions of the electron-density maps (in Å) for the respective structures (with PDB code) are stated as in the Protein Data Bank.

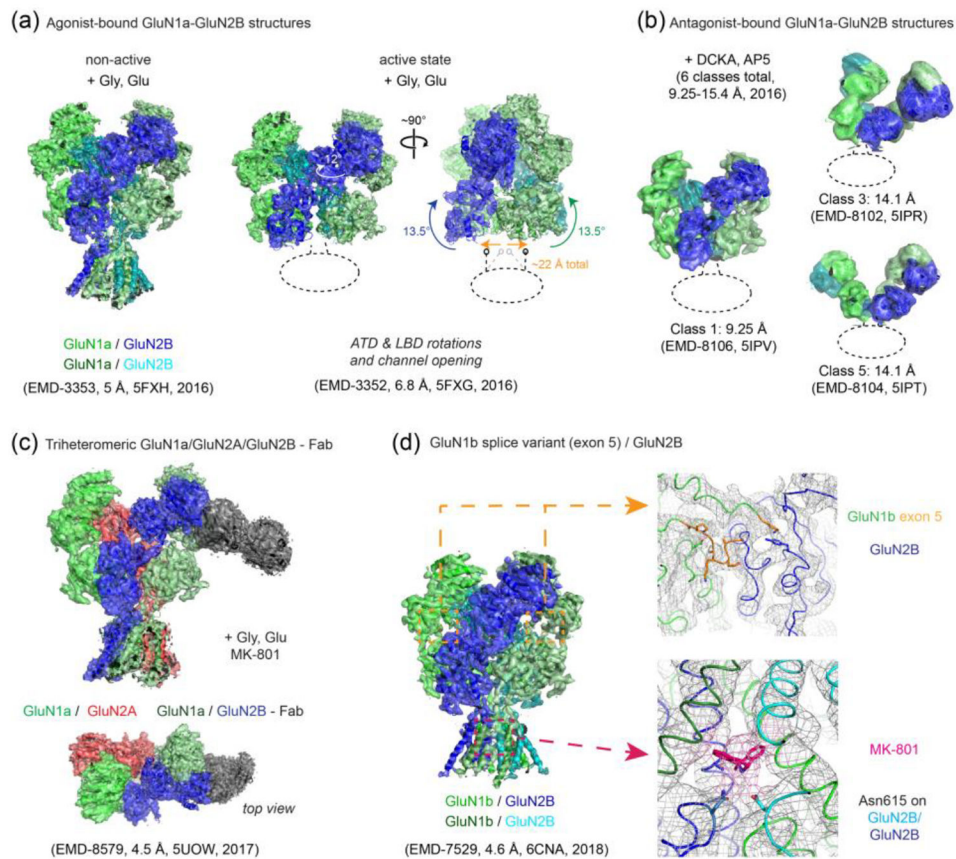


Figure 2. Single particle cryo-EM studies on the rat and *Xenopus* NMDARs.

(a) Multiple conformational states of the rat GluN1-GluN2B NMDARs bound to the agonists, glycine and glutamate. The cryo-EM density maps and the molecular models for the GluN1 subunits are colored green and light green whereas those for the GluN2B subunits are colored cyan and dark blue. Transitioning from the non-active state to the active state requires ‘opening’ of the bi-lobed structure of the GluN2B ATD and inter-subunit reorientation of the GluN1 and GluN2B ATDs by $\sim 12^\circ$, which translates to rotation movement of the GluN1-GluN2B LBD heterodimers by $\sim 13^\circ$ and onto the gate of the TMD channel (arrows show movement from “non-active 2” state to “active” state [[61]]). Unresolved TMDs are marked with dotted ellipses/lines. **(b)** Binding of the antagonists, DCKA and AP5, leads to large movements in the extracellular domains as shown by selected 3D classes of the *Xenopus* GluN1a-GluN2B NMDARs with different conformations and the disrupted pattern of the “dimer of dimers” assembly. The color code is as in *panel (a)*. **(c)** The overall assembly pattern of the tri-heteromeric *Xenopus* GluN1a-GluN2A-GluN2B NMDARs is similar to that of the di-heteromeric GluN1-GluN2B NMDARs in that the GluN1-GluN2-GluN1-GluN2 subunit arrangement is conserved. Binding of anti-GluN2B Fab (dark gray) facilitated single particle analysis by distinguishing the GluN2B subunit (dark blue) from the GluN2A subunit (red). **(d)** The cryo-EM structure of the GluN1b-GluN2B NMDAR splicing variant containing exon 5 in the GluN1b subunit shows that the exon 5-encoded motif (orange) is located at the ATDLBD interface and interacts with both the GluN1b LBD and GluN2B LBD to strengthen the hetero-dimeric

interaction. This structure also showed cryo-EM density for a channel blocker, MK-801, and interacting residues such as GluN2B Asn615 (shown as sticks) in the TMD channel. Similar density for MK-801 was also observed in the cryo-EM structure of the tri-heteromeric *Xenopus* GluN1a-GluN2A-GluN2B NMDARs. Resolutions of the EM maps (in Å) for any given class (with EMD code) are stated as in the EM Data Bank, which were determined by the current gold-standard Fourier Shell Correlation (FSC) cutoff at 0.143.

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