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...
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
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 states 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
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 C3, C2, and C1? A valuable clue is provided by the glycine insertion data (Figure 3D–F). They suggest that the early transitions (C3→C2 and C2→C1) along the activation pathway mostly involve motions within GluN2A whereas the late transitions (C1→O1 and O1→O2) involve concerted motions of both types of subunits. More specifically, as clarified by the theoretical

Key Figure

Conformational Models for NMDA Receptor Substates

![Key Figure](image)

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 C3. (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 ΔΔG. 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 C3→C2 and C2→C1 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 C3, but become closed in one of the two GluN2A subunits for C2, in both GluN2A subunits for C1, 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,
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.

Acknowledgments

This work was supported in part by National Institutes of Health Grants GM058187 and GM118091 (to H.X.Z.), and NS088479 (to L.P.W.).

Supplemental Information

Supplemental information associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tins.2017.01.001.

References

25. Yao, Y. et al. (2013) Conformational analysis of NMDA receptor GluN1, GluN2, and GluN3 ligand-binding domains reveals subtype-specific characteristics. Structure 21, 1788–1799