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Opinion

Advancing NMDA Receptor Physiology by Integrating Multiple Approaches

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NMDA receptors (NMDARs) are ion channels activated by the excitatory neurotransmitter glutamate and are essential to all aspects of brain function, including learning and memory formation. Missense mutations distributed throughout NMDAR subunits have been associated with an array of neurological disorders. Recent structural, functional, and computational studies have generated many insights into the activation process connecting glutamate binding to ion-channel opening, which is central to NMDAR physiology and pathophysiology. The field appears poised for breakthroughs, including the exciting prospect of resolving the conformations and energetics of elementary steps in the activation process, and atomic-level modeling of the effects of missense mutations on receptor function. The most promising strategy going forward is through strong integration of multiple approaches.

One Ion-Channel Protein, Many Conformations

Neurotransmitter-gated ion channels, including Cys-loop, purinergic, and ionotropic glutamate receptors, mediate fast signaling between cells in the nervous system [1,2]. These ion channels rapidly open in response to transient release of a neurotransmitter, generating an electrical and/or biochemical signal that impacts on cellular activity and hence on nervous system function. The allosteric linkage between the ligand-induced conformational changes and the rapid opening of the ion channel is central to the signal transduction process. Ionotropic glutamate receptors (iGluRs) are a key family of neurotransmitter-gated ion channels that mediate excitation throughout the nervous system. Recent functional, structural, and computational studies have resolved numerous features of the allosteric linkage in iGluRs [3,4]. Nevertheless, wide knowledge gaps remain [5]. One of the most challenging and elusive is to define the elementary steps, in terms of the conformations of the intermediate states involved and the transitions between them, along the pathway from agonist binding to ion-channel opening. These elementary steps are a key regulator of synaptic function and are often targets of allosteric modulators. Because of their transient nature, no single approach alone – functional, structural, or computational – will be capable of resolving these substates. In this Opinion we discuss multiple efforts to address this issue in NMDA receptors (NMDARs), a crucial class of iGluRs, and emphasize the need for strong integration of approaches.

NMDARs make unique contributions to synaptic physiology and dynamics in part because of their distinctive neurotransmitter transduction profile: they are activated slowly in response to transient glutamate but show persistent activity and slow deactivation. Further, their activity is modulated by an array of small ions and molecules (protons, Zn^{2+}, spermine) [1,6], membrane lipids [7], and post-translational modifications such as phosphorylation [8]. Missense mutations distributed throughout NMDAR subunits have recently been associated with devastating

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The number of iGluR structures, both for isolated domains and for C-terminal domain (CTD)-truncated constructs, has expanded drastically in the Protein Data Bank. The extracellular domains usually display ligand-induced structural changes, but the transmembrane layer has yet to be captured in an open-pore conformation.

Electrophysiological data, interpreted with the help of available structures, have defined the contributions of key gating elements, in particular the M3 helix and the M3–D2 linker, to channel activation. Single-channel data can further determine the energetics and kinetics, but not conformations, of substates along the activation pathway.

Computational studies have so far focused on motions within isolated domains or over very short timescales, but atomic-level modeling is becoming feasible to calculate gating energetics and kinetics.

The NMDAR field appears poised for breakthroughs, but urgently needs strong integration of multiple approaches.

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neurological disorders [9,10]. To define how these diverse agents and mutations mechanistically impact on receptor operation, it is essential to know the elementary steps in NMDAR activation. These will allow more-refined targeting of specific properties of NMDAR function, which has been the bottleneck in the development of clinically useful compounds.

Mechanistic Insights from Structures of Isolated Domains

A hallmark of iGluRs is their structural modularity, being composed of four largely independent domains: an amino-terminal domain (ATD), a ligand-binding domain (LBD), a transmembrane domain (TMD) forming the ion channel upon tetrameric assembly, and a disordered intracellular C-terminal domain (CTD) (Figure 1). All these domains are crucial to the allostery and hence physiology of iGluRs [1,6,11]. For NMDARs, the ATDs and LBDs form extensive structural contacts [12,13], and ATD deletion has a strong effect on properties including channel open probability, but the receptors remain functional [14,15]. We focus here on the two core domains: the LBD and the TMD.

Crystal structures of isolated extracellular domains started in 1998 [16] and continue to grow, reflecting their value in characterizing intra-domain interactions and motions. These domains were bound with various ligands, and their structures show response to ligand binding. Within the LBD, agonists bind at the cleft between two lobes (referred to as D1 and D2; Figure 1A). Indeed, one of the key mechanistic insights to emerge from the studies of the isolated LBDs was that ion-channel opening was driven by LBD lobe closure [1,17].

Partial agonists are extremely useful tools, as well as potential clinical therapies, because they perturb the conformations and relative stabilities of substates. For AMPA receptors (AMPARs), another major class of iGluRs, the degree of LBD lobe closure is correlated with agonist efficacy, with agonists inducing tight closure, competitive antagonists producing lobe opening, and partial agonists corresponding to intermediate closure (Figure 2A) [18,19]. This correlation implicates the degree of LBD lobe closure as a determinant of AMPAR partial agonism.

Surprisingly, for NMDARs, the degrees of LBD lobe closure are similar for full and partial agonists, although competitive antagonists still produce lobe opening (Figure 2B) [20–23]. The lack of structural response to NMDAR partial agonism is a manifestation of the limit to which structures can serve as reporters of functional properties. In the ideal case, crystal structures represent a thermally averaged snapshot, which should be close to the minimum position of the

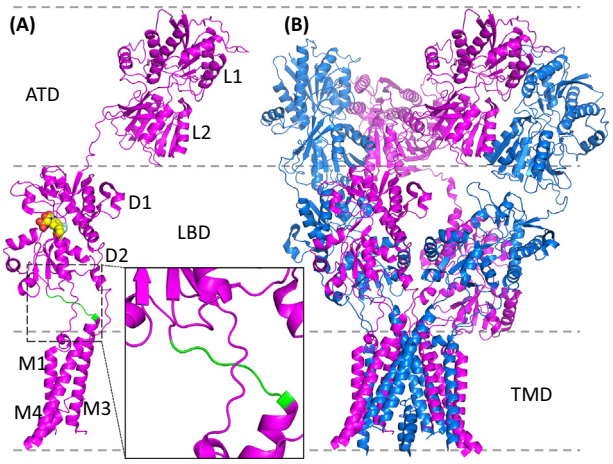

Figure 1. Modular Architecture of Ionotropic Glutamate Receptors. (A) Domains within a GluA2 subunit (C-terminal domain missing). Subdomains of the amino-terminal domain (ATD), L1/L2, and ligand-binding domain (LBD), D1/D2, and transmembrane helices of the transmembrane domain (TMD), M1/M3/M4, are indicated; a bound ligand is shown in space-filling mode and the M3–D2 linker is in green. (Inset) Enlarged view of the region around the linker. (B) Subunit organization within the homotetramer (Protein Data Bank 3KG2). A/C and B/D subunits are in blue and magenta, respectively. In NMDA receptors, the A/C and B/D subunits are GluN1 and GluN2, respectively.

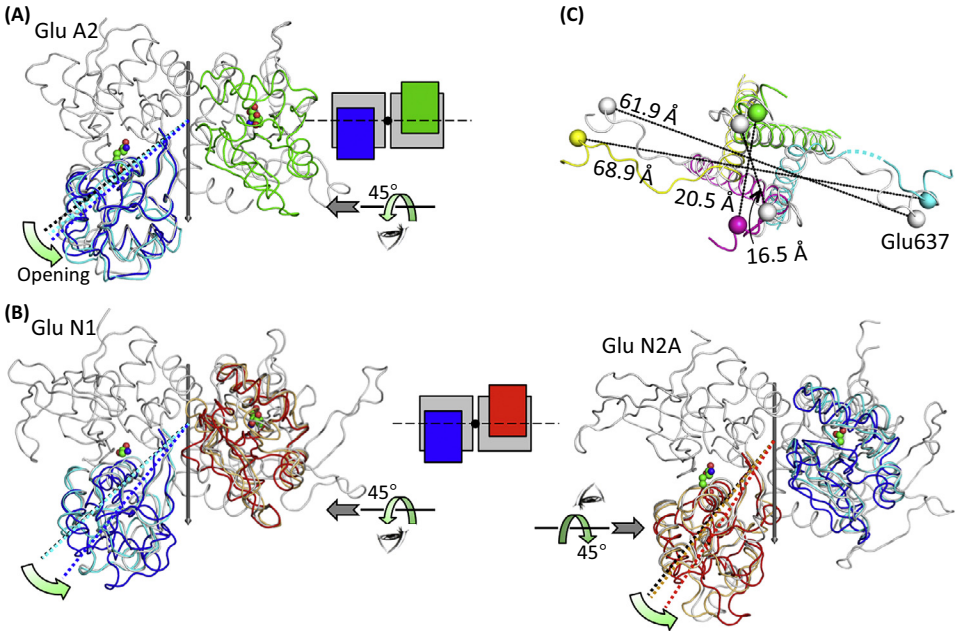

Figure 2. Differences in Partial and Full Agonist- and Competitive Antagonist-Bound Structures. (A) GluA2 ligand-binding domains (LBDs) bound with full agonist glutamate [grey dimer except green for the D2 lobe in one monomer, with ligand in space-filling mode; Protein Data Bank (PDB) 1FTJ], partial agonist kainate (one D2 lobe in cyan; PDB 1FTK), and antagonist 6,7-dinitroquinoxaline-2,3-dione (one D2 lobe in blue; PDB 1FTL). The view is 45° from the dimer symmetry axis (solid line with arrow); dashed lines indicate cleft opening angles. (B) GluN1/N2A LBDs bound with full agonists glycine and glutamate (grey dimer, with ligands in space-filling representation; PDB 4NF8), GluN1 partial agonist 1-aminocyclobutane-1-carboxylic acid (D2 lobe in cyan; PDB 1Y1Z) and antagonist 6,7-dinitroquinoxaline-2,3-dione (D2 lobe in blue; PDB 4NF4), and GluN2A partial agonist (2R)-amino(1-hydroxy-4-propyl-1H-pyrazol-5-yl)ethanoic acid (D2 lobe in orange; PDB 4JWX) and antagonist 1-(phenanthrene-2-carbonyl)piperazine-2,3-dicarboxylic acid (D2 lobe in red; PDB 4NF6). The views are 45° on either side of the dimer pseudo-symmetry axis. (C) Arrangements of the major pore-lining M3 helix and the M3–D2 linker in GluA2 AMPARs, in apo form (grey; PDB 4U2P) and a form, bound with agonist and allosteric modulator, that putatively represents the active state (color; PDB 4U1Y). Distances are measured between Cα atoms of Glu637 residues in diagonal subunits.

free energy landscape (although deviation from the position determined computationally can occur due to crystal packing and other factors [24,25]). Hidden from structure determination is the full free-energy landscape, not only the minimum position, that actually dictates functional properties. One hypothesis is that NMDAR partial agonism is determined not by the minimum position but by the curvature of the free-energy basin for LBD lobe closure [26]. This result demonstrates that the combination of computation and structure can provide novel insights into iGluR function. Computed LBD free energy landscapes can now be tested by single-molecule Förster resonance energy transfer (smFRET) measurements [27].

A Missing Conformation of iGluR Channel Pores

All ion-channel pores have two major conformations: closed and open. The pore-forming TMDs of iGluRs share structural homology to K^+ channels [28,29]. The second transmembrane helix (M3; Figure 1A) in iGluRs corresponds to TM2 or S6 in K^+ channels and was shown functionally to be a key determinant of ion-channel gating [30,31] and to harbor the activation gate [32]. These findings are supported by structures of CTD-truncated iGluRs (Figure 1) [29]. Together, functional and structural studies lead to the conclusion that pore opening of iGluRs involves the outward displacement of the M3 helix; displacements of key elements leading to pore opening have been visualized in molecular dynamics simulations [33].

The structures of CTD-truncated iGluRs provided the context to speculate how intra-domain motions and inter-domain couplings contribute to channel gating. The correlation between degree of lobe closure and agonist efficacy observed in isolated LBDs is also seen in CTD-truncated AMPARs [34]. For NMDARs, comparison of two cryo-electron microscopy (EM) structures, one bound with full agonists and one bound with competitive antagonists, showed that the latter produce LBD lobe opening in the GluN1 subunits but not in the GluN2B subunits [35]. In putatively active structures, the tips of the D2 lobes are usually farther separated between diagonal subunits (A/C or B/D) than in structures representing the resting state (Figure 2C). Histograms of distances between spin-labeled residues have also been measured by double electron–electron resonance experiments [34,35]. This technique, as well as other spectroscopic techniques like smFRET, may not have the resolution to decipher the subtle conformational changes that are likely to play key roles in receptor gating.

Unfortunately, the most important structural change, that is, words pore opening, is not captured by any of the putatively active structures. The pores either were closed (Figure 2C) [34–36] or could not be resolved [37,38]. Possibly the agonists (and allosteric modulators) used were not the best for capturing the open-pore structure; another possibility is that the solubilizing conditions in sample preparations did not model well the key biophysical properties of cell membranes which may be essential for maintaining TMD structural integrity [39]. Regardless, if structural techniques fail to even capture a stable functional state, then it would only be prudent to explore other strategies to seek conformational information on substates.

Identifying NMDAR Substates with Single-Channel Recordings

Functional approaches, most notably recordings of individual receptors, provide a powerful tool to extract the energetics of substates [40]. NMDARs composed of the GluN1/GluN2A subunits are particularly suitable for such studies because their activity is robust and displays a single conductance level, thus simplifying analysis. Single-channel current traces of GluN1/GluN2A normally show periods of high opening and closing activity, separated by longer quiescent periods (Figure 3A), revealing the distributions of five closed components (Figure 3B) and two to four open components [41–44]. The two longest closed components are thought to represent desensitized substates. While the desensitized substates in NMDARs remain unresolved, and may be important to the dynamics of synaptic and extrasynaptic receptors, we focus here on the briefer closed components which are thought to be on the activation pathway to channel opening [42,43].

Single-channel current traces can also be analyzed to reveal relative stability and transition rates between substates by subscribing to a specific kinetic model. Many kinetic models have been developed [42,44–46]. A prevalent model that can capture prominent features of NMDAR activity assumes that the activation pathway consists of sequential transitions along a set of substates (Figure 3C) [43,45–47].

We used this model to characterize the impact of glycine insertions in the M3–D2 linkers (Figure 3D–F) [48]. By integration with computation, we found that the allosteric linkage between the LBD and the M3 helix involved mechanical tugging. This insight motivated the development of a theoretical model for iGluR gating [49]. Agonist-induced outward expansion of the D2 tips leads to increased extensions, and consequently higher tensions, of the M3–D2 linkers. The would-be higher tensions drive the outward splay of the M3 termini and hence opening of the pore. In the resulting active state, the linker tensions recede and the linker extensions retract to be shorter than those in the resting state. The theoretical model has also clarified that a ‘pulling factor’, empirically defined to measure the effects of glycine insertions in

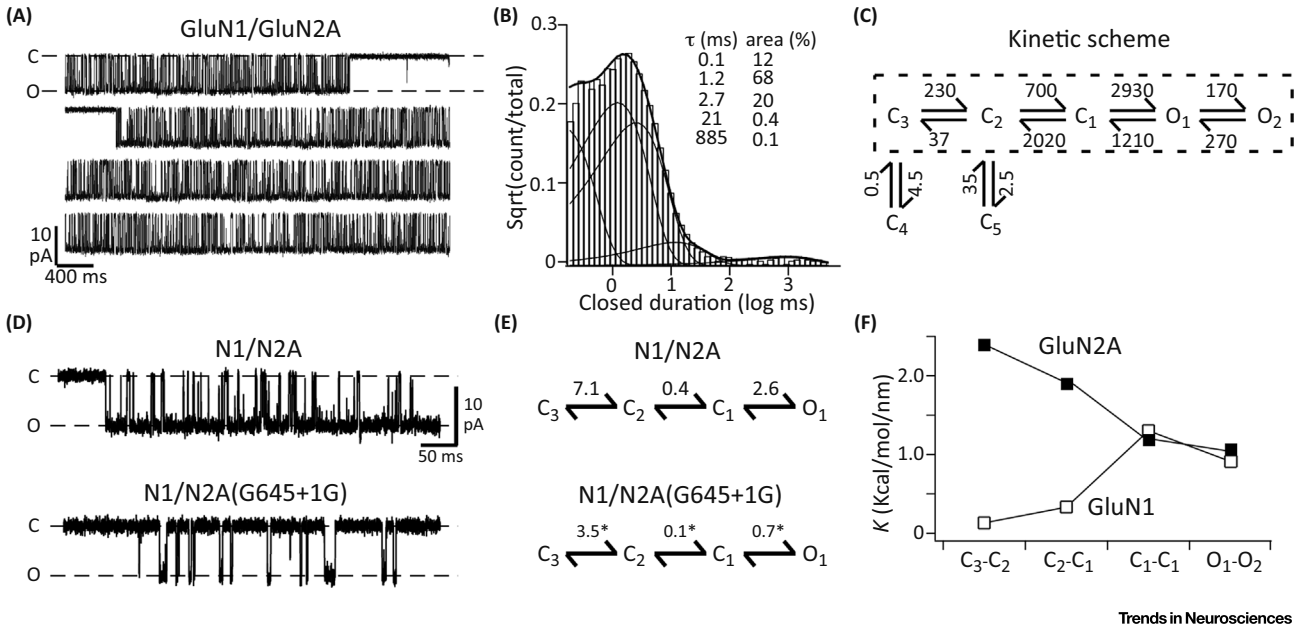

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Figure 3. Identification of NMDA Receptor Substates by Single-Channel Kinetic Analysis. (A) Single-channel current traces of wild-type GluN1/N2A recorded in the on-cell mode. Openings are downward. (B) Closed time distribution, fit with five exponentials (time constants and relative amplitudes shown). (C) Kinetic scheme for receptor activation (dashed box), with transition rates (in s^{-1}) shown; C_4 and C_5 are desensitized components. Values are for a single experiment. (D) Current traces of wild-type N1/N2A and a mutant with a single glycine inserted in the N2A M3–D2 linker. (E) Kinetic schemes of the constructs, with average equilibrium constants shown from pooled data. (F) Pulling factor (k), obtained as the slope of linear correlation between $\Delta\Delta G$ and insertion length, for each transition. Adapted from Kazi *et al.* [48]. See also Figure S1 in the supplemental information online.

the M3–D2 linkers (Figure 3F), is actually the decrease in linker tension when the receptor makes a transition from one substate to the next along the activation pathway.

The linear model has proven useful in characterizing the energetics and kinetics of NMDAR gating (Figure 3) [47,48,50–53]. Nevertheless, what this model lacks is a clear link to conformations, which is desperately needed to be able to move the field forward. Such a 'structure-based kinetic model' must first of all recapitulate the unequal contributions of the GluN1 and GluN2A subunits to channel activation (Figure 3F) [42,48,54]. It must also account for the roles of key gating elements, including the extracellular termini of the three transmembrane helices (M1, M3, and M4) and the associated linkers that connect them to the D2 lobe [55,56]. Indeed, the outer elements, M1 and M4, need to be displaced for efficient pore opening to occur [53]. Importantly, these outer elements contain numerous missense mutations (Figure S1 in the supplemental information online), some of which affect receptor gating [57]. Having a kinetic model that captures these conformational details would greatly help uncovering the mechanisms underlying the aberrant receptor functions.

What Should the Conformations of NMDAR Substates Look like?

Both structural and functional approaches face daunting challenges in producing information on the conformations of substates along the activation pathway. A promising and timely strategy for breakthrough is to use current knowledge, whether from electrophysiological, structural, or computational studies, to inspire hypotheses on functionally important conformations, and then to validate the consequent hypothesis-driven conformational modeling by targeted functional studies.

In the active state, the ion-channel pore is open while the LBDs have closed lobes. During stationary gating, with the LBDs saturated with agonists, the pore switches between closed

and open conformations (e.g., Figure 3A). There is little experimental information on the conformations of the agonist-bound LBDs during the pore closed periods. The aforementioned theoretical model [49] predicts semiclosed LBD lobes while the pore is closed because keeping the LBD lobes closed would result in over-stretched M3–D2 linkers and hence excessive tensions, which are relieved by reducing the degree of LBD lobe closure. Paradoxically, the hypothesized unstable state with both the LBD lobes and the channel pore closed might be what was captured by the agonist-bound structures [34–36], and this was perhaps stabilized partly by not closing the LBD lobes as tightly as in isolated LBD structures as well as partly by the solubilizing conditions overly favoring the pore closed conformation.

If semiclosed LBD lobes are indeed a characteristic of the pore-closed state during NMDAR stationary gating, what may distinguish the conformations of the three kinetic components C_3, C_2, and C_1? A valuable clue is provided by the glycine insertion data (Figure 3D–F). They suggest that the early transitions ($C_3 \rightarrow C_2$ and $C_2 \rightarrow C_1$) along the activation pathway mostly involve motions within GluN2A whereas the late transitions ($C_1 \rightarrow O_1$ and $O_1 \rightarrow O_2$) involve concerted motions of both types of subunits. More specifically, as clarified by the theoretical

Key Figure

Conformational Models for NMDA Receptor Substates

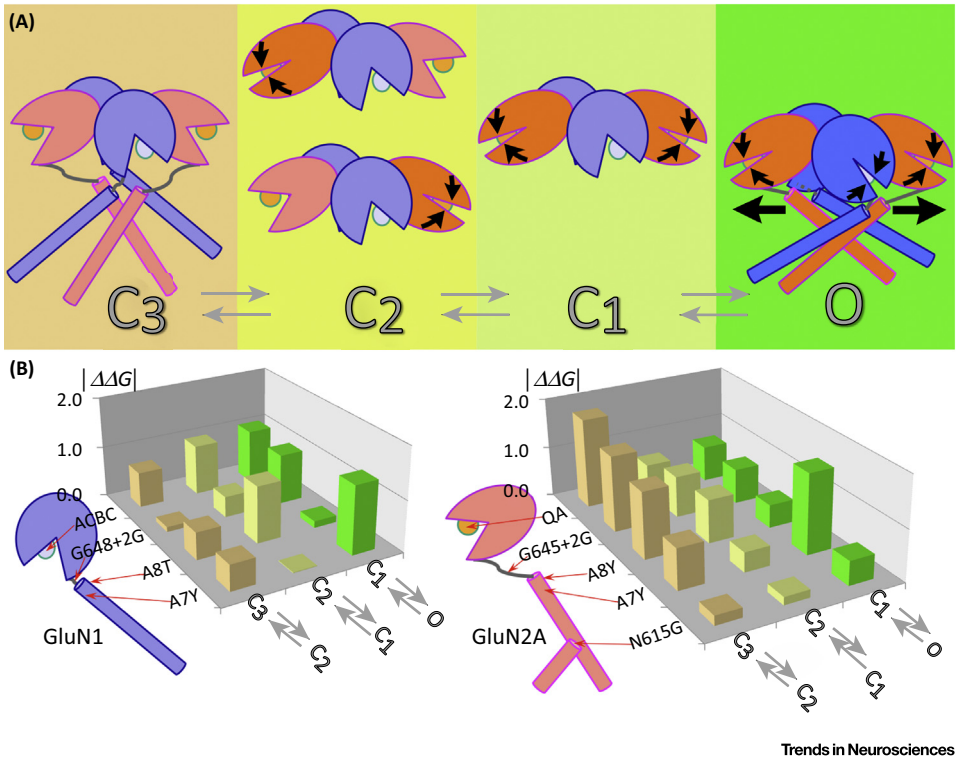

Figure 4. (A) Conjectured ligand-binding domain (LBD) and transmembrane domain (TMD) conformations in four substates. Black arrows indicate putative LBD and TMD motions relative to C_3. (B) Changes in free-energy differences between two substates, by mutations, and by GluN1 and GluN2A partial agonists 1-aminocyclobutane-1-carboxylic acid (ACBC) and quinolinic acid (QA), respectively. Bars represent magnitudes of $\Delta\Delta G$. For the present discussion, the LBD–TMD dividing line is assumed to be between the A7 and A8 positions at the M3 C-terminus; partial agonists are treated as LBD perturbations.

model [49], the greatest decreases in M3–D2 linker tension occur in GluN2A during the $C_3 \rightarrow C_2$ and $C_2 \rightarrow C_1$ transitions. Any change in linker tension likely involves D2 motion. It thus appears that the early transitions may have lobe closure of the GluN2A LBDs as a prominent feature, whereas the late transitions may have increased participation of the GluN1 subunits and of course the opening of the pore. A plausible scenario is that the LBD lobes are semiclosed in all the four subunits for C_3, but become closed in one of the two GluN2A subunits for C_2, in both GluN2A subunits for C_1, and in all the four subunits in the open components (Figure 4A, Key Figure).

This line of conjecture on the conformations of the NMDAR substates is generally consistent with results from other single-channel studies with mutations in different domains and agonists spanning a range of efficacies [47,50,52]. The useful quantity for the present purpose is $\Delta\Delta G$, the perturbation on the free-energy difference between two substates by a mutation or by a partial agonist, in reference to the wild-type receptor saturated with full agonists (glycine and glutamate). Specifically, the relative magnitudes of $\Delta\Delta G$ among the transitions along the activation pathway may provide a way to map these transitions to conformational changes in different domains of the receptor. The $\Delta\Delta G$ data can be summarized as follows (Figure 4B). First, perturbations in the LBDs mostly affect the early transitions whereas perturbations in the TMDs mostly affect the late transitions. Second, perturbations in the GluN2 LBDs more strongly affect the early transitions whereas perturbations in the GluN1 LBDs more strongly affect the late transitions. If we accept the assumption that a perturbation in a domain has the greatest effect on conformational changes within that domain, then the $\Delta\Delta G$ data serve as a solid basis for the foregoing conjecture on conformations of substates.

Conformational conjectures can be turned into atomic models through computation. In particular, semiclosed LBD conformations have been explored by molecular dynamics free-energy simulations [58]. Open conformations of the ion-channel pore can potentially be obtained from remodeling the closed conformations in crystal and cryoEM structures via *de novo* packing of the TMDs [59,60]. The atomic models can then be validated by electrophysiological studies. In particular, the accessibility of individual substituted cysteines and the formation of disulfide or metal bridges between pairs of substituted cysteines can be tested experimentally. $\Delta\Delta G$ values can be predicted from free-energy simulations and tested by single-channel studies.

Concluding Remarks

Resolving the conformations and energetics of the elementary steps along the activation pathway will constitute a breakthrough in NMDAR physiology. This knowledge will be invaluable for defining the operations of NMDARs at synapses and how they might be allosterically regulated by small molecules and post-translational modifications. In addition, there is a rapidly growing list of missense mutations in NMDAR subunits that are associated with neurological disorders [9,10]. The mutations could wreak havoc at any juncture in the life cycle of the receptors, including assembly, trafficking, and localization, as well as interactions with lipids and with other proteins. Many, however, presumably affect receptor gating, as has been shown for a dozen cases [9], but have yet to be tested. Ultimately what is needed is a model that can predict with reasonable accuracy whether a missense mutation affects receptor gating and, if so, the effect and mechanism thereof, without having to do an extensive functional characterization, thereby leading to a more rapid development of precision medicine (see Outstanding Questions).

At present, structural studies have yielded incredible insights into NMDAR function. Nevertheless, these structures require validation by functional studies, an issue that is particularly acute for the TMD and the linkers that connect them to the LBD. On the other hand,

Outstanding Questions

Capturing iGluR ion-channel pores in an open conformation is essential for defining their activation process and a prime target for structure determination. Solubilizing the TMD layer in lipid bilayers, for example, by using nanodiscs, may help to preserve native structures. Working with isolated TMD constructs may provide another avenue.

Integrated functional and computational studies have interrogated the crucial role of the M3–D2 linkers. The picture is far less clear for the outer elements, namely the M1 and M4 extracellular termini and the associated linkers, regarding how they participate in the elementary steps along the NMDAR activation pathway.

Domains outside the core gating domains, namely the ATD and the CTD, have prominent effects on NMDAR function at synapses. How do these domains mechanistically impact on the core gating process mediated by the LBD and the TMD? Only after a basic understanding is achieved from structural and electrophysiological studies can theoretical modeling be contemplated.

Prominent among the many important distinctions between NMDARs and non-NMDARs is the mechanism of desensitization. In non-NMDARs the center of action for desensitization has been located at the D1–D1 interface of the LBD dimer, whereas in NMDARs it is only starting to be investigated. Mechanistic understanding of NMDAR desensitization will again require the application of electrophysiological, structural, and computational approaches.

Integration of structural, functional, and computational approaches may lead to a formalism for linking single-channel data on NMDAR gating energetics and kinetics with atomic-level interactions and motions. Such a formalism will push the understanding of NMDAR physiology to the atomic level and help to realize the promise of precision medicine.

electrophysiologists have tended to be overly conservative, and must be willing to inject structures into kinetic models. This will be especially true for the intermediate states. Computational studies have so far focused on motions within isolated domains or over very short timescales, but atomic-level modeling is becoming feasible to calculate gating energetics and kinetics. A strong integration of multiple approaches, as illustrated by the prospective study on substates, will be necessary for moving the NMDAR field forward.

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Supplemental Information

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