Published in final edited form as:

Proteins. 2018 December ; 86(12): 1265–1276. doi:10.1002/prot.25595.

The GluN2B-Glu413Gly NMDA receptor variant arising from a *de novo* *GRIN2B* mutation promotes ligand-unbinding and domain opening

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Abstract

N-methyl-*D*-aspartate (NMDA) receptors are transmembrane glutamate-binding ion channels that mediate neurotransmission in mammals. NMDA receptor subunits are tetrameric complexes of GluN1 and GluN2A-D subunits, encoded by the *GRIN* gene family. Of these subunits, GluN2B is suggested to be required for normal development of the central nervous system. A mutation identified in a patient with developmental delay, E413G, resides in the GluN2B ligand-binding domain and substantially reduces glutamate potency by an unknown mechanism. GluN2B Gly413, though near the agonist, is not in van der Waals contact with glutamate. Visual analysis of the GluN2B structure with the E413G mutation modelled suggests that replacement of Glu with Gly at this position increases solvent access to the ligand-binding domain. This was confirmed by molecular modeling, which showed that the ligand is more mobile in GluN2B-E413G than WT GluN2B. Evaluation of agonist occupancy using random accelerated molecular dynamics (RAMD) simulations predicts that the glutamate exits the binding-site more rapidly for GluN2B-E413G than WT receptors. This analysis was extended to other binding-site mutations, which produced qualitative agreement between experimentally determined EC₅₀ values, deactivation time constants, and ligand motion within the binding-site. Furthermore, long sub-microsecond molecular dynamics simulations of the bi-lobed ligand-binding domain revealed that it adopted a cleft-open ligand-free state more often for GluN2B-E413G than wild-type GluN2B. This is consistent with the idea that L-glutamate binding is altered such that the ligand-binding domain occupies the open-cleft conformation associated with the closed channel.

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Competing Interests

SFT is a consultant for Janssen Pharmaceuticals Inc., is a member of the SAB for Sage Therapeutics, and is co-founder of NeurOp Inc. DCL is a member of the Board of Directors for NeurOp Inc. DCL, JPS, and SFT, and are co-inventors on Emory-owned intellectual property associated with allosteric modulators of NMDAR function.

Keywords

Ligand egress; *N*-methyl-*D*-aspartate; RAMD; Clam-shell; missense mutation

Introduction

Neurotransmission is mediated by the extracellular release of chemical neurotransmitters at the synaptic cleft between neurons. The amino acid L-glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system (CNS) and activates ligand-gated ionotropic receptors in the post-synaptic cell. Ionotropic glutamate receptors can be divided into three types by pharmacology and structure: kainate, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic-acid (AMPA), and *N*-methyl-*D*-aspartate receptors (NMDA-Rs) ¹. NMDA-Rs mediate a slow, Ca²⁺-permeable component of excitatory synaptic transmission that is important for normal brain development, synaptic plasticity, and long-term memory formation ¹. Furthermore, dysfunctional NMDA-R's have been implicated in a number of neuropathological conditions, including ischemia-induced cell death (Table S2 in Yuan *et al.* ²), depression ³, Parkinson's disease ⁴, schizophrenia ^{5,6} and neuropathic pain ⁷. Modulation of NMDA-Rs is therefore considered to be a therapeutically relevant strategy for treating these neurological conditions ⁸.

NMDA-Rs are hetero-tetrameric proteins composed of two glycine-binding GluN1 subunits and two glutamate-binding GluN2 subunits. There are 8 splice variants of the GluN1 subunit and four different GluN2 subunits (*A-D*) ¹. Variation in subunit composition results in NMDA-Rs with different characteristics for ligand binding, allosteric modulation, channel opening, and spatial and temporal expression in the CNS ⁹. Moreover, NMDA-Rs containing GluN2B, have been implicated in normal development of the mammalian brain ^{10–12}.

The architecture of a single NMDA receptor subunit comprises four semi-autonomous domains, including the amino terminal domain (ATD), agonist-binding domain (ABD), trans-membrane domain (TMD) and C-terminal domain (CTD) ¹³. Crystallographic have provided near-complete structures of tetrameric receptors lacking the CTD for the GluA2 AMPA receptor ^{13–15} and GluN1/GluN2B NMDA receptors ^{16–20}. The ATD of NMDA-Rs harbors multiple allosteric modulatory sites, in addition to controlling many receptor properties ^{21–23}. The neurotransmitters glutamate and glycine bind within the cleft of the bi-lobed or clam-shell-like ABD. Upon agonist binding, the clam-shell closes, which consequently leads to opening of the ion channel ^{24,25}. The ABD is formed by two separate portions of the polypeptide chain (termed S1 and S2) separated by part of the TMD. The TMD comprises three membrane spanning alpha-helices and a re-entrant loop on the cytoplasmic leaf of the plasma membrane.

Recently, a large number of disease-associated *de novo* mutations and rare variants have been identified ^{26–30}. Here we evaluate the structural basis by which a missense mutation (E413G) in *GRIN2B* in a patient with developmental delay reduces glutamate potency by 50-fold ³¹. Whole exome sequencing revealed the patient was heterozygous for this *de novo* mutation in the ligand-binding domain of GluN2B. We use molecular modeling and dynamics to gain insight into the effect of this amino acid substitution on structural basis of

agonist binding. We also modelled glutamate egress from the ligand binding domain via Random Accelerated Molecular Dynamics to explore the effect of multiple mutations in the ligand binding site.

Methods

Homology modeling

Homology models of the GluN1/GluN2B agonist binding domain dimer were generated with the GluN1/GluN2A ABD structure (PDB code 2A5T) as a template. During the preparation of this manuscript for publication, three full-length structures for GluN1/GluN2B became available^{16,17}. The ABD from the structure of Karakas *et al.*¹⁷ had an RMSD of < 1 Å with the homology model developed here, while the C_α RMSD of the ABD from structures of Lee *et al.*¹⁶ was similar for both our model and the structure of Karakas (1.7 – 1.8 Å). Due to the high similarity of the model built from 2A5T with the low resolution (3 – 4 Å), full length structures, we used the model as an appropriate starting point for these studies. The comparative modeling package Prime (Schrödinger)³² was used, as previously described^{33,34}. When aligned, the agonist binding domain of GluN2A shows 86% sequence identity and 92% sequence similarity with GluN2B. The GluN1/GluN2B interface was subjected to multiple rounds of side chain optimization and energy minimization using Prime (Schrödinger) to alleviate any strain introduced by homology modeling. Analysis with PROCHECK³⁵ revealed little difference in the overall G-factors between the crystal structure (0.34) and that of the GluN1/GluN2B (0.28) homology model; scores above –0.5 are considered acceptable.

Molecular dynamics

The molecular visualization program VMD 1.9³⁶ was used to prepare the system for molecular dynamics in NAMD³⁷. Hydrogen atoms were added with the Automatic PSF (AUTOPSF) Builder module. The GluN2B-E413G mutant with 6 alternative conformations and protonation states of His486 in the binding-site were prepared in VMD. Internal water molecules were placed using the dowser module of VMD. Visual inspection was used to retain water molecules in common with existing GluN2A structures if they overlapped by visual inspection (PDB ids: 2A5T, 2A5S), as well as with GluN2D structures (PDB ids: 30EK, 30EL, 30EM, 30EN). Dowser-placed waters that did not correspond with crystal structure waters were deleted. The waters within the agonist binding site were represented in all structures, except for one water in 30EM, which was excluded by the methyl moiety of *N*-methyl-*D*-aspartate. The remaining surface-positioned waters corresponded with a water in at least one of the structures, and were nonetheless freely exchangeable with the bulk solvent.

The system was parameterized with the CHARMM 27 force field³⁸ including CMAP corrections³⁹. Solvation by the TIP3 water model was completed using the AUTOPSF with a 12 Å padding to create an orthorhombic cell (± 82 Å $\times$ ± 69 Å $\times$ ± 78 Å). The AUTOIONIZE module was used to neutralize the system with 0.15 M NaCl. Ionizable residues (Asp/Glu/Lys/Arg) were prepared in their charged states and His residues (besides H486) were assumed to be in the neutral N_ϵ -protonated form. A cut-off of 12 Å was used for non-

bonded interactions with a switching function applied from 10-12 Å. Non-bonded interactions between atoms connected by three or fewer covalent bonds were excluded (with default scaling for 1-4 interactions). Periodic boundary conditions were simulated with Particle Mesh Ewald Sums for long-range electrostatic interactions using a grid spacing of 1 Å.

The solvent was first minimized for 1000 steps followed by minimization of the entire system for 50 steps. The solvent was then relaxed using molecular dynamics for 150 ps with 1 fs time-steps. The system was heated from 60 K to physiological temperature of 310 K in 5 K steps with velocity reassignment according to the Boltzmann distribution every 1 ps. A similar protocol was then used to relax the entire system for 500 ps from 10 K to 310 K. Multiple time stepping was used for force evaluation, with short-range interactions evaluated every step and full electrostatic interactions performed every two steps. A Langevin barostat was used to maintain pressure at 1.01325 bar with a period of 250 fs and a decay of 50 fs.

Data collection was carried out in the NPT ensemble at 310 K using a Langevin thermostat with a damping constant of 5, and at 1.01325 bar using a Langevin barostat with a decay of 100 and a period of 200. A time-step of 2 fs was used. Frames were collected every 2 ps (1000 steps).

Solvent accessibility

Based on the results of the simulations described above, the τ tautomer of His486 with a $\chi_2 > 0$ initial conformation was used to compare solvent accessibility of glutamate in the wild-type and mutant ABD. The G_SAS module of GROMACS^{40,41} was used to measure the solvent accessible surface area (SASA) of all protein residues across 100 ns.

In order to better visualize solvent accessibility in the binding pocket, CAVER⁴² was used to explore solvent access for the *apo* form of the protein. Snapshots of the protein from every 0.1 ns were used to explore the exit of a water-sized probe (1.4 Å) from within the ligand binding-site. As starting coordinates for tunnel prediction, the average of the C_α atom of the ligand over the entire trajectory was used.

Ligand egress with Random Accelerated MD

In order to simulate the effects of E413G on the unbinding of glutamate from GluN2B, the system was modeled with Random Accelerated Molecular Dynamics (RAMD, originally known as Random Expulsion Molecular Dynamics)⁴³. In the original implementation of this protocol, a random acceleration is applied to the ligand for a specified block of simulation time. If the ligand fails to move a minimum distance within that time, a new direction is randomly chosen. This technique was originally devised to explore long ligand access in tunnels with deeply buried active sites in cytochrome P450 in a tractable amount of simulation time^{43,44}. By contrast, in the present study a new direction for the acceleration was chosen for each RAMD time-block, since the binding-site of glutamate in GluN2B is located very near the surface. This strategy was chosen to enable a more thorough exploration of ligand egress from the binding-site. The RAMD simulations ended when the ligand was 10 Å from the protein center-of-mass (COM).

A number of parameters were optimized in order to use RAMD as a reliable tool for distinguishing the effect of the mutant vs the wild-type protein. These included the magnitude of ligand acceleration, enforced randomization of the acceleration vector, and starting co-ordinates of the protein/ligand complex. The results of this optimization are discussed in the Supporting Information.

The following protocol was chosen for RAMD on glutamate; 30 different simulations were initiated from a random snapshot of non-accelerated MD (10-20 ns) of the protein/ligand complex. Different initial coordinates were used to reduce any bias that could be introduced by a starting conformation more propitious for ligand egress. A constant acceleration of $0.225 \text{ kcal.}\text{\AA}.\text{amu.mol}^{-1}$ was applied to all atoms of the ligand, with a new direction randomly chosen every 0.2 ps (100 steps). Each simulation was run until the ligand was at least 10 Å from the protein center of mass. Such MD runs, took 1-10 ns and seldom exceeded 20 ns.

A similar RAMD protocol was also used to explore the egress of the cyclic glutamate derivative (*2S, 4S*)-2,4-piperidinedicarboxylate (PPC). Lower accelerations of $0.191 \text{ kcal.}\text{\AA}.\text{amu.mol}^{-1}$ PPC, and a 15 Å COM cutoff were used to correct for the larger momentum and size of the ligand compared to glutamate. General CHARMM force field parameters for the ligands were assigned by analogy using the Paramchem website^{38,45-47}.

Site-directed mutagenesis and two electrode voltage clamp recording

Site-directed mutagenesis was performed using the QuikChange protocol with Pfu DNA polymerase (Stratagene, La Jolla, CA, USA) on rat GluN2B (GenBank accession code: [U11419](#)) as previously described⁴⁸. The mutations were verified by sequencing through the region of the mutations. *Xenopus laevis* oocytes were obtained from Ecocyte (Austin, TX). Preparation and injection RNAs into oocytes as well as two-electrode voltage-clamp recordings were performed at room temperature (23 °C), as previously described⁴⁸. Briefly, oocytes were injected with 50 nl of water that contained 5–10 ng of cRNAs synthesized *in vitro* from linearized template cDNA at a ratio of 1:2 for GluN1:GluN2B (Genbank [U08261](#), [U11419](#)). Recordings were made 2–4 days after injection in an external solution that contained (in mM) 90 NaCl, 10 HEPES, 1 KCl, 0.5 BaCl₂ and 0.01 EDTA at pH 7.4. Voltage electrodes were filled with 0.3 M KCl, and current electrodes contained 3 M KCl. Recording solutions were prepared in external solution and contained glycine (30 μM) and glutamate (0.1–100 μM).

Whole cell voltage clamp recording from transfected HEK cells

HEK-293 cells (ATCC CRL 1573, Rockville, MD) were maintained at 37°C in humidified 5% CO₂ in DMEM with GlutaMax (Invitrogen, Carlsbad, CA), supplemented with 10% dialyzed fetal bovine serum, 10 μg/ml streptomycin, and 10 units/ml penicillin (see Yuan et al., 2009). Cells were removed from coverslips by treatment with trypsin, and subsequently re-plated in 0.5 ml per on glass coverslips that had been placed in 24 well plate (Warner Instruments, Hamden, CT); coverslips were coated with poly-D-lysine at 10 μg/ml. HEK cells were transiently transfected with NMDAR subunit cDNAs GluN1 and GluN2 plus GFP at a ratio of 1:1:1 using the calcium phosphate precipitation method (0.2 mg/mL total

cDNA). 200 μ M DL-AP-5 and 200 μ M 7-chlorokynurenic acid were included in the culture media to prevent excessive NMDAR activation and excitotoxic cell death. Following transfection, coverslips were transferred to a recording chamber and continuously perfused at 2 mL/min with the recording solution that contained (in mM) 150 NaCl, 3 KCl, 1 CaCl_2 , 10 HEPES, 10 D-mannitol with pH adjusted to 7.4 by addition of NaOH. Microelectrodes were made from thin-walled filamented borosilicate glass (World Precision Instruments catalog TW150F-4) and filled with the internal recording solution that contained (in mM) 110 D-gluconate, 110 CsOH, 30 CsCl, 5 HEPES, 4 NaCl, 0.5 CaCl_2 , 2 MgCl_2 , 5 BAPTA, 2 NaATP, and 0.3 NaGTP (pH 7.35). Pipettes filled with internal solution had resistances of 3–4 M Ω . Whole cell current recordings were made with the membrane potential held at -60 mV using an Axopatch 200B patch-clamp amplifier (Molecular Devices), and NMDAR current responses to brief (5 ms) or prolonged (1 sec) rapid external application of glutamate (1 mM) and glycine (30 μ M) were recorded at room temperature (23°C), filtered at 8 kHz (-3 dB, 8 pole Bessel filter,) and digitized at 20 kHz using a Digidata 1440A data acquisition system (Molecular Devices) controlled by Clampex 10.3 (Molecular Devices). Open tip junction potential currents were used to confirm the duration of the brief application of agonist. The current response deactivation time course was fitted to the sum of 1 or 2 exponential functions using non-linear least squares algorithms in ChanneLab (Synptosoft).

Results

Determination of the His486 orientation

Figure 1 shows the position of the bilobed agonist binding domain (ABD) within the tetrameric GluN1/GluN2B receptor complex^{16,17}. The agonist glutamate binds between an upper (D1) and lower (D2) lobe of the ABD, and occupancy of this site by agonist produces considerable closure of the two lobes⁴⁹. The binding site residues His486 and Glu413 are positioned near the agonist glutamate and each other. This prompted separate simulations to predict the correct protonation state and rotamer for His486, which could not be determined from existing crystallographic data. Neutral histidine is in equilibrium between two tautomers, alternating between protonation of N_ϵ and N_δ . According to IUPAC recommendations, these protonation states are designated as τ (“tele”/far) and π (“pros”/near) for N_ϵ and N_δ , respectively (Fig 1)⁵⁰. It is currently not possible to simulate proton transfer in popular MD software. Therefore, it was reasoned that simulation of all three protonation states to find the most stable combination with the two initial His486 starting rotamers would reveal the best option to use in further simulations. Substantial differences were observed in the mobility of His486 that were dependent on protonation state (N_T/N_π tautomerization or double protonation) and the starting conformation of the χ_2 -dihedral (Fig 2 a–b). With reference to individual torsion plots of Fig 2 (-180° - 180°), the $\chi_2 > 0$ starting conformation is mostly retained in both wild-type and E413G simulations for the $N_T(N_\epsilon)$ tautomer, and to a lesser extent for doubly protonated wild-type and E413G-mutant His486. However, the $N_\pi(N_\delta)$ tautomer is more mobile around χ_2 for approximately the latter half of 100 ns in both wild-type and mutant. When the starting conformation for χ_2 is < 0 , there is also increased mobility around this dihedral. In some of the simulations, His486 flips over and occupies the $\chi_2 > 0$ conformation for 30%-70% of the latter portion of 100 ns of MD

(e.g. $N_{T\text{wild-type}}$, N_T mutant, double-protonated wild-type). In those cases of $\chi_2 > 0$ simulations, N_π protonation conferred more side-chain mobility around the χ_2 dihedral. For the most part, mobility around the χ_1 dihedral was stable (Fig S1), except for the $N_T(N_e)$ tautomer in the $\chi_2 < 0$ initial conformation, which “swung out” into the solvent for a total of about 60 ns. The atomic contacts between His486 and the agonist glutamate remain the same between the different protonation and rotamer states, and the imidazole ring makes van der Waals contacts with the aliphatic backbone of glutamate. What differs are the potential interactions with Glu413, Ser512, Tyr731, as well as the waters near the binding-site cavity, which may in turn alter ligand binding. Specifically, in the $\chi_2 > 0$ conformation, the N_T tautomer of His486 can interact with Tyr731 via hydrogen bonding, or with Ser512 in the $\chi_2 < 0$ conformation (Fig 1), possibly contributing to the higher stability of this conformation and protonation state. In both conformations His586 can hydrogen-bond with Glu413 on alternating oxygens of the carboxyl side-chain.

Solvent accessibility of L-glutamate in WT and mutant

Because of the close proximity of GluN2B-E413G to the ligand binding-site, the ease with which glutamate could exit was investigated. Specifically, the effects of mutating this residue on solvent accessibility of the bound ligand were modeled. For the most stable state of His486 (N_{TH} , $\chi_2 > 0$), a greater average glutamate solvent accessible surface area of 17.8 Å² was found for ABD-bound glutamate in GluN2B-E413G compared to 14.0 Å² for the wild-type GluN2B. This suggests that L-glutamate can escape the binding site more easily in GluN2B-E413G, an idea consistent with the prediction that more exit paths exist for water from the binding site for GluN2B-E413G than WT GluN2B⁵¹. The increased solvent accessible surface will also increase water-agonist collisions, and possibly enhance water’s ability to compete for atomic contacts.

Ligand egress with Random Accelerated MD

During unbiased MD, it was not possible to observe large macroscopic differences between glutamate-bound GluN2B-E413G and wild-type GluN2B due to the difference in time scales on which the simulations could practically be run, and the time course with which agonists bind and unbind. However, when a small, randomly-directed acceleration was applied to the agonist glutamate, it was possible to observe its exit from the binding-site on a timescale that was compatible with MD simulations (ps - ns). We evaluated this approach for a range of acceleration values, choosing 0.225 kcal.Å.amu.mol⁻¹ as an acceleration that allowed completion of the simulations without distorting protein structure (See Supplementary Information, Table S1). The average ligand exit time from independent simulations was about 50% less in GluN2B-E413G compared to wild-type GluN2B (Fig 3, Table S2; see Supplemental Information). A decreased exit time for E413G from its binding site was observed consistently during evaluation of different protocols for the application of a randomly-directed acceleration to molecular dynamics simulation (RAMD), which varied the magnitude and direction of the acceleration vector. As expected, average exit time increased (i.e. residence time within the binding-site increased) with decreased acceleration. The reduced exit time from GluN2B-E413G is consistent with the 50-fold decrease in potency (EC₅₀) of glutamate³¹. This is also compatible with the 20-fold faster deactivation

rate⁵¹ for GluN2B-E413G compared to WT, since an increased unbinding rate appears to accompany reduced potency of NMDA receptors^{52,53}.

In the closed clam-shell agonist-bound conformation, glutamate contacts both lobes of the closed ABD, as shown in Figure 1. The α -carboxyl forms a salt-bridge with the side-chain guanidinium of Arg519 in D1, and the charged α -amino group hydrogen bonds with Ser512 (D1) and Asp732 (D2). In addition, the γ -carboxyl of the side-chain forms a hydrogen bond with Thr691 (D2) and a water-mediated interaction with Glu692. These residues comprise the *N*-terminus of α -helix E (Fig. S2)^{17,18}. Because α -helices have a net dipole moment, with the *N*-terminus being positively charged ($\sim +0.5$)⁵⁴, this set of interactions can be considered a weak salt bridge between the side-chain carboxyl and the *N*-terminus of helix E.

During simulated agonist unbinding and egress, the side-chain γ -carboxyl of the agonist glutamate first detaches from the *N*-terminus of helix E. In the case of GluN2B-E413G, a large void is present that can be occupied by the ligand side-chain, allowing for substantially reorganized ligand poses. This is also possible in the wild-type when the side-chain of Glu413 points into solvent, however, the latter moiety mostly interacts with His486 which minimizes detachment of the ligand (video S1)

Correlation between agonist potency and simulated egress

To further evaluate agonist unbinding and egress, additional GluN2B binding-site mutations were made and their effects on both the time course of glutamate deactivation and glutamate potency were assessed. We generated concentration-response curves for the GluN2B mutations E413G/Q/D/N/A, and H486G/F. These mutations all reduced glutamate potency at GluN1/GluN2B receptors and accelerated the deactivation time course (Table 1). We evaluated their potential effects on receptor function by performing RAMD simulations of ligand egress using the same method described above. For all mutations, there was a decrease in mean exit time relative to the simulated wild-type. There was a modest inverse correlation between mean exit time and measured $\log(\text{EC}_{50})$ ($R^2 -0.53$, $p < 0.04$, Fig. 4). In addition, we found an apparent direct correlation ($R^2 0.49$, $p < 0.06$) between ligand egress and the fastest component of the deactivation time constant, which is less likely to be influenced by entry into and exit from desensitized states. A relationship between ligand egress and tau deactivation became more apparent when the His486 mutations were analyzed independently from the Glu413 mutations (Fig 4). The fastest time constant describing deactivation and ligand egress showed an apparent linear relationship with an R^2 value of 0.99. The $\log(\text{EC}_{50})$ plotted as a function of mean exit time for His486 had an R^2 value of 0.91.

We found some mutations had similar effects on ligand egress and agonist potency. The iso-steric mutation GluN2B-E413Q increased EC_{50} by 16-fold (i.e. decreased potency), and modestly accelerated egress. Surprisingly, GluN2B-E413N/D increased EC_{50} more than GluN2B-E413G (128- and 78-fold, respectively). This result was consistent with RAMD for GluN2B-E413D, which predicted a shorter egress time (0.4 ns) compared to GluN2B-E413G (3.5 ns). By contrast, RAMD predicted a similar egress time for GluN2B-E413G (4.3 ns) and wild-type (6.8 ns) for GluN2B-E413N, despite large differences in potency. We

also found, as expected^{55,56}, that mutations at His486 had dramatic effects on glutamate potency and accelerated ligand egress time from the cleft. A large effect was observed for GluN2B-H486G with > 900-fold change in EC₅₀ and an exit time of 1.9 ns.

We next compared a measure of agonist flexibility, average RMSD for non-oxygen heavy atoms, to the fold shift in EC₅₀ for mutant receptors. Similar to egress time, there was a weak inverse correlation between RMSD and EC₅₀ ($R^2 = -0.26$). For each run, the RMSD for all ligand poses within 6.5 Å of the protein center of mass were determined relative to the first frame of the production run, and then averaged for the ensemble of runs (Table S2). Some mutations did not correlate with potency. H486F and H486G were predicted to slightly lower average agonist RMSD compared to the wild-type, although the average egress time was less in both. Potency (EC₅₀), deactivation rate, and chain flexibility are a function of many atomic-level properties, including microscopic agonist dissociation- and association-rates, ligand flexibility, and ligand binding domain conformation. Thus, these simulations are not expected to completely capture the full effect of mutations in the ligand-binding domain, which accounts for the weak correlations we observed.

We also evaluated the egress times for the conformationally restricted ligand (2*S*,4*S*)-2,4-piperidinedicarboxylate (PPDC). The egress time for PPDC was substantially shorter in the wild-type (1.44 ns) compared to E413G (3.93 ns).

ABD clam-shell dynamics

When the effect of GluN2B-E413G was modeled, it was expected that glutamate would unbind more rapidly in the mutant protein. However, the initial simulations with ligand showed no noticeable effect on opening of the bi-lobed clam-shell-like ABD within 100 ns. This was observed for all His486 states in wild-type and GluN2B-E413G. Bridging by L-glutamate between the two lobes of the clam-shell may have prevented opening during the time available for unbiased MD. Therefore, in addition to RAMD, we extended unbiased simulations of GluN2B-E413G and wild-type GluN2B in the *apo* state to assess whether clam-shell opening occurs faster without glutamate, and is altered by the mutation GluN2B-E413G. Three independent 200 ns *apo* simulations (N_{TH} , $C_2 > 0$) for each of the wild-type and E413G were observed to adopt open conformations of the “clam-shell”. Domain opening was represented by the distance between C_α of His486 of D1 and C_α of Val714 in D2 (Fig. 5). In the simulations of the closed, ligand-bound state, this distance varies between 13-15 Å, similar to the agonist-bound crystal structures of GluN2A and GluN2D^{25,52,57} in the *apo* simulations, this distance increases to 21 Å.

To simulate an intermediate state between the normal ligand-bound state and clam-shell opening, the effect of partially unbound glutamate was simulated without RAMD. From the E413G RAMD simulations, a frame was chosen with the γ -carboxyl of glutamate detached from helix E of D1 to initiate a long unbiased simulation. The mutant was chosen to increase the chance of observing ligand egress by limiting rebinding of the ligand in the more confined binding site of the wild-type. During 550 ns, a similar degree of clam-shell opening was observed compared to the *apo* simulations (Fig 6a). Furthermore, the ligand moved out of the binding-site into the surrounding solvent (Fig 6b). This suggests that the key event

leading to ligand unbinding and clam-shell opening is detachment of the γ -carboxyl from helix E.

From this, it is concluded that ligand binding, with interactions formed between both lobes, hampers the frequent observation of ligand unbinding during unbiased MD in a tractable amount of time. However, from a sufficiently unbound state in GluN2B-E413G, clam-shell opening and ligand egress was observed in one simulation. Therefore, the faster egress time for GluN2B-E413G predicted by RAMD suggests that mutations which facilitate ligand detachment which leads to increased clam-shell opening manifested as lower potency.

Discussion

The GluN2B-E413G mutation observed in a heterozygous patient exhibiting developmental delay strongly reduced the glutamate potency of GluN1/GluN2B by an unknown mechanism³¹. The proximity of this mutation to the glutamate binding-site suggests that mutations at Glu413 could alter agonist association and dissociation, and hence the synaptic NMDA-R response time course in patients harboring this mutation. Here we perform functional analysis of various mutations at or near Glu413, and evaluate atomic level molecular simulations to determine the mechanism underlying the functional effects of the *de novo* GRIN2B mutation on agonist potency, giving rise to GluN2B-E413G. Evaluation of egress time was consistent with the prediction that substitution at this position increases flexibility and solvent access, which contribute to agonist dissociation. We show that E413G accelerates glutamate unbinding in RAMD simulations, consistent with accelerated dissociation reported⁵¹. Furthermore, mutations that did not create additional tunnels for water to reach the cleft (H486F, E413Q) still reduced ligand potency by increasing the freedom of movement of ligand within the binding-site. These results suggest multiple effects of substitutions at Glu413 and His486 influence the stability of the agonist-bound state.

To test whether the effect of E413G also involved increased ligand flexibility, a conformationally restricted ligand ((2*S*,4*S*)-2,4-piperidinedicarboxylate) was modeled to replicate the key interactions in the closed ligand-binding domain. This ligand also egresses slower in the mutant simulations, suggesting that extra agonist rigidity compared to L-glutamate can oppose the effect of E413G. Finally, long simulations of the L-glutamate ligand-binding domain exhibited opening of the clam-shell that is expected upon channel closure, but has yet to be observed experimentally in a crystal structure for GluN2B.

Orientation of His486

Within the glutamate binding site, His486 is predicted to interact directly with Glu413. However, histidine has a pKa of 6.5 near physiological pH of 7.3, and thus, a significant fraction of both protonated (14%) and neutral forms (86%) of the histidine residues may be present in a cellular population of proteins provided the pKa is not shifted by local intra-protein interactions. Additionally, the similar X-ray-determined electron densities of carbon and nitrogen make it difficult to distinguish between the alternative side-chain rotamers. In typical molecular dynamics simulations, the bond connectivity is fixed, therefore protons cannot exchange. It was not possible to consistently predict His486 protonation by

optimization of the hydrogen-bond network in existing NMDA-R ABD structures. Therefore, since His486 interacts directly with a key residue of interest (Glu413) and with the ligand, we pursued a more thorough investigation to determine the correct orientation and protonation of His486. Both neutral tautomers and the rare doubly protonated state were simulated. Additionally, both possible side-chain rotamers were used to initiate simulations (differing by the χ_2 dihedral angle, designated $\chi_2 > 0$ and $\chi_2 < 0$, Fig. 1). From these twelve simulations, His486 was observed to settle on $\chi_2 > 0$ when initiated with $\chi_2 < 0$, and protonation of N_δ destabilizes His486. Therefore, a positive torsion angle with N_ϵ protonated was chosen for solvent accessibility predictions and further simulations (Fig. 1b).

Glutamate egress from the ABD

The GluN2B E413G mutation introduces a substantial void in the binding-site that could alter the agonist conformation, allowing additional conformations to be accessed while bound. In addition, this void increases solvent accessibility, allowing additional solvent-protein and solvent-agonist collisions. We hypothesized that both the ability of the ligand to adopt different conformations and the ability of water to enter this void accelerates the dissociation of atomic contacts from the pocket, until the ligand begins to undergo motion that leads to unbinding. During long unbiased (100 ns) MD runs, there were no observable effects on glutamate binding between the E413G mutant and the wild-type receptor; glutamate remained bound in all unbiased simulations. However, by applying a small acceleration to the glutamate ligand, it was possible to observe its exit from the binding-site over a pico- to nano-second timescale. Furthermore, with multiple trajectories of independent runs, the average exit time for the ligand was ~ 2 -fold faster for GluN2B-E413G than in the wild-type. This was observed for different acceleration and initial conditions (see Methods and Supplementary information)

The exit time for glutamate from several GluN2B-E413 mutations is faster than from wild-type GluN2B. While mutations that permit faster exit from the binding-site are expected to lower EC_{50} , the weak correlation indicate that other ligand-binding conditions need to be satisfied compatible with channel opening. Specifically, the mutations tested could disrupt or weaken the interaction between Glu413 and His486, which could result in the formation of a transient void that favors ligand unbinding, similar to GluN2B-E413G. These mutations are subtle compared to the large difference introduced by E413G, making it harder to capture the effects by RAMD. Furthermore, this method only measures exit-times as a proxy for the microscopic dissociation rate k_{off} , and does not consider other contributions to ligand binding such as the association rate k_{on} or increased affinity due to altered occupancy of a desensitized state.

When bound in the closed clam-shell conformation, glutamate makes non-covalent contacts with both D1 and D2 domains of the clam-shell. Thus, broken contacts with either of these domains may compromise channel gating by allowing the clam-shell to open. While decreased channel gating is not necessarily reflected by ligand egress times, it is suggested that compromised binding, nonetheless, allows the clam-shell to open and induce channel closure prior to egress (Fig 6e).

RAMD was initially devised to explore channels and ligand pathways where the binding site is deeply buried and possible access routes are not obvious^{43,58}. In the case of the ligand-binding domain of GluN2B, the ligand is located very near to the protein surface, and there is little doubt about the general direction of ligand access. Nonetheless, as the ligand did not egress during unbiased MD, we evaluated whether modified RAMD could be useful in exploring the effect the GluN2B-E413G and other mutations on ligand egress. During channel-exploration MD, it is normal to adjust the amount of acceleration on the ligand to a value that is appropriate for the ligand and protein^{59–63}. This was also the case for glutamate egress from GluN2B. Additionally, because the ligand is near the protein surface, random direction changes were applied to the acceleration to better approximate a random-walk, and thus better explore the effects of ligand-binding domain mutations. In normal RAMD, once the ligand encounters no resistance from the protein, it will traverse in a straight line away from the protein center of mass. To further improve the sampling, different initial conformations of the protein/ligand complex were used to initiate RAMD. This was observed to give better qualitative agreement with EC₅₀ values of the various mutations to Glu413 (see Supplemental Information). These results suggest that refinements to this methodology would be useful for studying ligand unbinding in other systems.

To test the hypothesis that GluN2B-E413G compromises receptor function by increasing the rate of glutamate egress, a conformationally-restricted ligand was simulated that was surmised to reside longer in the mutant binding-site. Visual inspection of the binding pose of glutamate suggested it would be straightforward to extend glutamate into a cyclic derivative by addition of methylene groups to give rise to (2*S*,4*S*)-2,4-piperidinedicarboxylate (Fig S1). The extra atoms can potentially take advantage of the void left by E413G in the mutant protein. Furthermore, the flexibility of the (former) gamma-carboxyl is reduced by this extension, enabling the ligand to maintain the bridging interaction between D1 and D2 of the ligand-binding domain.

ABD clam-shell dynamics

Previous structural studies suggest that glutamate binding mediates channel opening by inducing closure of the ligand-binding domain “clam-shell” structure. Conversely, ligand unbinding induces clam-shell opening and channel closure. In order to determine whether the model system used in this study can capture this behavior of the bilobed ABD, ligand-free wild-type and E413G GluN2B were simulated. In the absence of ligand, a more open conformation of the clam-shell was favored during molecular dynamics, for both E413G and wild-type. As a further test of whether partial ligand unbinding promotes clam-shell opening, a simulation with the gamma carboxyl of L-glutamate detached from helix E was used to initiate a long simulation without acceleration of the ligand. During 350 ns, a similar degree of clam-shell opening was observed (Fig 5). In combination with faster ligand egress predicted by RAMD, this suggests that E413G and other mutants compromise channel function by enabling channel closure by their rapid dissociation.

Previously reported attempts to simulate the apo-state of the ABD from glutamate receptors have observed a modest degree of clam-shell opening^{64–66,67}. To our knowledge, the largest degree of clam-shell opening in an unbiased simulation is that of Liu and Tang⁶⁸ where a

similar degree of opening is induced by Xenon binding. While it was not possible to observe clam-shell opening from a fully closed, ligand-bound state, it was possible to simulate ligand egress and clam-shell opening from an intermediate state on a sub-microsecond timescale. The degree of simulated clam-shell opening was considerably larger compared to existing experimental structures of the ABD. While there are still no structures for the apo-form of NR2x, a 17.1 Å opening has been observed for NR2A when bound by the inhibitor (2S, 3R)-1-(phenanthren-2-ylcarbonyl)piparazine-2,3-dicarboxylic acid ¹⁸. The larger degree of opening in simulation could be due to the absence of the entire membrane complex, which might be expected limit domain movement. Nevertheless, the ability to model opening with the ligand partially detached suggest a new approach to evaluation of agonist binding and unbinding at the atomic level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the National Institutes of Health-National Institute of Neurological Disorders and Stroke (SFT; NS065371), National Institutes of Health-National Institute for Child Health and Human Development (HY; HD082373), and the National Research Foundation of South Africa (78527)

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Figure 1 –.

Possible states of GluN2 His486. Two alternative conformations for the χ_2 dihedral angle are possible, designated in the main text as $\chi_2 < 0$ (a) and $\chi_2 > 0$ (b), respectively. Both conformations are represented in existing crystal structures of the glutamate ligand-binding domain of NMDAR. Furthermore, three possible protonation states are possible for each conformation: two singly charged conformations (protonation of N_ϵ or N_δ , yielding the N_T and N_π tautomers, respectively) and the positively charged doubly-protonated state. c) Overall architecture of the hetero-tetrameric NMDA receptor complex comprising two GluN2B subunits (grey) and two GluN1 subunits (orange). Each subunit is made up of an amino terminal domain (ATD), an agonist binding domain (ABD) and a transmembrane domain (TMD).

Figure 2 –.

His486 side-chain conformation is sensitive to protonation state and initial χ_2 rotamer. N_T/N_π . (a,b) When His486 is modelled as the N_π (N_δ protonated / orange) neutral tautomer, there is an increase in mobility around the χ_2 dihedral for both E413G (mt: ●) and wild-type (wt: ▼). In contrast, the N_T -tautomer (wt: ▼, mt: ●) is more stable. The doubly protonated charged state (wt: ▼, mt: ●) displays slight mobility around this bond. (b) The $\chi_2 > 0$ rotamer is also preferred, as demonstrated by a number of simulations initiated with $\chi_2 < 0$ that showed His486 flipping over to the $\chi_2 > 0$ rotamer. Little mobility is observed around χ_1 , irrespective of the starting conformation of χ_2 . (Fig S1)

Figure 3 –.

Box and whisker plot for the distribution of exit times of L-glutamate to reach 10 Å from the protein center of mass for 30 simulations. The same protein ligand conformation was used to initiate all simulations. At the relatively high acceleration of 0.25, resistance by the surrounding protein is negligible, resulting in only a slightly lower exit time for E413G. Exit time increases as acceleration on the ligand is decreased, and remains consistently higher for E413G. The boxes extend to the lower and upper quartiles with a line at the median. Whiskers extend to the inter-quartile range $\times 1.5$, with outliers represented as dots.

Figure 4:

Glu413 and His486 mutant analysis. a) Representative whole cell current responses recorded from HEK cells expressing GluN1/GluN2B with the indicated mutations introduced at either Glu413 and His486. Cells were exposed to a 1 second pulse of 1 mM glutamate in the continued presence of 30 μ M glycine (gray bar). b) The current response deactivation time course following rapid removal of glutamate was fitted by the sum of two exponential functions according to

$$I(\text{time}) = A_{\text{FAST}} \exp(-\text{time}/\tau_{\text{FAST}}) + A_{\text{SLOW}} \exp(-\text{time}/\tau_{\text{SLOW}})$$

Where τ_{FAST} and τ_{SLOW} are the time constants and A_{FAST} and A_{SLOW} the current amplitudes of each component. The weighted tau (T_W) was determined by

$$T_W = A_{\text{FAST}} \tau_{\text{FAST}} / (A_{\text{FAST}} + A_{\text{SLOW}}) + A_{\text{SLOW}} \tau_{\text{SLOW}} / (A_{\text{FAST}} + A_{\text{SLOW}})$$

for each mutant receptor and compared to wild-type receptor; see Table 1 for individual tau values. Asterisks (*) represent significant difference from WT (one-way ANOVA, $p < 0.0001$). c,d) The fast time constants (τ_{FAST}) for mutant and WT receptors are plotted as a function of mean exit time. Linear regression for all mutants yielded an R^2 value of 0.49. When analyzed independently, His486 mutant receptors yielded an R^2 value of 0.99. e,f) The log EC₅₀ for mutant and wild-type receptors is plotted as a function of mean exit time. Linear regression for all mutants yielded an R^2 value of 0.53. When analyzed independently, His486 mutants yielded an R^2 value of 0.91.

Figure 5 –.

Opening of the clam-shell domain in the Apo state. a) When the ligand binding domain is simulated without the L-glutamate ligand, the clam-shell is observed to open during the course of about 200 ns simulation. The degree of opening is measured by the distance between C_{α} of His486 and Val714 b) The clam-shell is observed to open sooner in the apo state (3 independent simulations, wt: ▼, ▲, ● mt: ▼, ▲, ●) than in the holo state (2 simulations, wt: ▲, ● mt: ▲, ●) for both the wild-type GluN2B (wt) and the GluN2B-E413G mutant (mt).

Figure 6 –.

Detachment of L-glutamate. a) The initial conformation is shown. b) One simulation was initiated with L-glutamate partially detached from helix E in GluN2B E413G. c-d) During the course of 550 ns, L-glutamate was observed to detach even more, breaking many of the interactions observed in the closed (open-channel) ABD conformation of (b). e) Stylized schematic of L-glu unbinding is shown. The GluN2B-E413G mutation increases ligand mobility and solvent access, promoting unbinding and ABD opening.

Table 1:

Agonist potency, deactivation and simulated egress times in wild-type and mutant GluN2B

	Exit Time (ns) ¹	Tau _{FAST} (ms)	Tau _{SLOW} (ms)	A _{FAST} %	Weighted Tau ² (ms)	EC ₅₀ (μM)
WT	6.8	220±15	740±34	55±2.5	440±17 (23)	1.8±0.18 (28)
H486F	4.3	95±7.4	840±381	94±1.3	120±9.9 (10)	14±1.2 (11)
E413Q	5.8	57±5.4	330±104	86±5.5	81±7.8 (8)	29±4.2 (12)
E413D	0.4	20±1.8	130±19	97±1.2	23±1.3 (8)	140±7.6 (11)
E413N	4.3	16±1.6	87±27	91±4.6	19±2.2 (12)	230±4.5 (11)
E413A	4.1	18±1.6	160±104	95±4.8	20±1.7 (11)	120±11 (12)
E413G	3.5	30±3.6	49±9.4	90±6.8	34±3.1 (8)	75±6.0 (7)
H486G	1.9	13±1.6	164±104	98±4.5	14±0.88 (10)	1800±61 (12)

All values are mean ± SEM.

¹Egress time was determined during RAMD with acceleration set to 0.225. Egress time was the minimum time taken for the ligand to be 10 Å from the protein center of mass (COM).

²Weighted tau was determined from the equation:

$$\text{Weighted Tau} = (A_{\text{FAST}} \text{ Tau}_{\text{FAST}} + A_{\text{SLOW}} \text{ Tau}_{\text{SLOW}}) / (A_{\text{FAST}} + A_{\text{SLOW}})$$