nature portfolio

# Peer Review File

# Bi-directional allosteric pathway in NMDA receptor activation and modulation

Corresponding Author: Dr Vasanthi Jayaraman

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

**Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.**

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This work addresses an important and yet unsolved question: what are the intramolecular movements that underlie electrical signaling through NMDA receptors. This is important because NMDA receptors mediate a substantial portion of excitatory transmission in CNS and because the kinetics of their activation, which is a direct reflection of intramolecular dynamics, is critical for normal brain function.

The authors use smFRET, which is the state-of-the art approach for estimating changes in intramolecular distances in large proteins such as the NMDA receptors. This technique offers information that is complementary to that provided by molecular imaging of fixed receptors, such as single particle CryoEM, or functional measurements provided by single-channel electrophysiology. Moreover, the 5 ms resolution reported in this paper is – to my knowledge – the highest in the field, which allows authors to compare distances calculated from FRET efficiencies with those reported from atomic-resolution structural studies.

The data are of high quality and the conclusions are well supported by the results presented. Given that this is one of only two labs that are reporting results in NMDA receptors with this approach, I am eager to see it in print.

The results afford two notable advances. First, the actual measurements (in Table 1) provide robust evidence of separation among populations, and quantitative sorting by distance. The result with GNE is particularly novel and provides evidence for direct coupling (bidirectional communication) between the amino terminal domain and the linkers adjacent to the pore. This is an often ignored property of allostery! IN other words, if manipulating the geometry/dynamics of site A (with drugs or mutations) affects the geometry/dynamics of site B, then the reverse must be true! This is done here cleverly by using a positive allosteric modulator that binds at the linker region.

Therefore, I find that this article reports rigorous work and important results. All my comments below reflect my preference to improve readability. Therefore, are suggestions only, in no order.

• It would be more valuable to show primary data (traces) and histograms in the main portion of the paper (for at least one condition) rather than in the supplementary material, as it is now. The e-phys controls, while necessary, do not advance the field and can be easily moved from fig 1 to supplementary material.
• The table, which is now in supplementary material, presents a nice summary of all the results and I would prefer to see it in the main text, as it is likely to be more valuable than the cartoon in the last figure.
• Speaking of cartoons, the representations of labeling sites (in Fig 1) while useful because it is very intuitive can be done in only one structure, perhaps labelling the sites, or coloring them as LBD, TMD, ATD. Or such. Which could save space for primary data and table.
• The measurements reported here for GluN1/GluN2D receptors mirror those reported previously by this group for GluN1/GluN2A receptors (Nat chem Biol, 2017). And the authors refer frequently to values reported there. This is a valuable comparison. It would be easier on the reader if the table in this manuscript would follow the same structure as in their previous article (columns/rows).

Reviewer #2

(Remarks to the Author)
see attachment, Nature Comm Review.071624

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have incorporated all my suggestions in their revision. This work is fantastic, and now contains all the info that structural and functional experts can learn from. The results connect qualitative structural information as captured by X-ray and CryoEM with quantitative functional information as captured by single molecule kinetics, a gap that will continue to require attention.

Reviewer #2

(Remarks to the Author)
The authors has modified the manuscript to address all of my concerns.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank the reviewers for their detailed comments and suggestions. We are pleased to see their enthusiasm for our work, noting that they consider it “a valuable study that will add to the currently missing knowledge of NMDA receptor conformational dynamics and its correlation with function,” and that it “addresses an important and yet unsolved question: what are the intramolecular movements that underlie electrical signaling through NMDA receptors.”

We have addressed all the concerns raised and made the requested edits. Specifically, we have included the controls requested by Reviewer 1. We added anisotropy measurements for each site and fluorophore, showing no significant changes between the apo, glutamate/glycine, and glutamate/glycine/modulator-bound states. Additionally, we included a discussion of the orientation factor (k) in the Methods section. We also present data using Alexa 555 and Alexa 750 as donor and acceptor (with an $R_0$ of 44 Å) attached to the site at the amino-terminal domain. This data shows trends consistent with those reported for Alexa 555 and Alexa 647. We believe these controls should address Reviewer 1’s concerns regarding the smFRET data.

The detailed point by point response is provided below:

<u>Reviewer 1:</u>

1. It would be more valuable to show primary data (traces) and histograms in the main portion of the paper (for at least one condition) rather than in the supplementary material, as it is now. The e-phys controls, while necessary, do not advance the field and can be easily moved from fig 1 to supplementary material.

This is a great idea, and we thank the reviewer for this suggestion. We have changed the figures in main text to include primary traces (Figure 2, Figure 3, Figure 4, and Figure 5).

2. The table, which is now in supplementary material, presents a nice summary of all the results and I would prefer to see it in the main text, as it is likely to be more valuable than the cartoon in the last figure.

We thank the reviewer for this suggestion and have moved the data to the main text (Table 1).

3. Speaking of cartoons, the representations of labeling sites (in Fig 1) while useful because it is very intuitive can be done in only one structure, perhaps labelling the sites, or coloring them as LBD, TMD, ATD. Or such. Which could save space for primary data and table.

We think this is a great suggestion, so we have made a single figure showing all the sites for labeling, this is now Figure 1A.

4. The measurements reported here for GluN1/GluN2D receptors mirror those reported previously by this group for GluN1/GluN2A receptors (Nat chem Biol, 2017). And the authors refer frequently to values reported there. This is a valuable comparison. It would be easier on the reader if the table in this manuscript would follow the same structure as in their previous article (columns/rows).

We appreciate the reviewer’s suggestion to reformat the table. We have edited Table 1 to follow the same structure as our most recent paper on the GluN1/GluN2A variant (Durham et al., PNAS,

2020). We decided to follow the format of this table as most of the GluN1/GluN2A data we make comparisons with came from this paper and this is a more recent paper from our lab.

Reviewer 2:

1. For FRET analysis, the amount of energy transfer depends on the distance between the fluorophore and also their relative orientation (displayed by the $\kappa$ term.) Was the orientation assumed to be random or was any orientation factor taken into account? (This can be particular problematic in smFRET, although the range in energy transfer can possibly give some limits to this.) The methods section does not mention it. Could this difference between various states account for the differences seen, as opposed to changes in distance? This is particularly significant because of the next point.

This is an excellent question, and we thank the reviewer for suggesting that we include this data in the paper. When selecting sites for labeling, we chose those that are solvent-exposed and expected to be anisotropic. To confirm this, we performed polarizability measurements for each site using different fluorophores. We typically observed polarizability values in the range of 0.07–0.2, indicating that the fluorophores are relatively anisotropic. More importantly, there was no significant change in polarizability between the apo, liganded, and modulator-bound states, suggesting that this factor is unlikely to account for the changes observed in the smFRET data. We have now included this data in Supplementary Table 1 and added the following section on the orientation factor ($\kappa$) in the Methods section on page 14 of the revised manuscript:

*"To determine the error associated with the orientation factor ($\kappa$), we measured the polarization ratio for donor (Alexa 555) and acceptor (Alexa 647) fluorophore-labeled protein for each of the constructs studied in smFRET. For these experiments, we followed the same sample preparation steps as for smFRET experiments. Following ultracentrifugation, an antibody or affinity pull down was performed on the supernatant. Fluorescence polarization measurements were performed at room temperature using PTI QuantaMaster polarization accessory. The polarization ratios were calculated from the equation[67]:*

$$p = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}$$

*Where $I_{VV}$ and $I_{VH}$ are the measured fluorescence intensities with the excitation polarizer vertically oriented and emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor for the detection system. Polarization ratios were compared using Kruskal-Wallis and Dunn's multiple comparisons tests. P values < 0.05 are considered significant. The calculated polarization ratio values are provided in Supplementary Table 1. The values range from 0.07 to 0.26, and there are no significant changes in the polarization ratios between the conditions measured (apo, liganded and modulator bound) for a given site. Thus, it is unlikely that the changes in smFRET between the different conditions (apo, liganded and modulator bound) measured here for a given site arise due to differences in the orientation factor[68]."*

2. The FRET Efficiencies they measured are awfully high, ranging from 0.8 to 1. FRET is most sensitive when the distance is = 0.5 energy efficiency. Presumably it was so high because of the spectral overlap of the donor fluorescence and acceptor's absorption. Why

wasn't another pair chosen that the Ro was less, and therefore E ~ 0.5? While I know this is rather painful and involves much more work, unless they can convincingly convince the reader of point 1 and especially point 2, it raises questions about the validity of their conclusions. (If course, having another dye pair then leads to questions about $\kappa^2$ again.)

We agree that FRET efficiencies closer to 0.5 provide greater sensitivity to changes in distance. However, in our single-molecule FRET experiments, we found that higher FRET efficiencies help us better identify FRET molecules in the FRET channel due to the higher photon count. Additionally, based on prior structures of other NMDA receptor subtypes, our hypothesis was that the addition of glutamate-glycine would lead to decoupling at the amino-terminus. We anticipated that such decoupling would shift the smFRET histogram towards lower FRET states, which justified starting with a high smFRET distribution. Nonetheless, we appreciate the reviewer's concern, and to demonstrate consistency, we performed smFRET using Alexa 555 and Alexa 750, which has an $R_0$ of 44 Å, for the GluN1/GluN2D amino-terminal domain site. This site exhibits the most significant changes and is critical for showing bi-directionality with the modulator. The new data is now included in Supplementary Figure 3 and is addressed as follows in the Results on page 8 of the revised manuscript:

*"To verify that the changes at the amino-terminal domain are not fluorophore dependent, we performed additional smFRET experiments using Alexa 555 and Alexa 750 as the donor and acceptor fluorophores, which have an $R_0$ of 44 Å. The smFRET histograms show similar trends as those observed with Alexa 555 and Alexa 647, with a shift towards lower FRET upon binding glutamate and glycine relative to apo conditions (Supplementary Figure 3a), and a shift back towards higher FRET upon binding GNE-9278 (Supplementary Figure 3b). These data show the robustness of the smFRET data and further confirm our results that allosteric communication between the amino-terminal and transmembrane domains is conserved even in the modulatory mechanism of positive allosteric modulators and, more importantly, that the pathway is bidirectional."*

3. The authors mention in the main text about modifications at GluN2A and GluN2D amino-terminal domains and refer to Fig. 1D. However, Fig. 1D only shows GluN1/GluN2D-ATD and its corresponding whole-cell recording trace. Was the electrophysiological recording performed for the GluN1/GluN2A-ATD construct with the cysteine modification at Thr174?

We thank the reviewer for pointing out this error. We have now included the data, which is shown in Figure 1D.

4. For statistical significance, it would be good to provide the actual p-values as well, instead of just mentioning $p<0.05$. Given that less than 100 molecules were observed for almost all the FRET constructs, it is possible to improve the statistical significance of the results by increasing the sample size if the p-values are close to 0.05.

We have included p values for all data in the figures (Figure 2, Figure 3, Figure 4, and Figure 5).

5. Half the data in Fig. 2 and Fig. 3 is from a different paper, presumably from their own lab. This should definitely be cleared up.

The focus of the paper is GluN1/GluN2D variant and the data from previous paper is on GluN1/GluN2A variant. It was included to make it easier for the reader to make the comparison. We have indicated this in the Figure Captions.

Figure 2 Caption has this sentence: "*Data for GluN1-TMD/GluN2A are from Dolino et al.[47].*"

Figure 3 Caption has the following sentence: "*Data for GluN1/GluN2A-ABD and GluN1-ABD/GluN2A are from Durham et al.[46].*"

Minor concerns of axes labels (Figure 2, Figure 4, Figure 5) have been addressed. Additionally, excitation intensities have been included on page 13 of the Methods section of the revised manuscript as follows:

"*For experiments with Alexa 555 and Alexa 647, the laser intensity of 532 nm was approximately 0.8 μW, and for experiments with Alexa 555 and Alexa 750, the laser intensity of 532 nm was approximately 5 μW.*"

Nature Comm Review:

NMDA receptors are part of the family of glutamate receptors and play an important role in excitatory synaptic transmission. While the structure of these receptors and their various subtypes is known, there are still gaps in our understanding of their conformational states and how they can affect their function. In this article, Bender et. al. utilize single molecule Forster Resonance Energy Transfer (smFRET) technique to study conformation dynamics of NMDA receptors. The authors focus specifically on GluN1/GluN2A and GluN1/GluN2D receptors.

Using single-molecule pull down experiments, the authors are able to measure the FRET efficiencies at various sites including GluN1 trans-membrane domain (TMD), GluN1 glycine binding domain, GluN2 glutamate binding domain and GluN2A and GluN2D amino terminal domains (AMTD. They also look at conformation dynamics by extracting transition probabilities from smFRET traces. The authors observe a higher FRET efficiency as measured at the GluN1 TMD in GluN1/GluN2D receptors in apo state, suggesting that they are more tightly packed compared to GluN1/GluN2A receptors. The FRET efficiency reduces upon glutamate and glycine binding. The transition probability maps show that the TMD is more dynamic for GluN1/GluN2A compared to GluN1/GluN2D. The dynamicity increases for both in the ligand bound state compared to apo state. For the glutamate and glycine-binding domains, no significant difference was observed between GluN1/GluN2A and GluN1/GluN2D in either apo or bound states.

The GluN2 ATDs in the GluN1/GluN2A and GluN1/GluN2D receptors show a difference in both apo and ligand bound states, with GluN1/GluN2D having lower FRET efficiencies. There is a larger fraction of low FRET efficiency states observed for GluN1/GluN2D receptors correlates with their lower $P_{open}$, thus correlating function and conformation. The authors also study the effect of positive allosteric modulator GNE-9278 on ATD of GluN1/GluN2D receptors in the presence of glutamate and glycine. They observe an increased FRET efficiency and decreased conformational dynamics. This correlates with an increase in activation of GluN1/GluN2D receptors.

Overall, this is a valuable study that will add to the current missing knowledge of the NMDA receptor conformational dynamics and its correlation with their function. The value to the neuroscience community would be better addressed by a biologist/neuroscientist, rather than a technique-type of person as we are. I have some technical questions (points 1 and 2 below) which have to do with the interpretation of the very high energy transfer that they saw and consequently whether their results are robust (or even correct, in the worst case).

Following are some comments about the paper.

Major concerns:

1. For FRET analysis, the amount of energy transfer depends on the distance between the fluorophore and also their relative orientation (displayed by the $\kappa^2$ term.) Was the orientation assumed to be random or was any orientation factor taken into account? (This can be particular problematic in smFRET, although the range in energy transfer can possibly give some limits to this.) The methods section does not mention it. Could

this difference between various states account for the differences seen, as opposed to changes in distance? This is particularly significant because of the next point.

2. The FRET Efficiencies they measured are awfully high, ranging from 0.8 to 1. FRET is most sensitive when the distance is = 0.5 energy efficiency. Presumably it was so high because of the spectral overlap of the donor fluorescence and acceptor's absorption. Why wasn't another pair chosen that the $R_o$ was less, and therefore E ~ 0.5 ? While I know this is rather painful and involves much more work, unless they can convincingly convince the reader of point 1 and especially point 2, it raises questions about the validity of their conclusions. (If course, having another dye pair then leads to questions about $\kappa^2$ again.)
3. The authors mention in the main text about modifications at GluN2A and GluN2D amino-terminal domains and refer to Fig. 1D. However, Fig. 1D only shows GluN1/GluN2D-ATD and its corresponding whole-cell recording trace. Was the electrophysiological recording performed for the GluN1/GluN2A-ATD construct with the cysteine modification at Thr174?
4. For statistical significance, it would be good to provide the actual p-values as well, instead of just mentioning $p<0.05$. Given that less than 100 molecules were observed for almost all the FRET constructs, it is possible to improve the statistical significance of the results by increasing the sample size if the p-values are close to 0.05.
5. Half the data in Fig. 2 and Fig. 3 is from a different paper, presumably from their own lab. This should definitely be cleared up.

Minor concerns:

1. The axes labels for all the state transition probability maps are extremely small and very hard to read. It would be helpful for the readers to increase their font size.
2. For facilitating accurate replication of the results, it would be helpful to mention the excitation laser intensities in the methods section.

Paul Selvin

University of Illinois, Urbana-Champaign