Structure of the Zinc-bound Amino-terminal Domain of the NMDA receptor NR2B subunit

Erkan Karakas, Noriko Simorowski and Hiro Furukawa

Corresponding author: Hiro Furukawa, Cold Spring Harbor Laboratory

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 29 September 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. You will see that the referees are generally positive about your work and that they would support its ultimate publication in The EMBO Journal after appropriate revision. I would thus like to invite you to prepare a revised manuscript in which you need to address the issues raised by the referees in an adequate manner. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFeree COMMENTS

Referee #1 (Remarks to the Author):

This nicely written manuscript describes the resolved structure of the NR2B amino-terminal domain in the absence and presence of zinc. It complements similar structures for kainate and AMPA receptor subunits also published recently by other laboratories. In some sense the NMDA receptor
ATD is the most functionally relevant structure, though, because of the presence of several allosteric modulatory sites, including a zinc binding site. This also represents the site of action of modulators in clinical trials, and therefore the resolved structure could be of use in drug design efforts. The structure itself contains a surprising twist in architecture that was not predicted in homology modeling with the AMPA or kainate receptor ATDs. Finally, the authors explore predictions regarding the zinc and phenylethanolamine binding site using mutagenesis and physiological analyses, which yield data generally consistent with the structural model. A very nice piece of work that merits publication. It will unquestionably open up new avenues for exploring structure and function in this NMDA receptor domain.

Minor issues
1. The reference for Clayton et al. is incomplete in the bibliography.
2. p. 10, line 2: citation in support of "GluR6 ATD" should be Kumar et al., not Clayton et al., 2009.

Referee #2 (Remarks to the Author):

This work describes the crystal structure of the aminoterminal domain (ATD) of NMDA receptor subunit NR2B. ATD structures have recently been published for homologous AMPA and kainate receptor subunits. However, in these glutamate receptor subclasses, the functional role of ATD is unclear. In contrast, in NMDA receptors, ATD regulates channel activity by mediating inhibitory actions of Zn2+ ions and ifenprodil. The data presented by Karakas et al. are solid and provide structural explanations for zinc binding and demonstrate striking differences to AMPA/kainate receptors in the relative orientation of the two ATD lobes and in the oligomerization state. The manuscript is well-written and the methods and experimental findings are presented and discussed in a logical and clear manner. This is an important contribution to the field and will provide an essential starting point for further detailed structural studies.

I have the following remarks:

1. A minor weakness of the study is that it does not show (predicted) closure or any other conformational change in ATD upon Zn2+ binding, but the authors suggest that this is due to saturation of nonspecific ion binding sites between ATD lobes by Na+ and Cl- ions. If binding of sodium and chloride stabilizes the closed state of the ATD, and if the closed state is indeed essential for binding of idenprodil and Zn2+, experiments testing the effects of monovalent ions (or mutations of the binding residues) on the binding of ifenprodil and zinc would be informative and might strengthen the author's hypothesis.

2. Elimination of Zn1 site by mutations leads to a quite modest decrease in the IC50 value (from to). Why is this so and what are the causes of the remaining inhibition by zinc?

3. In Fig. 2, the amino acid residues forming the hydrophilic and hydrophobic pockets and the ion binding site are difficult to distinguish. Alternative coloring or side chain representations might work better. Another Figure issue: Figure 4 (and Fig. 4 legend) seems to lack the color code (for the degree of hydrophobicity).

4. The terms homology and homologous are used incorrectly (p. 4, p. 15) and should be replaced by similarity / similar.

5. The authors should consider adherence to the revised nomenclature for ionotropic glutamate receptors (Collingridge et al., 2009, Neuropharmacology 56: 2-5), which is rapidly gaining popularity. Hence, NMDA receptor subunit NR2B should be "GluN2B".

6. Typos: On page 17, line 7, the word "bind" should be removed; on page 19, line 3, "Van der Waal" should be "van der Waals".

Referee #3 (Remarks to the Author):

The glutamate receptor ion channels which mediate excitatory synaptic transmission in the brain,

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and NMDA receptors in particular, have received decades of attention due to their role in synaptic plasticity, development and disease. Karakas and colleagues present the first structure of the amino terminal domain of an NMDA receptor subunit, and use this to design mutants for functional analysis to test aspects of allosteric modulation. The work is of significance because, despite a long history of site directed mutagenesis based on models of NMDA receptor ATDs, an experimental structure has never been obtained. Perhaps not surprisingly (at least to structural biologists), the NR2B ATD structure has unique features not revealed by previous modeling studies, and adopts a conformation which is strikingly different from that found for the GluR2 and GluR6 ATDs, but which makes good sense in light of the known ligand binding properties and anticipated conformational changes in the ATD produced by allosteric modulators. Although much remains to be done, this paper paves the way for a deeper investigation into analysis of NMDA receptor function, and is a major advance in the field.

The structural work is sound, indeed impressive, with experimentally phased maps from 4 heavy atom derivatives used for model building, combined with anomalous difference maps for identification of cation and anion binding sites; this is a substantial body of experimental work, which unfortunately only crystallographers will appreciate. The major findings of the study are (i) identification of a Zn binding site underlying the 'low affinity' modulation of NR2B by Zn; (ii) identification of novel Na and Cl binding sites in the interdomain cleft that MIGHT play a role in setting the conformation and resting tone of NR2B ATD modulation of NMDA receptor gating; (iii) identification of a large twist between domains R1 and R2 that is unique amongst GluR ATD structures solved to date.

The paper is already strong, but there are three areas in which the impact of the study could be substantially increased,

1) The characteristic features of the NR2A and NR2B ATDs are high affinity modulation by Zn and drugs like ifenprodil respectively. In the present study, somewhat perversely given the unique ligand binding properties of NR2A and NR2B, the low affinity Zn binding site of NR2B is identified, and a mechanism proposed to account for the much higher affinity binding of Zn to NR2A. This could easily be tested, by swapping the NR2A loop containing the proposed additional Zn ligands into NR2B, and or by a small series of point mutants, and then testing either by ITC, or functional assays.

2) An unexpected result of the present study is the finding that cations (probably Na) and anions, almost certainly Cl, bind to the NR2B cleft, in between the proposed binding site for ifenprodil and the low affinity Zn binding site. The authors propose that the binding of these ions modulates the open/closed equilibrium of the NR2B ATD. This really needs to be experimentally tested because the ion concentrations used in the crystallization experiments are in the Molar range, raising the question as to whether these sites are occupied under physiological conditions to produce the proposed modulation. This could be easily tested in experiments on oocytes, using a series of ion substitution experiments, i.e. replacing Cl, by NO3, MeSo3, acetate, I, F etc; and likewise replacing Na by K, Rb and Cs. In prior work on allosteric Na and Cl ion binding sites in kainate receptors such substitutions produced dramatic changes in ion channel activity even though a series of cation structures was solved using ion concentrations much higher than 150 mM. It is likely that for NR2B the same series of ions will also have different affinity and efficacy for the Na and Cl sites, and thus allow to probe the proposed mechanism. The cation substitution experiments will have the complication that the major permeant ion is being replaced, but prior work has already established that NMDA receptors are non selective, so this is only a minor consideration. If no effects are seen, then it is unlikely that the proposed mechanism of modulation occurs.

3) The sedimentation properties of the isolated NR2B indicate that it forms monomers, in contrast to the GluR2 and GluR6 ATDs which form dimers, indicating that in intact NMDA receptors, the ATD likely assembles as NR1/NR2 heterodimers. The crystallographic analysis focuses on the conformation of a single NR2B ATD, for which there is one copy in the asymmetric unit, but does not report what packing is observed in the P3(1)21 lattice. This should be reported and discussed in terms of whether there are likely biological assemblies or not.

Minor points.
1) The discussion of the Na binding site needs to be extended to include ligand distances (are these consistent with Na binding) and geometry: Na is usually 6 or 5 fold coordinated. It is confusing that on page 17 the Na ion is identified as Na1, which is unnecessary as there is only a single Na ion identified.

2) Page 19 para 2: the discussion of two modes of ifenprodil binding is hard to follow; how does this relate to the interpretation elsewhere that ifenprodil also produces channel block.

3) For the most part the paper is well written, but a few typos need correction,

Page 2 line 4 change to: ... of NMDA receptors is that their ion channel ... 
Page 3 line 3 delete 'the': ... of L-glutamate from nerve terminals ... 
Page 3 line 4 from end delete 'the': ... The opening of NMDA receptor ion channels 
Page 4 line 6 from end insert 'the': ... small ligands that bind the ATD of ... 
Page 7 line 1 insert 'an' ... structure of an NMDA receptor ATD ... 
Page 10 line 3: the GluR6 ATD reference should be Kumar not Clayton 
Page 11 line 4 change dimer to homo dimer 
Page 11 para 2 missing The: The NR2B ATD also has ... 
Page 17 line 7 where bind Na ions ... should read ... where bound Na ions

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Referee #1:

This nicely written manuscript describes the resolved structure of the NR2B amino-terminal domain in the absence and presence of zinc—-A very nice piece of work that merits publication. It will unquestionably open up new avenues for exploring structure and function in this NMDA receptor domain.

Comment 1 and 2

Minor issues
1. The reference for Clayton et al. is incomplete in the bibliography. 2. p. 10, line 2: citation in support of "GluR6 ATD" should be Kumar et al., not Clayton et al., 2009.

Response

We are extremely delighted with the positive comments provided by Referee 1. We have put the complete reference for Clayton et al and replaced the citation on page 10, line 2 from Clayton et al to Kumar et al as suggested.

Referee #2:

This work describes the crystal structure of the amino terminal domain (ATD) of NMDA receptor subunit NR2B.---The data presented by Karakas et al. are solid and provide structural explanations for zinc binding and demonstrate striking differences to AMPA/kainate receptors in the relative orientation of the two ATD lobes and in the oligomerization state. The manuscript is well-written and the methods and experimental findings are presented and discussed in a logical and clear manner. This is an important contribution to the field and will provide an essential starting point for further detailed structural studies.

Comment 1

A minor weakness of the study is that it does not show (predicted) closure or any other conformational change in ATD upon Zn2+ binding, but the authors suggest that this is due to saturation of nonspecific ion binding sites between ATD lobes by Na+ and Cl- ions. If binding of sodium and chloride stabilizes the closed state of the ATD, and if the closed state is indeed
essential for binding of idenprodil and Zn2+, experiments testing the effects of monovalent ions (or mutations of the binding residues) on the binding of ifenprodil and zinc would be informative and might strengthen the author's hypothesis.

Response
The Na+ and Cl− bindings at the clamshell cleft are mediated mainly by main chain oxygens and nitrogens. There are three residues, S131, Q153, and R292, whose side chains participate in binding of Cl− ions. We have mutated those residues to alanine to test ifenprodil and zinc effects as suggested by Referee2. Mutations of S131 and Q153 to alanine have little or no effect on zinc sensitivity or ifenprodil sensitivity. In contrast, the R292A mutant has a modest effect on zinc sensitivity and robust effect on ifenprodil sensitivity (Data is added to Supplementary Table SIII). R292 is responsible for binding of both Cl2 and Cl3 but it is also involved in hydrogen bonding network with other residues within the clamshell cleft. Therefore the observed effect might be due to the disruption of the hydrogen bonding network perturbing the architecture around the clamshell cleft.

A similar point was raised by Referee 3 (Comment 2) suggesting to conduct ion replacement experiments using two-electrode voltage clamp on Xenopus oocytes to assess whether or not Na+ and Cl− modulate NMDA receptor activity at physiological concentrations (approximately 150 mM). Substitution of Na+ with Rb+ and Cs+ and Cl− with NO3− did not cause any major shift in the current-voltage (I-V) plot indicating that the NR2B NMDA receptors are not modulated by physiological concentration of NaCl.

In light of these new evidences, we revised the manuscript not to emphasize Na+ and Cl− binding to be a major factor in inducing the closed cleft conformation. We made the following revisions in the manuscript:

1) We changed the section title from "Ion Binding Stabilizes NR2B ATD in Closed Conformation" to "NR2B ATD in Zinc-free form" on page 16.

2) We added the following sentences to the end of the first paragraph on page 18:
"Can physiological concentration of Na+ and Cl− induce clamshell closure? To date, there has not been any known mechanism for a functional modulation of NMDA receptors by monovalent ions such as Na+ and Cl− like the ones observed in kainate receptors (Plested and Mayer, 2007; Plested et al, 2008). Indeed, we have not observed any significant change in the current-voltage relationship by substitutions of Na+ by Li+ or Rb+ or Cs+ and Cl− by NO3− at 150 mM salt concentration (data not shown). Thus, while binding of Na+ and Cl− can take place at the NR2B ATD clamshell cleft, it is likely not a driving force for the clamshell closure."

3) We changed the last sentences of the first paragraph on page 18 from
"We suggest that binding of Na and Cl ions promotes stabilization of the closed conformation. In the present crystal structure, the closed conformation of NR2B ATD is substantially favored because the crystallization condition contains 3 to 3.5 M of NaCl and this fully occupies the ion binding sites at the cleft."

to
"Thus, it is plausible that the current crystallographic study on the zinc-free form may have simply captured the closed conformation-a more favorable conformation than the open conformation in NR2B." at the end of the second paragraph on page 18 of the revised manuscript.

4) We have changed the last sentences of the second paragraph on page 18 from
"Based on our current crystal structure, it appears highly possible that the MTS modification of Tyr 282 perturbs the binding of Na, CI1 and CI2 and destabilizes closed conformation, thereby
shifting the conformational equilibrium of ATD to the open-cleft form and favoring the opening of the ion channels."

to

"Based on our current crystal structure, it appears possible that the MTS modification of Tyr 282 or the Arg292Ala mutation perturbs the architecture around the clamshell cleft and destabilizes the closed conformation, thereby shifting the conformational equilibrium of ATD to the open-cleft form and favoring the opening of the ion channels or the lowering potency of allosteric inhibition. Taken together, the cleft residues at and around the ion binding site are important structural motif that controls the opening and closing of the NR2B ATD clamshell and the ion channel activity."

at the end of the second paragraph on page 19 of the revised manuscript.

Comment 2
Elimination of Zn1 site by mutations leads to a quite modest decrease in the IC50 value (from to). Why is this so and what are the causes of the remaining inhibition by zinc?

Response
We appreciate this important point and agree with Referee2 that it has to be mentioned and discussed. Indeed, the "residual" zinc effect was also observed in Paoletti's study (Rachline et al, 2005) where they mutated H127 in NR2B. Furthermore, a similar effect was observed in the mutational analysis of NR2A by Neyton's group (Fayyazuddin et al, 2000). Thus, as suggested by Neyton, the NR2 subunits contain low affinity zinc inhibition site in addition to high-affinity sites observed in NR2A and NR2B. The exact position of the zinc binding site is not known and is an open question at the moment. To address the point above, the following sentences are added to page 15:

"In all of the cases above, the mutants are only able to shift the IC50 values to 5-6 μM. A similar effect has been observed for the analysis of the high-affinity zinc inhibition site in NR2A where mutations of the binding residues were only able to shift the IC50 values to the low micromolar range (Fayyazuddin et al, 2000). This is most likely attributed to the presence of the low-affinity Zn2+ inhibitory site conserved across the NR2 subtypes as previously suggested by Neyton (Fayyazuddin et al, 2000)."

Comment 3
In Fig. 2, the amino acid residues forming the hydrophilic and hydrophobic pockets and the ion binding site are difficult to distinguish. Alternative coloring or side chain representations might work better. Another Figure issue: Figure 4 (and Fig. 4 legend) seems to lack the color code (for the degree of hydrophobicity).

Response
We agree with Referee 2 that the original Fig. 2 lacks clarity especially at the hydrophilic and hydrophobic pockets, and ion binding site. We have changed the stick representation of the amino acid residues to ball and stick representation so that they are much easier for readers to distinguish. We also added the color code to the legend of Fig. 4 on page 29.

Comment 4
The terms homology and homologous are used incorrectly (p. 4, p. 15) and should be replaced by similarity/similar.

Response
As suggested by Referee 2, we changed homology/homologous to similarity/similar in the text on page 4 and removed homologous from the text on page 15 (page 16 on the revised manuscript).
Comment 5
The authors should consider adherence to the revised nomenclature for ionotropic glutamate receptors (Collingridge et al., 2009, Neuropharmacology 56: 2-5), which is rapidly gaining popularity. Hence, NMDA receptor subunit NR2B should be "GluN2B".

Response
We are also aware of the trend in nomenclature. However, the recently published GluR2 and GluR6 ATDs papers, use conventional nomenclature (GluR). For that reason, we would like to adhere to the 'traditional' nomenclature to be consistent with others. However, we are still flexible to change NR2B to GlnN2B if EMBO Journal prefers to use the nomenclature.

Comment 6
Typos: On page 17, line 7, the word "bind" should be removed; on page 19, line 3, "Van der Waal" should be "van der Waals".

Response
We have fixed the typos on pages 17 and 19.

Referee #3:
The glutamate receptor ion channels which mediate excitatory synaptic transmission in the brain, and NMDA receptors in particular, have received decades of attention due to their role in synaptic plasticity, development and disease. Karakas and colleagues present the first structure of the amino terminal domain of an NMDA receptor subunit, and use this to design mutants for functional analysis to test aspects of allosteric modulation. The work is of significance because, despite a long history of site directed mutagenesis based on models of NMDA receptor ATDs, an experimental structure has never been obtained. ---Although much remains to be done, this paper paves the way for a deeper investigation into analysis of NMDA receptor function, and is a major advance in the field. The structural work is sound, indeed impressive, with experimentally phased maps from 4 heavy atom derivatives used for model building.---The paper is already strong, but there are three areas in which the impact of the study could be substantially increased,

Comment 1
The characteristic features of the NR2A and NR2B ATDs are high affinity modulation by Zn and drugs like ifenprodil respectively. In the present study, somewhat perversely given the unique ligand binding properties of NR2A and NR2B, the low affinity Zn binding site of NR2B is identified, and a mechanism proposed to account for the much higher affinity binding of Zn to NR2A. This could easily be tested, by swapping the NR2A loop containing the proposed additional Zn ligands into NR2B, and or by a small series of point mutants, and then testing either by ITC, or functional assays.

Response
We have made the NR2B mutant construct suggested by Reviewer 3 and measured IC50 for zinc-inhibition. We have only observed little or no change in IC50 (0.6 ± 0.05 µM) compared to the wild type NR2B (IC50 = 0.8 µM). The experiment could mean that there may be a subtle difference in the shape of the binding pocket between NR2A and NR2B subunits. Addition of two histidine residues to bring sub-micro molar affinity to zinc in NR2B to nano molar range observed in NR2A is a challenging task because a slight difference in the residue orientation can interfere with the Zn coordination geometry. Thus, while the experiment shows that there is more causes than two histidine residues (H42 and H44) for the high-affinity zinc inhibition in NR2A, we still think it is reasonable to suggest that the presence of those two histidine residues in NR2A is, at least partly, a factor for mediating zinc inhibition at a nano molar potency. Indeed, the H42A and H44A mutations in NR2A abolish high-affinity zinc inhibition. The first paragraph on page 16 in the revised manuscript was fixed as follow:
...The equivalent region of NR2A has additional two amino acid residues and contains two histidine residues, His 42 and His 44, which are critically involved in zinc sensitivity (Fayyazuddin et al, 2000). Consequently, His 42 and His 44 may be able to position themselves in close proximity to Zn1 and form more ideal coordination along with His 128 (His 127 in NR2B), and Asp 283 (Glu 284 in NR2B). While the architecture of the zinc binding site between NR2A and NR2B may be distinct from each other, we propose that a difference in the number of direct coordination (four for NR2A and two for NR2B) may be one of the factors underlying the large difference in zinc sensitivity between the two subunits.

Comment 2
An unexpected result of the present study is the finding that cations (probably Na) and anions, almost certainly Cl, bind to the NR2B cleft, in between the proposed binding site for ifenprodil and the low affinity Zn binding site. The authors propose that the binding of these ions modulates the open/closed equilibrium of the NR2B ATD. This really needs to be -----. The cation substitution experiments will have the complication that the major permeant ion is being replaced, but prior work has already established that NMDA receptors are non selective, so this is only a minor consideration. If no effects are seen, then it is unlikely that the proposed mechanism of modulation occurs.

Response
We appreciate this important point made by Referee 3. We have conducted the ion replacement experiment suggested above using two-electrode voltage clamp on Xenopus oocytes in the similar ion exchange protocol to the one in Plested et al (2007 and 2008: Mark Mayer's group). In this experiment, we have substituted Na\(^+\) by Rb\(^+\) or Cs\(^+\) and Cl\(^-\) by NO\(_3\)\(^-\), respectively, at 150 mM, and measured the current-voltage (I-V) relationship.

At physiological salt concentration (150 mM), we did not observe any major shift in the slope of I-V plot indicating that the NR2B NMDA receptors are not modulated by physiological concentration of NaCl. Thus, while Na and Cl ions in the crystal structure appear to be a stabilizer in for the closed conformation, the above experiment implies that the ion bindings are not driving forces for the clamshell closure of NR2B ATD at physiological concentration. They are perhaps incorporated into the clamshell cleft during the closure process.

Taken together, we now revise the manuscript not to emphasize Na\(^+\) and Cl\(^-\) binding to be a major factor in inducing the closed cleft conformation. The changes in the manuscript are listed in response to Comment 1 by Referee 2.

Comment 3
The sedimentation properties of the isolated NR2B indicate that it forms monomers, in contrast to the GluR2 and GluR6 ATDs which form dimers, indicating that in intact NMDA receptors, the ATD likely assembles as NR1/NR2 heterodimers. The crystallographic analysis focuses on the conformation of a single NR2B ATD, for which there is one copy in the asymmetric unit, but does not report what packing is observed in the P3(1)21 lattice. This should be reported and discussed in terms of whether there are likely biological assemblies or not.

Response
For referees' evaluation, we included a figure representing the protomer arrangement within the unit cell. On the basis of the crystallographic observation and the analytical ultracentrifugation result that the NR2B ATD proteins exist as monomers in solution, we are certain that the NR2B subunit does not form homodimers and that the contacts observed in the P3\(_1\)21 crystal packing do not represent physiological arrangement. To some degree, we predict that the dimeric arrangement of NR2B ATD retains a feature observed in the dimeric arrangement observed in GluR2 or GluR6 ATD even thought the NR2B ATD and GluR2 or GluR6 ATD structures are fundamentally different. As evident in the attached crystal packing figure, there is no resemblance between the crystal packing of NR2B ATD to the GluR2 or GluR6 ATD dimeric arrangement. To address this point, we have added the following sentences on page 8 to address this point:
"Consistent with the result from sedimentation velocity, there is no apparent evidence for a formation of physiological dimers. This is in contrast to GluR2 and GluR6 ATDs, which form dimers in solution as well as in crystals (Clayton et al, 2009; Jin et al, 2009; Kumar et al, 2009). The packing pattern of the NR2B ATD protomers in the P3\textsubscript{2}1\textsubscript{2}1 crystal does not share any common feature observed in GluR2 ATD or GluR6 ATD."

Comment 4 (Minor Point)

The discussion of the Na binding site needs to be extended to include ligand distances (are these consistent with Na binding) and geometry: Na is usually 6 or 5 fold coordinated. It is confusing that on page 17 the Na ion is identified as Na1, which is unnecessary as there is only a single Na ion identified.

Response

We agree with Referee3 on this point. To address these points, we did the followings:

1) We have included distances between ions and the coordination ligands in Fig. 6B
2) We have changed Na1 to Na on page 17
3) We have added extensive discussion on coordination geometry of Na and Cl on page 18. Specifically, we have added a few sentences on page 18: "The Na ion is located in between Cl1 and Cl2 and is coordinated by main chain oxygen of Ser 131 and Phe 146 with the coordination distance of 2.2 Å and 2.3 Å, respectively (Figure 6B). These values are within an appropriate range of the mean carbonyl-Na$^+$ and carboxylate-Na$^+$ distance observed in the Protein data bank (2.42 Å) (Harding, 2002). Sodium ions are most commonly coordinated by six ligands (Harding, 2002), thus, we anticipate that several water molecules are also involved in the Na$^+$ coordination even though they are not clearly visible in the current structure due to insufficient resolution of the current structures."

Comment 5 (Minor Point)

Page 19 para 2: the discussion of two modes of ifenprodil binding is hard to follow; how does this relate to the interpretation elsewhere that ifenprodil also produces channel block.

Response

To clarify the discussion about the two modes of ifenprodil binding and avoid the confusion with the channel block, we changed the discussion about I133A and I133S mutations on page 20 as follows:

"Consistent with the crystal structure, the Ile133Ala or Ile133Ser mutation has a significant effect on ifenprodil sensitivity (Figure 7B). For both Ile133Ala and Ile133Ser, data points can be fitted to two-site model, but not one-site model Hill equation. It has been shown previously that the NR2B subunit contains two ifenprodil binding sites: a voltage-independent high-affinity site at ATD and voltage-dependent low-affinity site at the ion channel pore (Perin-Dureau et al, 2002; Williams, 1993). Thus, the high- and low-affinity components in the Ile 133 mutants likely represent the ATD-mediated inhibition and the ion channel block present at -20 mV holding potential, respectively (Figure 7B). The efficacy of the ATD-mediated ifenprodil inhibition is 40% of the peak current in these mutants whereas the efficacy is approximately 90% in wild type. Of interest to note is the two modes of ifenprodil binding suggested recently by homology modeling of NR2B ATD and docking of ifenprodil (Mony et al, 2009). One possible explanation for the half reduction of the ATD-mediated inhibition is that the mutation may have hampered one of the two binding modes."

Comment 6 (Minor Point)

For the most part the paper is well written, but a few typos need correction,

Page 2 line 4 change to: ... of NMDA receptors is that their ion channel ...
Page 3 line 3 delete 'the': ... of L-glutamate from nerve terminals ...
Page 3 line 4 from end delete 'the': ... The opening of NMDA receptor ion channels Page 4 line 6 from end insert 'the': ... small ligands that bind the ATD of ...
Page 7 line 1 insert 'an' ... structure of an NMDA receptor ATD ...
Page 10 line 3: the GluR6 ATD reference should be Kumar not Clayton Page 11 line 4 change dimer to homo dimer Page 11 para 2 missing The: The NR2B ATD also has ...
Page 17 line 7 where bind Na ions ... should read ... where bound Na ions

Response
We truly thank Referee 3 for pointing out our grammatical mistakes and typos. We have fixed the text accordingly.

Other minor changes

1) We have changed the numbering of supplementary figures (S2 to S3 and S3 to S2) to improve the flow of the manuscript.

2) In the original manuscript, 3\textsubscript{10} helices have not been displayed in all of the structural figures. We have now included the structural motifs and labeled them as $\eta_1$ and $\eta_2$ in all of the figures to improve the accuracy of the information included in the current manuscript. There is no change in the interpretation of structure-function relationship.

Referee comments
20 October 2009

Referee #3 (Remarks to the Author):
The revised paper addresses all of the issues raised in my review. The study is an important advance in the field and will likely be highly cited.