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Mapping the conformational landscape of glutamate receptors using single molecule FRET

David M MacLean^{1,*}, Ryan J Durham^{2,3}, and Vasanthi Jayaraman^{2,*}

¹Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, New York 14642, USA.

²Center for Membrane Biology, Department of Biochemistry and Molecular Biology, University of Texas Health Science Center at Houston, Houston, Texas 77030, USA.

³Biochemistry and Cell Biology Graduate Program, Graduate School of Biomedical Sciences, University of Texas Health Science Center at Houston, Houston, Texas, 77030, USA.

Abstract

The ionotropic glutamate receptors mediate excitatory neurotransmission in the mammalian central nervous system. These receptors provide a range of temporally diverse signals which stem from subunit composition and also from the inherent ability of each member to occupy multiple functional states, the distribution of which can be altered by small molecule modulators and binding partners. Hence it becomes essential to characterize the conformational landscape of the receptors under this variety of different conditions. This has recently become possible due to single molecule fluorescence resonance energy transfer measurements along with the rich foundation of existing structures allowing for direct correlations between conformational and functional diversity.

Keywords

Glutamate receptors; AMPA; NMDA; TARP; single molecule FRET

Significance of understanding the conformational landscape of glutamate receptors.

Ionotropic glutamate receptors (iGluRs) mediate the majority of excitatory neurotransmission in the central nervous system and are critical players in synaptic plasticity, which underpins the processes of learning and memory formation [1, 2]. In addition to its physiological functions, the glutamate receptor family is also implicated in a

*Correspondence to: Vasanthi Jayaraman, Department of Biochemistry and Molecular Biology, Center for Membrane Biology, University of Texas Health Science Center, MSB 6.528, 6431 Fannin St, Houston, Texas 77030 Tel.: (713) 500-6236, vasanthi.jayaraman@uth.tmc.edu.

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variety of neuropathologies, including epilepsy and ischemic stroke, due to its roles in glutamate excitotoxicity and neural circuit coordination [1, 3]. Mutations and dysfunction of the receptor are also implicated in learning disorders and autism [4–7]. The myriad of processes involving glutamate receptor activity underscores the importance of gaining a fuller understanding of the receptor's function, including to better inform drug development [see Box 1].

A critical step in understanding how the receptor mediates the plethora of functions, and to modulate the receptor function in disease states, is to understand the structural states underlying the different functional states of the receptor. The recent increase in the cryo-electron microscopy based structures along with high resolution X-ray structures provide a rich structural foundation. Building on these structures it has now become possible to use single molecule fluorescence-based methods to study the conformational landscape and transitions across the landscape with the hope of being able to tie this to the single channel-based functional states. In this Review we summarize the current state of the structure-dynamic control of glutamate receptor function gained from a combination of these methods with particular emphasis on the recent single molecule FRET investigations.

From structure to conformational-dynamics analyses of glutamate receptors.

As expected of receptors with wide-ranging roles, ionotropic glutamate receptor signals can be modulated at different levels. At the level of genetics and pharmacology, they can be broadly divided into α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl D-aspartic acid (NMDA), and kainate (KA) receptors. AMPA receptors and KA receptors mediate the fast component of glutamatergic signaling while NMDA receptors mediate the slow component. There is also genetic heterogeneity within a given receptor subtype as subunit composition, alternative splicing [8, 9], and RNA editing [10] can all lead to subtle or substantial changes in excitatory postsynaptic current and ionic selectivity. A large array of potential function-modifying auxiliary subunits adds another level of modulation, which is especially pronounced amongst the AMPA subtype [11–13], as do post-translational modifications including phosphorylation of receptors [14, 15] and a range of physiological small molecule modulators such as polyamines [16–19], zinc [19–22], and protons [23–27].

The overall architecture is similar across these receptors, with all the subtypes being tetrameric, arranged as a dimer of dimers [28–31]. Each subunit has an extracellular amino-terminal domain and an agonist-binding domain, as well as a transmembrane domain and a structurally unresolved intracellular C-terminal domain (Figure 1a). The first structural insight into these receptors was obtained from structures of the isolated agonist-binding domains that showed a bi-lobed structure [32]. The antagonist-bound structure showed an open cleft and the agonist-bound form a closed cleft, suggesting cleft closure of the agonist-binding domain as the mechanism for activation (Figure 1b) [32, 33].

Structures of the agonist-binding domain dimers, along with mutational and crosslinking studies, showed desensitization of the AMPA and KA type iGluRs involved the decoupling

of the agonist-binding domain dimer interface [34]. While the roles of the agonist-binding domain are largely shared across iGluR subtypes, the function of the amino-terminal domain varies. The amino-terminal domains of NMDA receptors set the channel's basal open probability and also act as sites of allosteric modulation by protons, polyamines, and exogenous small molecules [35–37]. The amino-terminal domains of AMPA and KA receptors have a small impact on channel gating, but analytical ultracentrifugation experiments on the soluble amino-terminal domains reveal the important role this domain plays in driving subtype-specific assembly, with KA receptor amino-terminal domain dimers being weaker than AMPA receptors [38] and heteromeric receptors having a tighter dimer assembly relative to homomeric receptors [38–40]. Similar studies with the agonist-binding domain showed that the agonist-binding domain had very weak interactions between the dimers, indicating that the agonist-binding domain layer is highly dynamic [41] and highlighting the need to study the dynamic motions of the receptor in order to gain a complete understanding of the structure-function correlates.

The more recent X-ray and cryo-EM structures of the full length receptors have provided further insight into the arrangement of these domains and allosteric communication across them. These structures show that the amino-terminal domain and agonist-binding domain exhibit a two-fold symmetry, assembling as a dimer of dimers, but with crossover between the amino-terminal domain and agonist-binding domain (Figure 1a). Additionally, the full length structures show a higher degree of interactions between the amino-terminal domain and agonist-binding domain in the NMDA subtype, highlighting the mechanism of allosteric modulation in NMDA receptors which is mediated across these domains.

The extensive structural information currently available for the glutamate receptors makes them ideal candidates for investigating their conformational dynamics in the protein. Normal model analysis [42–44] and NMR-based methods have shown microsecond and millisecond dynamics in the agonist-binding and amino-terminal domains, further highlighting the dynamic nature of the receptor [45, 46]. However, these methodologies are not amenable for investigating the full length receptor. Luminescence resonance energy transfer [19, 22, 47–55] and double electron-electron resonance spectroscopy [56–58] have been used to study the conformational changes occurring in full length glutamate receptors, albeit limited to equilibrium conditions. Biochemical crosslinking and state-dependent trapping, in conjunction with electrophysiology, have also provided insight into the overall conformational changes associated with activation and desensitization [41, 59, 60].

The above discussed structural, biochemical, and functional methods provide a rich foundation on which it is now possible to map the complete conformational landscape, including the low occupancy states, using single molecule based methods. The extensive single channel functional studies on these receptors [2, 25, 61–66] have provided the functional states and the function-based mechanism of the receptor under varying liganded and modulated states. Being able to tie these functional states to conformational changes in the protein would ultimately complete the structure-function correlations in this protein. Single molecule FRET (smFRET) is best suited for such investigations as it is able to provide the spectrum of conformational states that the receptor occupies as well as map the transitions between these states. Using the available end state structures, sites exhibiting the

largest conformational changes can be identified and labeled with fluorophores allowing for investigation of the conformational landscape at this site. By following the changes at the donor and acceptor fluorophore at a single molecule level at this site (Figure 2) the conformations that the protein explores under varying liganded conditions can be identified along with the kinetics of transitions between the states. While smFRET measurements are ideal for single molecule level structure-function correlations, there are several limitations of the smFRET-based measurements that need to be taken into account while making such correlations. Unlike single channel functional recordings that allow for measurements for long periods of time, smFRET measurements are relatively short, tens to hundreds of seconds, due to photobleaching of the fluorophores. Thus, tens of molecules have to be studied in smFRET in order to obtain kinetics of transitions. Additionally, the signal to noise ratio limits the time resolution to millisecond time scales when the measurements are performed in the tethered configuration. Higher microsecond time resolution is possible when probing the protein in diffusion configurations, however, the ability to watch changes in a given molecule is lost in this method [67]. Additionally, the sites that can be labeled in the protein are limited to those that do not alter the function of the receptor and to sites that are highly mobile to reduce the bias stemming from orientation factor [68]. Working within the constraints of these limitations, smFRET measurements have been able to provide correlations between conformation and function at the single molecule level in the glutamate receptors, and the following sections aim to summarize these results.

Conformational landscape of the agonist-binding domain and partial agonism.

The process of understanding the conformational landscape of the glutamate receptor began with crystallographic studies on the isolated agonist-binding domain [32, 45, 47, 69–71]. In the case of the AMPA receptor, the structures indicated that the extent of cleft closure in the agonist-binding domain correlated with agonist efficacy [72, 73] (Figure 1b). However, several additional studies disputed this simple hypothesis. In particular the L651V [47, 69, 74] and T686S mutants [63] had a fully closed cleft when bound to glutamate, similar to what is seen with full agonists, but glutamate is a partial agonist with these mutants [62]. NMR investigations of the soluble agonist-binding domain suggested that orientation and dynamics may also play a role in partial agonism [46, 75]. These studies highlighted the need to study the complete conformational landscape as can be best afforded by single molecule methods such as smFRET. smFRET measurements on the agonist-binding domain of wild type and T686S mutant revealed that the most probable state for the glutamate-bound T686S is a closed cleft form similar to wild type receptor, consistent with the crystal structures [76]. However, the T686S mutant traversed a wider range of cleft closure states unlike the wild type receptor (Figure 3a) [76]. Thus, the fraction of receptors in the closed cleft state was significantly less for the T686S mutant when bound to glutamate relative to wild type receptor when bound to glutamate. This led to the revised hypothesis that the fraction of the receptor population in the closed cleft state dictates the extent of activation. This hypothesis held up for a series of agonists and mutant receptors (Figure 3b) [77], thus highlighting the strength in understanding the complete conformational landscape even if limited to measurements along a single axis defined by two labeling sites. Importantly, the

smFRET results were consistent with molecular dynamic simulations performed in conjunction with an umbrella sampling strategy. These simulations also found both wild type and mutant agonist-binding domains probed a wide range of energy states in the apo and liganded conditions [78], thus highlighting the complementarity between these methods.

Conformational landscape at the amino-terminal domain and desensitization.

While the investigations on the isolated agonist-binding domain provided significant insight into the mechanism of activation, a crucial advance is to evaluate how well the observations of the soluble agonist-binding domain hold in the full length receptor. By adapting an *in situ* purification strategy (Figure 2) as described by Vafabakhsh et al., [79, 80] for AMPA receptors, satisfactory concentrations of double-labelled purified AMPA receptors could be obtained [81]. This made it possible to directly observe conformational states of the full protein (or at least between two positions) upon ligand binding. An ideal position to study the conformational landscape was across the dimer-dimer (tetramer) interface at the amino-terminal domain (Figure 4a inset). This position could be used to address a discrepancy emerging in the structural literature. Cryo EM structures under desensitizing conditions from two laboratories found that the amino-terminal domain of AMPA receptors can splay apart or ‘fall over’ during desensitization or agonist binding [31, 56]. Similar large separations were also observed in the EM structures of native receptors [82]. In contrast, crystallographic studies from Yelshanskaya et al. did not report such splaying upon agonist binding and presumptive desensitization [83], possibly due to deletion of several amino acids in the linker between the agonist-binding and amino-terminal domains. The smFRET investigations showed multi-component efficiency distributions, with three distinct resolvable populations, indicating the amino-terminal domain of the full length channel populates at least three different conformations (Figure 4) [81]. The high FRET efficiency population was consistent with the crystal structure distance, while the lower FRET efficiency states correlated well with the decoupling observed in the cryo EM structures. Thus it is likely that differences in methods (crystallography versus cryo EM), sample and construct preparation, full length versus truncated linker constructs, and analysis within a single method may account for a tendency to observe one class over another. Interestingly, addition of cyclothiazide to inhibit desensitization collapses the smFRET state distribution into two much more compact conformations [81]. This collapse is also echoed in the structural literature where open states tend to have much more homogeneity than resting or desensitized conformations [31, 84]. While the smFRET data have been able to provide insight into the inconsistencies in the structures of the amino-terminal domain of the homomeric receptors, given that the native receptors are heteromeric, future studies would need to focus on these subtypes. Currently there is one structure of the heteromeric AMPA receptors [85] which shows a more compact arrangement of the amino-terminal domain, different from what is seen with the homomeric receptors. smFRET investigations will be able to provide further insight into this conformation and its dynamics.

Thus far, only the conformational landscape of the AMPA receptor amino-terminal domains in the full length receptor has been probed using smFRET. This method could readily be

applied to the agonist-binding domain clam shell or dimer interface within the full receptor. There are several unanswered questions with regards to dimer interface at the agonist-binding domain. There is considerable evidence for some resting state desensitization or separation of the dimer interface [47]. Also, functional crosslinking experiments across the AMPA dimer are consistent with a basal level of dimer separation [59]. The full length structures show primarily a coupled dimer interface [28]. Thus smFRET investigations of the full length receptor specifically looking at the dimer interface should be able to bridge the gap between the states seen by spectroscopy, crosslinking, and structure. Full length smFRET would provide a fount of insight into the extent of resting state separation and how this equilibrium is modified by ions, auxiliary proteins, allosteric modulators, and other factors. Furthermore, smFRET on the agonist-binding domain or dimer interface has the potential to open altogether new roads of inquiry. AMPA receptors desensitize with far less glutamate than required for activation [61, 86]. What happens to the dimer interface at these sub-activation levels of glutamate? Does the separation of one dimer strongly destabilize the other? Could this be accomplished by a single molecule of glutamate as expected based on kinetic models [61]? Mutli-color smFRET or other advances may enable us to ask these detailed long lingering questions by simultaneously monitoring each dimer pair in a single channel.

Modulation by auxiliary subunits.

In the brain, AMPA receptors co-assemble with a variety of auxiliary proteins, including transmembrane AMPA receptor regulatory proteins (TARPs) [11]. These TARPs not only play a role in trafficking and localization of the AMPA receptors, they also alter the biophysical properties of the receptor. For example, the TARP stargazin, or $\gamma 2$, increases agonist efficacy, slows deactivation and desensitization, accelerates recovery from desensitization, and attenuates polyamine block [11–13, 87, 88]. The structures of the AMPA receptor in complex with stargazin showed extensive interactions between the agonist-binding domain of the AMPA receptor and the extracellular domain of stargazin [89, 90]. Fast perfusion electrophysiology measurements and luminescence resonance energy transfer methods addressed the mechanism underlying the increase in efficacy in the presence of stargazin and suggested that this could be a result of the agonist-binding domain of the AMPA receptors being in a more closed cleft state in the presence of stargazin [91]. In order to address the underlying differences in the conformational landscape that could be related to changes in desensitization, the distance across the dimer in the amino-terminal domain was probed [81]. These experiments found that the range of conformational states occupied by the receptor in the presence of stargazin was biased towards closer, more compacted structures than with the receptor only (Figure 4). This suggests that some gain of function effects of stargazin may arise by constraining the conformational landscape of the complex to a more productive set of states, where agonist-induced motions are more effectively coupled to the gating machinery. Such smFRET experiments have only been used for stargazin, the prototypical TARP. However, there are a number of other TARPs which differ in the strength of modulation as well as a host of structurally dissimilar auxiliary proteins which act as either gain or loss of function modulators. The experiments pioneered

using stargazin set the ground work for future investigations on this assortment of AMPA auxiliary proteins [92].

Correlation of conformational and functional states: probing the transmembrane segments.

A full account of how agonist binding controls channel gating requires mapping the diversity of agonist-binding domain states unto conformations of the transmembrane segments. Single channel recordings reveal multiple closed and open states in this receptor family, distinguishable by lifetime and conductance. Therefore there must also be a range of conformational states for the transmembrane segments. For such investigations, NMDA receptors provide a unique advantage for smFRET as they are obligate heteromers (Figure 5a). Thus it becomes easier to obtain unique smFRET distances within a tetramer as they have two glycine-binding and two glutamate-binding subunits arranged in a checkerboard pattern. The retention signal masking methods used for triheteromeric studies may allow further refinement of smFRET site labels [93, 94]. The full length structures of the NMDA receptors primarily exhibit inactive closed channel packing of the transmembrane segments [58, 95]. Recent computational studies presented a model of an open state structure obtained with molecular dynamic simulations which was correlated with prior cysteine accessibility data to ensure its correctness [96], and coarse grain modeling of the activation [66]. These structures provide an initial framework for the detailed smFRET investigations of the motions in the transmembrane segments that can be correlated to the multiple functional states [55]. The smFRET investigations at the first transmembrane domain of the NMDA receptor showed multiple states (Figure 5b and 5c). The assignment of the states to possible closed and open channel states was done based on structures of closely related NaK channel for which open and closed structures were determined [97, 98]. In addition, the fraction of the states assigned to presumptive open channel states correlated well with the fraction of steady state currents in these proteins. Based on these assignments, the smFRET-based state connectivity and energy profile were strikingly similar to those determined from single channel current recordings, providing the conformational correlates to the functional landscape [64, 65]. While the 5 ms resolution of these experiments does not allow for direct correlations between the state transitions in the smFRET and the single channel recordings, the two long-lived desensitized states predicted from the single channel recordings as well as the tail end longer time scale fractions of the open states could be correlated to the smFRET states. The smFRET studies also showed that the closed inactive states were not all the same [55]. The apo resting state was more tightly packed with shorter distances across the transmembrane segments relative to the closed (assigned to desensitized states) observed in the presence of agonists. Additionally, these studies showed that the zinc-bound inhibited receptor had smFRET-based distances and states similar to those seen in the resting apo state, which is consistent with a tight packing of the transmembrane region [55].

Concluding remarks and future perspectives.

Ion channels have a rich and profitable history of deriving insight from single molecule methods, and there are many questions which have yet to be addressed [see Outstanding

Questions Box]. If the field can combine this predisposition with the improvements in protein purification produced by the structural biology revolution and with advances in optical methods that make it possible to study the single molecule conformational landscape, the next decade is poised to be enormously fruitful.

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Highlights

- Ionotropic glutamate receptors, isolated segments and full length, have over 1000 structures available in different liganded states and in complex with auxiliary subunits
- The functional parameters of ionotropic glutamate receptors are well characterized by macroscopic and single channel electrophysiological recordings
- Single molecule fluorescence resonance measurements are beginning to map site-specific transitions between known structural end points at temporal resolutions approaching that of electrophysiology
- These studies are particularly illuminating for transitions amongst electrically silent conformations and for providing single molecule explanations for macroscopic observations

Box1-**Conformation and Energy landscape and Glutamate receptor function**

The multiplicity of states that glutamate receptors occupy has been known for several decades [2, 61, 64, 99, 100], but associating conformations to these functionally identified states and correlating kinetics of transitions between the conformational states has been proven to be a challenge. smFRET is unique in being able to address this challenge as it is able to provide the conformational landscape and map transitions between these conformational states. The large number of X-ray- and electron microscopy-based structures that are becoming available lay the required foundation for such smFRET investigations as they provide the specific sites where the large conformational changes occur. Correlating the conformational states identified by smFRET to the functional states determined by single channel recordings and understanding how these are altered by ligands, drugs, or disease conditions provides the necessary foundation for future translational studies where selective modulation of specific states can be achieved to address pathological and genetic disease states.

Outstanding Questions

- How can we directly relate the conformational landscape in one domain to that of the other?
- How are the allosteric communications between the subunits within the dimer different from that across the dimer? How does this relate to negative cooperativity that has been previously shown between glycine and glutamate?
- What is the role of the intracellular carboxy terminal domain in dictating the conformation-energy landscape of the transmembrane segments?
- How do intracellular signals such as calmodulin, phosphorylation etc., alter the conformational landscape?

Figure 1:

AMPA receptor structure and agonist-binding cleft. A) The tetrameric AMPA receptor structure which exhibits the modular domain arrangement inherent to all ionotropic glutamate receptors. Each subunit is distinctly colored with the modular domains, R1/R2 lobes of the ATD, and D1/D2 lobes of the ABD labelled. The position of residue T686 is shown by a red sphere on the green subunit. PDB: 3KG2 [28]. B) The different degrees of AMPA receptor agonist-binding domain cleft closure when bound to: competitive antagonist ZK 200775 (blue, PDB: 3KG2); apo condition (cyan, PDB: 1FTO) [32]; antagonist DNQX (green, PDB: 1FTL) [32]; partial agonist iodo-willardiine (yellow, PDB: 1MQG) [72]; and full agonist glutamate (red, PDB: 1FTJ) [32].

Figure 2: smFRET methodology. A) Scheme for attachment of glutamate receptors to glass coverslip for observation. A genetically-encoded Twin-Strep tag is added to the C-terminus of the glutamate receptor which allows for the direct attachment of the receptor to the streptavidin-coated coverslip. B) Representative donor and acceptor intensity measurements from a single molecule and efficiency trace calculated from the donor-acceptor traces. The traces are shown before and after being subjected to denoising. C) Histogram of occurrence versus FRET efficiency determined from the section of the efficiency trace shown in B prior to acceptor photobleaching.

Figure 3:

Role of smFRET in developing the hypothesis of agonist-binding domain control of AMPA receptor activation. A) smFRET histograms showing the effect of the T686S mutation on the behavior of the AMPA receptor. Note the wide range of conformations sampled by the mutant receptor. Figure 3a extracted from [76]. B) Activation of AMPA receptors versus agonist-binding domain closed-cleft occupancy for a variety of ligands and mutants. The data reveal that the fraction of the proteins exhibiting FRET efficiency higher than 0.76 correlates with the degree of activation of the receptor. Figure 3b extracted from [77].

Figure 4:

smFRET measurements of the AMPA receptor inter-ATD distance. smFRET histograms showing the states probed by the AMPA receptor when the receptor is labeled at residue 23 of the amino-terminal domain under conditions of A) desensitization (1 mM glutamate), C), open channel (1 mM glutamate + 100 mM CTZ), and D) desensitization in presence of stargazin (1 mM glutamate). B) Representative traces of the AMPA receptors studied in A). Inset in A) Cartoon representations of the four states of the receptor identified in the desensitized state. Figure extracted from [81].

Figure 5: NMDA receptor structure and state identification. A) smFRET labeling sites for measuring the NMDA receptor transmembrane segments shown by green and red spheres, with fluorophore-accessible volumes shown by green and red shaded areas. Note the heterotetrameric arrangement of the NMDA receptor subunits (GluN1 in magenta and green, GluN2 in yellow and cyan). PDB accession code: 5IOU [58]. B) and C) Identification of multiple states of the transmembrane segments of the NMDA receptor bound to B) no agonists (apo condition) or C) agonists glutamate and glycine and channel blocker MK-801. The efficiency of each identified state is shown above the histogram. Figures extracted from [55].