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Cryo-EM structures of the triheteromeric NMDA receptor and its allosteric modulation

Wei Lü¹, Juan Du¹, April Goehring, and Eric Gouaux^{1,2}

¹Vollum Institute, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239

²Howard Hughes Medical Institute, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239

Summary

N-methyl-D-aspartate receptors (NMDARs) are heterotetrameric ion channels assembled as diheteromeric or triheteromeric complexes. Here we report structures of the triheteromeric GluN1/GluN2A/GluN2B receptor in the absence or presence of the GluN2B-specific allosteric modulator Ro 25-6981 (Ro), determined by cryo-EM. In the absence of Ro, the GluN2A and GluN2B amino terminal domains (ATD) adopt “closed” and “open” clefts, respectively. Upon binding Ro, the GluN2B ATD clamshell transitions from an open to a closed conformation. Consistent with a predominance of the GluN2A subunit in ion channel gating, the GluN2A subunit interacts more extensively with GluN1 subunits throughout the receptor, in comparison to the GluN2B subunit. Differences in the conformation of the pseudo 2-fold related GluN1 subunits further reflect receptor asymmetry. The triheteromeric NMDAR structures provide the first view of the most common NMDA receptor assembly and show how incorporation of two different GluN2 subunits modifies receptor symmetry and subunit interactions, allowing each subunit to uniquely influence receptor structure and function, thus increasing receptor complexity.

Introduction

The NMDAR (1) is a molecular coincidence “detector” that transduces binding of glycine (2) and glutamate (3), together with the voltage-dependent unblock of magnesium (4, 5), into the opening of a transmembrane ion channel, resulting in depolarization of the postsynaptic membrane potential and entry of calcium, thereby initiating both electrical and chemical signals in the postsynaptic cell (6). Spread throughout the central nervous system (7, 8), NMDARs are integral for fast excitatory signal transmission, essential for normal brain development, and function and are implicated in multiple neurological injuries, diseases and disorders (9). NMDARs play particularly crucial roles in learning and memory and are the targets of clinically relevant drugs for treatment of Alzheimer’s disease (10), schizophrenia (11), depression (12) and epilepsy (13).

Diversity in NMDAR function arises in part from a spectrum of NMDAR subunits that can assemble within the heterotetrameric complex and that include the glycine-binding GluN1 and GluN3 subunits and the glutamate-binding GluN2A-D subunits (9). The prototypical NMDAR harbors two GluN1 subunits and two GluN2 subunits, whereby the identity and

count of the GluN2 subunit are dictated by cell-specific conditions. The GluN1/GluN2A or GluN1/GluN2B receptors are canonical representatives of diheteromeric receptors (14) and the GluN1/GluN2A/GluN2B receptor is the paradigm triheteromer, the most common NMDAR receptor spread throughout the hippocampus and cortex (15–19). While a great deal is known about the physiology, pharmacology and structure of the GluN1/GluN2B diheteromeric receptor, there is a relative dearth of knowledge about the GluN1/GluN2A/GluN2B triheteromer. This receptor is uniquely modulated by the GluN2A and GluN2B allosteric antagonists divalent zinc (Zn) (20) and the phenylethanamines ifenprodil or Ro 25-6981 (Ro) (21), respectively, and exhibits ion channel gating kinetics, as well as pharmacology, that are distinct from either the GluN2A or GluN2B diheteromeric receptors (22–24). While there are multiple structures of the diheteromeric GluN1/GluN2B receptor (25–27), there is no experimental structure of a full-length GluN2A-containing NMDAR, and thus the conformation and interactions of the GluN2A subunit remain unknown.

To define how two different GluN2 subunits are incorporated into the NMDAR heterotetrameric assembly and to elaborate the structure of the full-length GluN2A subunit, we elucidated the structure of the GluN1/GluN2A/GluN2B triheteromeric receptor by single particle cryo-electron microscopy (cryo-EM), using a high affinity GluN2B-specific Fab to unambiguously distinguish the GluN2B subunit from the GluN2A subunit. We further carried out single particle cryo-EM studies in the presence or absence of the GluN2B-specific allosteric antagonist, Ro, in order to understand the structural basis for the action of GluN2B specific antagonists in the context of a triheteromeric receptor. Our structural studies illustrate the architecture of the triheteromeric receptor complex, define the molecular action of Ro, and show how the GluN2 subunits participate in distinct interactions throughout the receptor assembly.

Receptor isolation and structure determination

The wild-type triheteromeric GluN1/GluN2A/GluN2B receptor from *Xenopus laevis*, deemed triNMDAR_{wt}, expresses poorly and is biochemically unstable, thus hindering single particle cryo-EM studies. To improve expression level and stability we exploited two sets of constructs derived from previously published studies, the triNMDA_{EM} construct and the triNMDA_{EM}-G610 construct (Fig. S1 A–C), where the latter construct has the G610 to R substitution in the GluN1 subunit reverted to the wild-type glycine residue (26, 27). In comparison to triNMDA_{wt} (Fig. S1D), the triNMDA_{EM}-G610 preserves small but measureable glycine/glutamate-induced conductances as well as inhibition by Zn or Ro. We carried out additional studies using constructs where we reverted an additional mutation in GluN1 M4 helix to its wild-type identity (GluN1_{EM}-G610-M816) or where we simply used wild-type GluN1 subunit, in combination with GluN2A_{EM} and GluN2B_{EM} constructs (Fig. S1 E–H; see Methods). These two closely related constructs showed larger currents that were also inhibited by Zn or Ro, thus supporting the conclusion that the functional receptor is a triheteromeric assembly. Agonist-induced currents of the triNMDA_{EM} construct are not measurable, either due to low conductance or to a low open probability or both.

Receptor expression in mammalian cells was monitored by fluorescence-detection size-exclusion chromatography (FSEC) (28) and enhanced by utilization of a bicistronic Bacmam

virus harboring the GluN1 and GluN2A subunits, together with a monocistronic GluN2B virus (29). A systematic screening of fusion partners showed that a SRC homology 3 (SH3) domain fused to the C-terminus of the GluN2B subunit further increases receptor expression (Fig. S1 I). Coexpression of the GluN1, GluN2A and GluN2B subunits yields triheteromeric GluN1/GluN2A/GluN2B receptors in addition to diheteromeric GluN1/GluN2A and GluN1/GluN2B receptors. To isolate the triheteromeric complex, we exploited Strep-II tagged GluN2A, His₈ tagged GluN2B and untagged GluN1 subunits (Fig. S1 I) combined with a two-step affinity purification strategy. Dual-affinity purified triheteromeric receptor eluted from a size-exclusion chromatography column (SEC) as a single sharp peak and showed three protein species on a SDS-polyacrylamide gel (Fig. S1 J).

To probe the biochemical integrity of the triheteromeric receptor preparation and to label the GluN2B subunit for cryo-EM reconstruction, we developed the 10B11 and 11D1 monoclonal antibodies to the GluN1 and GluN2B subunits, respectively (Fig. S1 K). These antibodies recognize three-dimensional epitopes and do not bind under denaturing conditions, thus allowing us to selectively distinguish subunits within the assembled complex. To evaluate the extent to which the triheteromeric receptor preparation was a homogenous population and not a mixture of diheteromeric receptors, we analyzed the shifts of the receptor upon binding of GluN1 and GluN2B-specific Fabs using FSEC. The 10B11 (GluN1) Fab shifts both the diheteromeric GluN1/GluN2A and triheteromeric GluN1/GluN2A/GluN2B receptors to earlier elution volumes, whereas 11D1 (GluN2B) only shifts the triheteromeric receptor (Fig. S1 L–M). In addition, the shift of the triheteromeric receptor by 11D1 is about half of that promoted by 10B11, consistent with the presence of two GluN1 and one GluN2B subunits in the purified receptor, as opposed to the preparation being a mixture of diheteromeric receptors (Fig. S1 M).

To define the arrangement of subunits in the triheteromeric NMDAR and to understand the structural basis for receptor modulation by GluN2B-specific modulators, we elucidated structures of the glycine and glutamate-bound triNMDAR_{EM} in complex with the 11D1 Fab in the absence or presence of Ro, termed the non-Ro-bound and Ro-bound states, respectively (Fig. S2, S3, S4). Ambient zinc, a nanomolar-affinity allosteric inhibitor acting at the GluN2A subunit (30), was chelated by EDTA in the receptor preparation. We also carried out single particle cryo-EM studies on the functional triNMDAR_{EM}-G610 receptor-11D1 Fab complex to compare to the triNMDAR_{EM} (Fig. S5, Fig. S6). To determine the effects of Fab binding, we determined a low-resolution structure of the triNMDAR_{EM} in the absence of the Fab (Fig. S7), finding that the triNMDAR_{EM}-G610 structure is indistinguishable, at the present resolution, from that of triNMDAR_{EM} (Fig. S6, Fig. S8). Because the triNMDAR_{EM} yielded the highest resolution reconstructions, we use it as the basis for the overall structural analysis.

The GluN2A and GluN2B subunits share 73% sequence identity and have similar predicted secondary and tertiary structure. We thus employed the GluN2B-specific Fab, 11D1, to distinguish the GluN2A and GluN2B subunits for particle alignment in image processing (Fig. S9 A–C). After reference-free two-dimensional (2D) classification we observed a strong signal for a single Fab on one side of the receptor in most classes of the non-Ro-bound and Ro-bound receptor (Fig. 1 A, Fig. S3 B, Fig. S4 B). In some classes, however, we

observed a second Fab with variable intensity or apparent occupancy. Nevertheless, in subsequent 3D classification, a Fab-free reference model resulted in five 3D classes showing an unambiguous signal for only a single Fab (Fig. S3 C, Fig. S4 C). Therefore, the apparent second Fab signal in some of the 2D classes was due to the pseudo 2-fold symmetry of the complex and an imperfect alignment of particles. The 3D classes each occupy a similar percentage of particles and share similar overall shape; the differences in the reconstructions are mainly due to the flexibility of the Fab and the intrinsic flexibility between the extracellular domains and transmembrane domain (TMD) of the receptor (Fig. S3 C, Fig. S4 C). Three-dimensional refinement of individual classes yielded low-resolution reconstructions. By combining all of the particles and masking out the mobile Fab, we obtained reconstructions of the non-Ro-bound and Ro-bound forms of the triNMDAR_{EM} at nominal resolutions of 4.5 and 6.0 Å, respectively (Fig. S3 D–F, Fig. S4 D–F).

In the non-Ro-bound form, the densities for the ATDs, LBDs, ATD-LBD linkers, and TMD including M2, P loop and M3 are continuous and mostly well defined, with some bulky side chains visible (Fig. 1 B, Fig. S9, Movie S1). By contrast, the densities of the LBD-TMD linkers, M1 and M4, although less well defined, are of sufficient strength and connectivity to trace the main chain. The loops connecting M1 and M2 are not visible in the density map. The position of the GluN2B subunit is determined by the density of Fab bound primarily to the R1 lobe of GluN2B ATD, along with a minor interface to the R2 lobe of the adjacent GluN1 ATD (Fig. 1B–E, Fig. S9 A–C). The quality of the density maps and the validity of the structure is supported by the observation of 9 and 7 N-linked glycans in the non-Ro and Ro-bound structures, respectively (Fig. S9 D–G). Despite a lower resolution of the Ro-bound structure, we visualized the densities of ATD, LBD, and ATD-LBD linkers, along with the M3 helix, and were able to fit molecular models derived from the non-Ro-bound structure into these density features (Fig. 1 C, Fig. S4). We defined the structure of the GluN2A ATD (Fig. S10) by exploiting a homology model derived from high-resolution GluN2B ATD structures (25, 26, 31) in combination with flexible fitting (32). In comparison to the unliganded GluN2B ATD, the GluN2A ATD ‘clamshell’ possesses more pronounced cleft closure and a more extensive interface – ~30% larger – with its paired GluN1 ATD, as discussed below.

Overall architecture

The triheteromeric NMDAR adopts a bouquet-like shape, with ATD, LBD and TMD arranged in layers from top to the bottom, assembled as a GluN1/GluN2A/GluN1/GluN2B heterotetramer (Fig. 1 B–D) (25, 26). The receptor displays a “dimer of dimers” arrangement (33–35), first seen in the GluA2 AMPA receptor structure (36), with a swapping of domains between the ATD and LBD layers. The two GluN1 subunits occupy the A/C positions and the GluN2A and GluN2B subunits occupy the B/D positions, respectively. Within the ATD layer, the GluN1 (A)/GluN2A (B), and GluN1 (C)/GluN2B (D) subunits associate as ‘local’ ATD heterodimers, respectively, whereas in the LBD layer, GluN1 (C)/GluN2A (B), and GluN1 (A)/GluN2B (D) interact to form local LBD heterodimers (Fig. 1 E).

There are extensive subunit-subunit interactions within and between GluN1/GluN2A and GluN1/GluN2B heterodimers that meld the tetrameric assembly together. Within the ATD layer the most intensive subunit-subunit interactions occur within each ‘local’ heterodimer, involving interactions between subunits at the A/B and C/D positions. Inter-heterodimer interactions between the R2 interface of GluN2A and GluN2B ATDs (Fig. 1 E) suggest a structural basis for transduction of conformational movements between the GluN2A- and GluN2B-containing ATD heterodimers. Even though the Ro-bound structure exhibits a similar overall subunit arrangement to the non-Ro-bound structure, there is a “compression” between the R1 lobes of the two GluN1 ATDs along with an increase in the separation between the R2 lobes of the GluN2 ATDs (Fig. 1 C–D), demonstrating that binding of Ro to the GluN1/GluN2B ATD heterodimer promotes conformational rearrangement throughout the entire ATD layer.

In the transition from the ATD layer to the LBD layer, the distance between ATD and LBD in GluN1/GluN2A is shorter, resulting in a more compact ATD-LBD interface than in GluN1/GluN2B (Fig. 1 F). At the level of the LBD, GluN2A (B) and GluN2B (D) represent the distal subunits, while GluN1 A and C are in proximal positions (Fig. 1 E), consistent with the greater role of the GluN2 subunit in receptor gating (1, 37–39). As a consequence of domain swapping, the LBD heterodimers are formed by A-D and B-C subunits with extensive heterodimer contacts, termed the ‘major’ interface. By contrast, the ‘minor’ interfaces are formed by fewer, yet still significant, interactions between LBD heterodimers (Fig. 1 E). The TMDs of four subunits show a similar arrangement as observed in AMPA, kainate and NMDA receptors (25, 26, 36, 40–43). However, the organization of the four transmembrane helices are distinct between the two GluN1 subunits, particularly the orientation of M4, a helix that participates in extensive interactions with an adjacent GluN2 subunit, relative to the rest of the TMD (Fig. 1 F). The differences within the TMD layer, along with the distinct arrangements throughout the extracellular domains, demonstrate that triheteromeric NMDARs harbor an overall asymmetric architecture distinct from diheteromeric NMDARs which, by contrast, have an approximate overall 2-fold axis of symmetry in their resting and activated states (25–27, 44). The asymmetry of the triheteromeric NMDAR is thus consistent with non-equivalent functional roles of the GluN2A and GluN2B subunits (22, 45, 46).

Asymmetry of ATD and ATD-LBD interfaces

NMDA receptors harbor binding sites for subunit-specific allosteric modulators on the GluN2 subunits (1, 47, 48), and conformational changes are conveyed to the LBD layer via the ATD-LBD interface and the ATD-LBD linker (38). In the triheteromeric NMDAR, the GluN1/GluN2A ATD makes multiple interactions with the LBD layer, whereas the GluN1/GluN2B ATD is separated from the LBD layer by a solvent-filled gap (Fig. 2 A–B). We hypothesize that these differences are largely the consequence of distinct conformations of the ATD-LBD linkers. Whereas the GluN2A ATD and LBD interface is occupied by a contracted linker, which mediates extensive ATD-LBD interactions, the GluN2B linker adopts an extended conformation and does not ‘fill’ the space between the ATD and LBD, resulting in a contact area that is ~40% of that of GluN2A.

Inspection of the ATD-LBD interface shows that the GluN2A ATD not only interacts with its own LBD, but also with its neighboring GluN1 subunit. The protruding $\alpha 5$ - $\alpha 6$ loop on the “bottom” of the GluN2A ATD is embedded within an ATD-LBD crevice formed by loop 1 of the GluN2A LBD together with loop 2 and helix F of an adjacent GluN1 LBD (Fig. 2 C). By contrast, the $\alpha 5$ - $\alpha 6$ loop of the GluN2B ATD is positioned to make only a few interactions with loop 2 of the GluN1 LBD (Fig. 2 D). More extensive GluN2A ATD to LBD contacts are consistent with studies that show triheteromeric NMDARs are less sensitive to GluN2B-specific modulators while retaining substantial modulation by GluN2A-specific small molecules (22, 23).

To further explore the conformations of the GluN2A and GluN2B subunits in the triheteromeric receptor, we compared the ATD heterodimers and individual subunits with each other (Fig. 2 E–G) and also with the isolated non-Ro-bound GluN1/GluN2B ATD heterodimer and the Ro-bound diheteromeric GluN1/GluN2B receptor structures (Fig. S11 A–D) (26, 44). First, upon superposition of the R1 lobes of the two GluN1 subunits from the triheteromeric NMDAR, we see that the GluN1/GluN2A ATDs exhibit a clockwise rotation relative to the GluN1/GluN2B ATDs. Second, when the individual GluN2A and the GluN2B ATDs are superimposed via their R1 lobes, we observe that the GluN2A ATD adopts a more closed-clamshell conformation in comparison to the apo GluN2B ATD. Indeed, the conformation of the GluN2A ATD is more similar to that of the Ro-bound GluN2B ATD structure in the intact diheteromeric GluN1/GluN2B receptor (Fig. S11 A–D), where the GluN2B ATD adopts a closed-clamshell conformation due to the binding of phenylethanolamine allosteric modulators at the interface of the GluN1/GluN2B ATDs (26, 27, 44, 46).

The triheteromeric receptor, prepared with zinc chelator, should not have zinc bound to the receptor and thus it is striking that the ‘apo’ GluN2A ATD adopts a closed cleft conformation (38, 49). We thus compared the conformation of the GluN2A ATD from the triheteromeric receptor structure with the structure of the isolated GluN2A ATD in its zinc-bound form by superposition of their respective R1 lobes. This analysis shows that the isolated, zinc-bound GluN2A ATD adopts a different conformation with an “outward” twisted R2 lobe (Fig. S11 E–F) (50). We speculate that in the intact receptor, zinc might induce a conformation of the GluN2A ATD that is different from what is seen in the current triheteromeric receptor structure, perhaps a conformation similar to that of the isolated, zinc-bound ATD structure, with a ‘twisted’ conformation of the R1 and R2 lobe. The zinc-bound ATD, in turn, could stabilize the LBD layer in a conformation that is not compatible with an open ion channel gate. We note, nevertheless, that protons also inhibit NMDAR activity (51) and it is possible that the pH of the triheteromeric receptor preparation (6.5) stabilizes the GluN2A ATD in a closed-cleft conformation, in the absence of zinc. Most importantly, further structural studies are required to unambiguously resolve the effects of protons and zinc on the structure of the triheteromeric receptor.

The closed clamshell of the GluN2A ATD leads to more extensive interactions with its paired GluN1 ATD through two loops – the upper part of the $\alpha 5$ - $\alpha 6$ loop in the R2 lobe and post- $\alpha 8$ loop in the R1 lobe (Fig. 2 F). This suggests an important role of the $\alpha 5$ - $\alpha 6$ loop that connects the ATD heterodimer interface with the ATD-LBD interface. A portion of the

long post- $\alpha 8$ loop in the GluN2A R1 lobe adopts an α -helix conformation in GluN2B, which effectively compresses its length and reduces the extent to which the GluN2B subunit can interact with the GluN1 subunit. Lastly, the GluN2B ATD of the triheteromeric NMDAR exhibits an open clamshell conformation, similar to that of the diheteromeric non-Ro-bound GluN1/GluN2B (Fig. S11), thus indicating that the GluN2A subunit does not profoundly alter the GluN1/GluN2B ATD conformation and thus modulator binding (22).

The conformational differences between GluN2A and GluN2B subunits at the ATD-LBD interfaces are reminiscent of the difference between NMDAR and non-NMDAR glutamate receptors. In non-NMDARs, direct interactions between the ATD and LBD layers are minimal. Therefore, even if the ATDs undergo ligand-induced movements, there are few molecular routes for transduction of conformational changes and thus the ATDs of non-NMDARs do not markedly modulate ion channel gating (36, 41–43, 52). By contrast, there are multiple distinct contacts between the ATD and LBD layers in NMDARs, and thus a number of mechanisms by which movements of the ATDs can be transduced to the LBD layer and, subsequently, to the TMD to modulate ion channel activity (38, 53, 54). More extensive contacts of the GluN2A ATD with both the GluN1 ATD and LBD layers suggests that the GluN2A subunit is positioned to more profoundly influence the activity of triheteromeric NMDARs in comparison to the GluN2B subunit, a structural observation consistent with analysis by electrophysiological studies of triheteromeric NMDARs (22).

Organization of LBD layer

Comparison with the agonist-bound LBD crystal structures reveals that all four of the LBD clamshells in the triheteromer adopt a “closed” conformation, consistent with complete occupancy by the full agonists (Fig. S12) (34). Indeed, we visualized density for glutamate in the GluN2A LBD (Fig. S12 A), but not in the GluN2B subunit, likely due to a lower local resolution of the map for the GluN2B LBD. Overall, the LBD layer shares a similar organization with diheteromeric GluN1/GluN2B receptors. The GluN2 LBDs form two interfaces with their adjacent GluN1 subunits: a major interface within the heterodimer formed by the D1-D1 interface, and a minor interface between the heterodimer formed by the D2 of the GluN1 subunit and D1 of the GluN2 subunit (Fig. 1 E, Fig. 3 A). While the major interfaces are similar, the minor interface of the GluN2A subunit buries ~2.6-fold larger solvent accessible surface than that of the GluN2B subunit, thus disrupting the pseudo two-fold symmetry at the LBD layer (Fig. 3 B–C). Specifically, in the GluN1/GluN2A minor interface, loop 1 of GluN2A is coupled to the loop connecting helix F and G in the GluN1 subunit; helix K and the downstream S1-M4 linker in the GluN2A subunit are also in closer contact with helix E and the M3-S2 linker in the GluN1 subunit (Fig. 3 C). Thus, the more extensive interactions of GluN2A with both GluN1 subunits within the LBD layer are consistent with the dominant role of the GluN2A subunit in the gating of triheteromeric NMDARs (22).

Subunit rearrangement upon allosteric modulation

The presence of a GluN2A subunit markedly decreases the inhibition rate, efficacy and binding affinity of phenylethanolamine allosteric antagonists, such as ifenprodil or Ro, at

triheteromeric GluN1/GluN2A/GluN2B receptors, in comparison to diheteromeric GluN1/GluN2B receptors (22, 23). However, the molecular basis by which the Ro-insensitive GluN2A subunit perturbs phenethylamine modulation is unclear. The triNMDAR_{EM} constructs bind Ro with submicromolar affinity (Fig. 4 A), ~3-fold weaker than that of the diheteromeric GluN1/GluN2B receptor (26). To probe the structural underpinnings for differences in phenethylamine binding and modulation between diheteromeric and triheteromeric NMDARs, we solved the structure of the triheteromeric NMDAR in the presence of Ro (Fig. 1 C, Fig. S4). In the Ro-bound structure, the ATD layer of the GluN2B subunit undergoes a substantial rearrangement, leading to a more compact ATD-LBD interface (Fig. 4 B). The GluN1/GluN2B ATD undergoes a clockwise rotation (Fig. 4 C) due to an identical clamshell closure of the GluN2B ATD as in the Ro-bound GluN1/GluN2B diheteromer (Fig. 4 D, Fig. S13, Movie S1). By contrast, the GluN1/GluN2A ATD heterodimer remains largely unaffected as does the Ro binding pocket at the GluN1/GluN2B ATD interface (Fig. S13). However, the binding of Ro does remodel the ATD layer from asymmetric to pseudo two-fold symmetric by repositioning the two ATD heterodimers (Fig. 4 E–F) which, in turn, diminishes the contacts between the two GluN2 ATDs (Fig. 4 G–H). Our results suggest that phenylethanolamine modulators perturb the overall receptor structure, at least in part, by alterations in the interface between GluN2 ATDs. This interface is less extensive in the diheteromeric GluN1/GluN2B receptor (27, 44), explaining reduced Ro affinity on triheteromeric NMDAR, because the Ro-induced clamshell closure requires overcoming the interactions at the GluN2 ATD interface. In the Ro-bound state, the diminished GluN2 ATD interface precludes the GluN2B ATD from affecting the GluN2A ATD, consistent with an increase of zinc inhibition via the GluN2A subunit in the presence of ifenprodil, akin to the enhanced ifenprodil inhibition in the presence of divalent zinc (45).

In addition to the ATD layer, contacts of the GluN2B ATD with the LBD layer are altered upon Ro binding. In particular, the lower part of the α 5- α 6 loop moves apart from loop 2 of the GluN1 LBD, resulting in few interactions with the GluN2B LBD (Fig. 4 I). We speculate that, in the absence of Ro, the lower part of the α 5- α 6 loop is in a position that hinders the GluN1/GluN2A LBD heterodimer from restoring its “resting” position after channel activation. Binding of Ro decouples the α 5- α 6 loop from GluN1 LBD, facilitating the GluN1/GluN2A LBD heterodimer movement toward the pore center, promoting channel closure.

Asymmetric transmembrane domain

The M3 helix is a crucial structural determinant linking agonist binding to channel gating. In the non-Ro-bound structure, M3, M2 and the pore loop (P loop) have well defined densities. The M3 helix and the tip of the P loop form a pyramidal vestibule harboring an elliptical density feature representing a trapped MK-801 molecule, with its two aromatic rings positioned toward the M3 helices of GluN2A and GluN2B (Fig. 5 A–B), consistent with experimental measurements of MK-801 binding to the triNMDA_{EM} construct (Fig. 5 C) despite a low ion channel activity. Based on the observation that the M3 bundle crossing occludes the ion channel pore, the non-Ro-bound structure is an agonist-bound, closed-blocked state.

By comparing the M3 helix of the triheteromeric GluN1/GluN2A/GluN2B and diheteromeric GluN1/GluN2B receptor, we observed that, unlike diheteromeric NMDAR with pseudo 4-fold symmetry (25, 26), the TMD of triNMDAR deviates substantially from pseudo 4-fold symmetry due to an extension of the GluN1 (C) subunit from the pore axis (Fig. 5 D). To understand why the two GluN1 subunits are asymmetric, we traced their interactions through the LBD to the ATD-LBD interface and the ATD. The GluN1 M3 helix is connected to its D2 LBD lobe, which in turn interacts with the GluN2 R2 ATD lobe at the ATD-LBD interface (Fig. 5 E). Thus, we suggest that the arrangement of the GluN1 M3 helix is indirectly altered by the asymmetric GluN2 ATD and ATD-LBD interface, specifically, the interactions between GluN1 (A) and GluN2A, and GluN1 (C) and GluN2B. Compared to the compact interaction between GluN2A ATD and GluN1 (A), the contacts between GluN2B ATD and GluN1 (C) are nearly ruptured (Fig. 2 A–D, Fig. 5 E), which promotes movement of the D2 lobe of the GluN1 (C) subunit toward the TMD, thus “pushing” the M3 helix away from the central pore axis.

Mechanism

The presence of GluN2A and GluN2B subunits in triheteromeric NMDAR disrupts the 2-fold symmetry in the ATD and LBD layers and the pseudo 4-fold symmetry in the TMD layer of diheteromeric NMDARs. The GluN2A ATD adopts a “closed” cleft in the absence of the allosteric inhibitor zinc and at low pH, while the GluN2B ATD cleft transitions from “open” to “closed” upon binding of the allosteric modulator Ro. The GluN2A and GluN2B subunits have similar structures yet they make distinct contributions to receptor structure and function (Fig. 6 A–B). First, the GluN2A ATD interacts extensively with the GluN1 R1 lobe within the ATD heterodimer, and thus is poised to modify the conformational properties of its GluN1 ATD partner to a greater extent than GluN2B. Second, the GluN2A ATD resides on top of its cognate LBD, participating in extensive interactions that include insertion of the $\alpha 5$ - $\alpha 6$ loop into a pocket located on top of the GluN2A/GluN1 LBD minor interface. By contrast, the GluN2B ATD is loosely connected to its LBD and is not positioned to make as extensive of contacts with the corresponding LBD layer. Lastly, the GluN2A LBD interacts extensively with the GluN1 LBD at both minor and major interfaces, whereas the GluN2B LBD is primarily coupled to the GluN1 subunit at the major interface. Taken together, the presence of two different GluN2 subunits endows the NMDA receptor with enhanced diversity in structure and function, enriching the complexity of the receptor for signaling at chemical synapses and increasing its effectiveness as a potential target for therapeutic agents.

Methods

Construct design

GluN1, GluN2A and GluN2B constructs used here, deemed GluN1_{EM}, GluN2A_{EM} and GluN2B_{EM}, were designed based on the previously reported *Xenopus laevis* NMDA GluN1-1 and GluN2B-2 constructs (26, 27). GluN1_{EM} is generated by removal of the C-terminal of GluN1-2, including the enhanced green fluorescent protein (eGFP), 3C cleavage site (Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro) and an octa-histidine tag. GluN2B_{EM} reverts the

K216C mutation in GluN2- 2 to a wild-type lysine residue, with a GFP11, SH3 fusion protein and an octa-histidine tag placed at its C-terminus. GluN2A_{EM} was designed based on the GluN2B_{EM} construct, but including the C-terminal 3C cleavage site, eGFP and strepII tag. Due to the coexistence of diheteromeric GluN1/GluN2A and GluN1/GluN2B, we used split GFP to monitor the expression level of triheteromeric NMDAR over diheteromeric NMDAR. Briefly, the GFP can be cleaved into two parts including GFP1-10 and GFP11 and these can be reassembled into a functional intact GFP by fusing them into interacting protein subunits (55). We fused GFP1-10 and GFP11 into the C-terminal of bicistronic GluN1-GluN2A and GluN2B, respectively. As a result, only triheteromeric GluN1/GluN2A/GluN2B containing both GFP1-10 and GFP11 will fluoresce, and the diheteromeric GluN1/GluN2A or GluN1/GluN2B containing either GFP1-10 or GFP11 will not, which was useful for initial construct screening. We replaced the GFP1-10 in the bicistronic GluN1-GluN2A construct with an intact GFP for large-scale expression because monitoring the GFP fluorescence was important to control the quality of virus production for the bicistronic construct. The final constructs we used for EM experiments are listed in Fig. S1 I. To boost expression levels, GluN1_{EM} and GluN2A_{EM} constructs were cloned into the same pEG BacMam vector, with the result deemed the GluN1-GluN2A_{EM} construct.

Purification of tri-NMDARs

Bacmid and baculovirus of GluN1-GluN2A_{EM} and GluN2B_{EM} in BacMam vector were generated (29) and P2 viruses were used to infect suspension HEK293 GnT⁻ cells at a multiplicity of infection (M.O.I.) of 1:1 (GluN1-GluN2A_{EM}:GluN2B_{EM}) and then incubated at 37 °C. At 12 h post-transduction, 10 mM sodium butyrate and 2.5 μM MK-801 were added to the culture and the temperature was set to 30 °C. The cells were collected and resuspended in a buffer containing 150 mM NaCl, 20 mM Tris 8.0 in the presence of 1 mM PMSF, 0.8 μM aprotinin, 2 μg/ml leupeptin, and 2 mM pepstatin A (protease inhibitors). The receptor was extracted from whole cell with a buffer containing 150 mM NaCl, 20 mM Tris 8.0, 1% MNG-3, protease inhibitors and 2 mM cholesteryl hemisuccinate (CHS) for 2 h at 4 °C. The solubilized receptors containing GluN1/GluN2A, GluN1/GluN2B and GluN1/GluN2A/GluN2B were incubated with TALON resin to remove strepII-tagged GluN1/GluN2A. The bound GluN1/GluN2B and GluN1/GluN2A/GluN2B receptors were then eluted with 250 mM imidazole at pH 8.0 to streptactin resin. His-tagged GluN1/GluN2B passed through the column and only GluN1/GluN2A/GluN2B was bound to streptactin resin and eluted with buffer containing 5mM desthiobiotin. The receptor was concentrated, mixed with Fab 11D1 at a molar ratio 1:1.2 and was further purified by size-exclusion chromatography in the buffer containing 400 mM NaCl, 20 mM MES pH 6.5, 0.5 mM n-dodecyl β-D-maltoside (DDM), and 0.2 mM CHS. Peak fractions containing the receptor were pooled and concentrated to 4 mg/ml.

Antibody production

Monoclonal antibodies against GluN1 and GluN2B (10B11 and 11D1, respectively) were raised by Dan Cawley (Vector and Gene Therapy Institute, OHSU) using standard methods. GluN1/GluN2 was purified as described previously (26) in 1 mM DDM and in the presence of 1 mM glutamate, 1 mM glycine, and 0.2 mM CHS. Purified GluN1/GluN2 was reconstituted into liposomes for immunization as described previously for SERT (56) (57),

except 400 mM NaCl and 0.8% Na deoxycholate was used for reconstitution of GluN1/GluN2 and excess salt and detergent was removed using PD-10 desalting columns. Mice were immunized with 30 µg of reconstituted GluN1/GluN2B to generate hybridoma cell lines. Antibodies were screened by fluorescence-based size-exclusion chromatography (28) and western blot to select clones that recognized natively folded GluN1/GluN2B and GluN1/GluN2A protein. The 10B11 and 11D1 monoclonal antibodies were purified from hybridoma supernatants using 4-mercapto-ethyl-pyridine chromatography resin. Fab was generated by papain cleavage of 10 mg mAb at 1 mg/ml final concentration for 2 h at 37 °C in 50 mM NaPi, pH 7.0, 1 mM EDTA, 10 mM cysteine and 1:100 w/w papain. The digest was quenched with 30 mM iodoacetamide at 25 °C for 10 min and Fc was removed from the MAb digest using Protein A. Fab 10B11 was purified by anion exchange using a Hi Trap Q HP column in 20 mM Tris pH 8 and 200 mM NaCl. Fab 11D1 was purified by cation exchange using a Hi Trap SP HP column in 20 mM NaOAc pH 5 and 200 mM NaCl.

EM data acquisition and processing

Purified GluN1/GluN2A/GluN2B was mixed with 2 mM glycine, 2 mM glutamate, 20 µM MK-801, 0.5 mM EDTA and/or 1 mM Ro (in DMSO) a few hours before grid preparation. Double blotting of a 1.3+2.5 µl sample at a concentration of 4 mg/ml was applied to a glow-discharged Quantifoil holey carbon grid (gold, 1.2/1.3 µm size/hole space, 300 mesh), blotted using a Vitrobot Mark III using 3s blotting time with 100% humidity, and then plunge-frozen in liquid ethane cooled by liquid nitrogen. Images were taken by an FEI Titan Krios electron microscope operating at 300 kV with a nominal magnification of 85k. Images were recorded by a Gatan K2 Summit direct electron detector operated in super-resolution counting mode with a binned pixel size of 1.70 Å or 1.33 Å for Fab-bound data or non-Fab-bound data, respectively. For the non-Ro-bound data, each image was dose-fractionated to 70 frames with a total exposure time of 21 s with 0.3 s per frame. For the Ro-bound data, each image was dose-fractionated to 50 frames with a total exposure time of 20 s with 0.4 s per frame. The images were recorded using the automated acquisition program SerialEM. Nominal defocus values varied from 1.3 to 2.5 µm.

For the Ro-bound data, super-resolution counting images were 2x2 binned in Fourier space, motion corrected and summed using MotionCor2 (58). Defocus values were estimated using Gctf (59). Approximately 3000 particles were manually picked and subjected to an initial reference-free 2D classification using Relion (60). Eight representative 2D class averages were selected as templates for automated particle picking for all the Fab-bound data using Gautomatch (<http://www.mrc-lmb.cam.ac.uk/kzhang/Gautomatch/>). The auto-picked particles were visually checked and false positives were removed. The particles were further cleaned-up by two rounds of 2D classification using Relion. The CTF values of individual particles from selected 2D class averages were estimated using Gctf (59). For 3D classification in Relion, a Fab-free reference model was generated from the GluN1/GluN2B crystal structure (PDB code: 4TLM) and low-pass filtered to 50 Å using EMAN2 (61). The 3D classes (5 for Fab-bound data or 6 for non-Fab-bound data) each occupy a similar percentage of particles and share similar overall shape with differences mainly due to the flexibility of the Fab and the intrinsic flexibility between the extracellular domains and transmembrane domain of the receptor. Three-dimensional refinement of individual classes

yielded low-resolution reconstructions. By combining particles from all the classes, we obtained a reconstruction at higher resolution. Initial 3D refinement was carried out using Relion. Particles were further refined using FREALIGN's local refinement (62). Subsequently, for all the Fab-bound data, a soft mask around the receptor was calculated and supplied for final refinement using FREALIGN. The final resolutions reported in Table S1 are based on the gold standard FSC 0.143 criteria (60). No symmetry was applied during the image processing. A similar procedure was used for the other three datasets with the exception that no additional soft mask was used during 3D refinement of non-Fab-bound data.

Model building

A homology model of the triheteromeric GluN1/GluN2A/GluN2B receptor was generated with the crystal structure of the diheteromeric GluN1/GluN2B receptor (26) (PDB code: 4TLM) as a template using the SWISS-MODEL online server (63). A homology model for the Fab was made using the Fab from an existing crystal structure (PDB code: 4M48) by mutating all the residues to alanine. Both homology models were first rigid-body fitted into the non-Ro EM density map using Chimera (64), followed by molecular dynamics flexible fitting (MDFF) (32), which improves the model to map correlation coefficients (CC, without Fab, the same below) from 0.803 to 0.911 (backbone, 0.775 to 0.876). This model was then subjected to Rosetta refinement and a total number of 100 models were generated. The top 10 models with best geometry statistics were inspected for map agreement using Coot (65). The chosen model has slightly improved CC (0.919 and 0.883 for all atoms and backbone, respectively). In addition, the positioning of loops/linkers is improved and some errors related to secondary structure assignments and geometry are corrected. The model from Rosetta was further manually adjusted in Coot, guided by the crystal structures of intact diheteromeric GluN1/GluN2B (PDB code: 4TLM) (26) and GluN1/GluN2A LBD domains (PDB code: 2A5T) (34), as well as by the densities of bulky side chains. The final model has a CC of 0.929 and 0.892 for all atoms and backbone, respectively. For the Ro-bound data, the structure of the non-Ro-bound state was first rigid-body fitted into the EM density map, followed by MDFF. Subsequently, each subdomain of the non-Ro-bound structure, including the R1 and R2 lobes of ATD, D1 and D2 lobes of LBD, and TMD, were aligned to the Ro-bound model and combined to a new Ro-bound model. This model, and the linkers between subdomains in particular, was inspected and manually adjusted in Coot, guided by the non-Ro-bound structure, the crystal structure of intact diheteromeric GluN1/GluN2B (PDB code: 4TLM) and the crystal structure of the LBD of diheteromeric GluN1/GluN2A (PDB code: 2A5T). For validation, FSC curves were calculated between the final models and EM maps. The geometries of the atomic models were evaluated using MolProbity (66). All figures were prepared using UCSF Chimera and Pymol (Schrödinger) (67).

Saturation binding experiments

MK-801 and Ro binding affinity was determined by scintillation-proximity assay (SPA). SPA experiments were set up in triplicate wells of a 96-well plate. Affinity-purified triheteromeric NMDARs (20 nM) saturated with 2 mM glycine and 2 mM glutamate was incubated with 1 mg/ml copper yttrium silicate (Cu-YSi) beads (Perkin Elmer) and ^3H -labelled MK-801 or Ro (1:9 ^3H : ^1H) in SEC buffer (20 mM Tris pH 8, 150 mM NaCl, 1 mM MNG-3 and 0.2 mM CHS) with a final volume of 100 μl . Non-specific binding was

determined by the addition of 1 mM ifenprofiol (for ^3H -Ro) or 1 mM PCP (for MK-801). Plates were incubated at room temperature until the counting was stable and were read using a MicroBeta TriLux 1450 LSC and luminescence counter. Data were analyzed using GraphPad Prism.

Two-electrode voltage clamp electrophysiology (TEVC)

The GluN1, GluN2A, and GluN2B constructs for TEVC experiments in the pGEM vector are engineered with the C-terminal tags according to methods developed by the Hansen lab for selective cell-surface expression of recombinant triheteromeric NMDAR (22). The RNAs were transcribed using the mMessage mMachine T7 Ultra Kit (Ambion). *Xenopus laevis* oocytes were injected with a total 30–200 ng of mRNA with a ratio of GluN1:GluN2A:GluN2B 2:1:1 and were incubated at 16 °C for 2–3 days in the presence of 50 μM competitive antagonist D-APV and 10 $\mu\text{g}/\text{ml}$ gentamicin. Borosilicate pipettes were filled with 3 M KCl. The recordings were performed in a buffer containing 100 mM NaCl, 0.3 mM BaCl_2 , 5 mM HEPES 7.3 and 0.05 mM heavy-metal chelator ethylenediaminetetraacetic acid (EDTA) at -60 mV. All recording experiments were carried out at least 3 times independently.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Architecture of triheteromeric NMDARs

(A) Representative 2D class averages where red arrows indicate bound Fab. (B-C) Cryo-EM maps of triheteromeric NMDAR in the non-Ro-bound or Ro-bound states, respectively, viewed parallel to the membrane. The distances between the center of mass (COM) of the upper (R1) lobes of both GluN1 ATD, and between the COM of the lower (R2) lobes of both GluN2 ATDs, are indicated. The Fab was excluded during refinement and the residual density is in green. The four subunits, GluN1 (A), GluN2A (B), GluN1 (C) and GluN2B (D), are in yellow, red, orange and blue, respectively. (D) Cartoon representation of the

model of triheteromeric NMDAR in the non-Ro-bound state, viewed parallel to the membrane plane. The distances between the COM of the R1 lobes of both GluN1 ATDs, and between the COM of the R2 lobes of both GluN2 ATDs, are indicated. N-linked carbohydrates are in stick representation. **(E)** Structures viewed from the extracellular side of the membrane representing the subunit arrangement in the ATD (top), LBD (middle) and TMD (bottom) layers. **(F)** Density with the atomic model for individual subunits, viewed parallel to the membrane. The distances between the COM of ATD and LBD are indicated.

Figure 2. Asymmetric ATD and ATD-LBD interfaces

Surface and cartoon representations of the GluN1/GluN2A (**A**) and GluN1/GluN2B (**B**) heterodimers of the non-Ro-bound state, viewed parallel to the membrane. GluN1/GluN2A and GluN1/GluN2B ATDs are highlighted in black frame (**C-D**), showing interaction of GluN2A ATD and GluN2B ATD with the LBD layer, respectively. The C_{α} atoms of several key residues in the ATD-LBD crevice are shown as spheres, and distances between C_{α} atoms are indicated ($\text{\AA}$). (**E-F**) Superimposition of GluN1/GluN2A and GluN1/GluN2B ATD heterodimers using the R1 lobe of GluN1 subunits. The relative rotations of the R2 lobes as well as the COM of R2 lobes are indicated. (**G**) Superimposition of GluN2A and GluN2B ATDs using their R1 lobes illustrates the clamshell closure of GluN2A ATD. The relative rotation of their R2 lobes, as well as the distances between the COM of R1 and R2 lobes, are indicated.

Figure 3. Asymmetry in the LBD layer

(A) The LBD major and minor interface of the GluN2A subunit. (B-C) The LBD minor interface of GluN2A (B) and GluN2B (C). Two interfaces showing the major differences between GluN2A and GluN2B are highlighted in green circles, with one between loop 1 (GluN2) and helices F/G (GluN1) and another one between helix K and the S2-M3 linker (GluN2) and helix E (GluN1). GluN2A LBD makes extensive interactions with an adjacent GluN1 LBD.

Figure 4. Consequences of Ro 25-6981 binding

(A) Saturation binding of ^3H -Ro to triNMDAR_{EM} in the presence of glycine and glutamate. Results are the mean of three biological replicates and the error bars represent s.e.m. (B) Surface and cartoon representation of the GluN1/GluN2B heterodimer in triNMDARs in the Ro-bound state, viewed parallel to the membrane. The ATD heterodimers are outlined in black frames. (C) Superimposition of the GluN1/GluN2B ATD heterodimer in the non-Ro-bound and Ro-bound state using the R1 lobe of GluN1 subunits. Relative rotation angles of the R2 lobes, as well as the distances between the COM of R2 lobes, are indicated. (D) Clamshell closure of GluN2B ATD induced by Ro illustrated by superimposing GluN2B ATDs using the R1 lobes. Relative rotation angles of the R2 lobes and the distances between the COM of R1 and R2 lobes are indicated. (E-F) Subunit arrangement of the ATD layer in the non-Ro-bound (E) and Ro-bound state (F), respectively. The COMs of the ATD are shown as filled circles, with their distances in Å. The interfaces between the GluN2 ATDs are boxed in green and details are shown in (G-H). The C $_{\alpha}$ of four residues representing $\alpha 6$ and $\alpha 7$ are shown as spheres, with their distances in Å. (I) Interaction of GluN2B ATD with the LBD layer in the Ro-bound state. The C $_{\alpha}$ of the same residues in Fig. 2D are shown as spheres, with their distances in Å.

Figure 5. MK-801 binding and asymmetric TMD organization

(A) Densities of MK-801, M2, P loop and M3. Two GluN2 subunits are shown for clarity, viewed parallel to the membrane. (B) The MK-801 binding site viewed from the extracellular side of the TMD with the density map in blue mesh. The C_{α} of the residues that might interact with MK-801 are shown as spheres. (C) Saturation binding of ^3H -MK-801 to the triNMDAR_{EM} constructs in the presence of glycine and glutamate. Results are the mean of three biological replicates and the error bars represent s.e.m. (D) Arrangement of the M3 helices of the triheteromeric NMDAR (colored closed circles) in comparison with diheteromeric NMDAR (gray dashed circles), superimposed using COM of M3 with their distances in Å, showing the deviation from two-fold symmetry. (E) Arrangement of the R2 lobes in GluN2 ATD with the D2 lobes in GluN1 LBD and M3. The COMs of each domain are shown as colored closed circles with distances in Å.

Figure 6. Conformational asymmetry and allosteric modulation

(A–B) Conformational differences between GluN1/GluN2A and GluN1/GluN2B heterodimers in the non-Ro-bound state. The $\alpha 5$ - $\alpha 6$ loop (purple) participates in two major interactions: the upper part interacts with GluN1 ATD, together with the post- $\alpha 8$ loop (in black) of GluN2 ATD; the lower part is poised to interact with GluN1/GluN2A and GluN1/GluN2B LBD heterodimers. C, Conformational changes in the GluN1/GluN2B heterodimer upon binding of Ro (green).