Functional Evaluation of a De Novo GRIN2A Mutation Identified in a Patient with Profound Global Developmental Delay and Refractory Epilepsy

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ABSTRACT

The N-methyl-D-aspartate receptor (NMDAR), a ligand-gated ionotropic glutamate receptor, plays important roles in normal brain development and a wide range of neurologic disorders, including epilepsy. Here, we evaluate for the first time the functional properties of a de novo GRIN2A missense mutation (p.M817V) in the pre-M4 linker in a child with profound global developmental delay and refractory epilepsy. Electrophysiological recordings revealed that the mutant GluN2A(M817V)-containing receptors showed enhanced agonist potency, reduced sensitivity to endogenous negative inhibitors (Mg²⁺, proton, and zinc), prolonged synaptic-like response time course, increased single-channel mean open time, and increased channel open probability. These results suggest that the gain-of-function M817V mutation causes overactivation of NMDAR and drives neuronal hyperexcitability, which may contribute to the patient’s observed epileptic phenotype. Molecular modeling of the closed channel conformation reveals that this mutation weakens the interaction between GluN2 transmembrane helix M4 and two GluN1 transmembrane helices, and increases atomic fluctuation or movement of the pre-M1 region of GluN1 subunit, suggesting a mechanism by which channel function is enhanced. The functional changes of this mutation on agonist potency occur when the mutation is introduced into all other GluN2 subunits, suggesting a conserved role of this residue in control of NMDAR function through interactions of membrane spanning GluN2 and GluN1 helices. A number of NMDAR-targeted drugs including U.S. Food and Drug Association–approved NMDAR channel blockers were evaluated for their ability to inhibit receptors containing GluN2A(M817V) as a first step to exploring the potential for rescue pharmacology and personalized medicine.

Introduction

N-methyl-D-aspartate receptors (NMDARs), a subtype of ionotropic glutamate receptor, are ligand-gated cation channels that mediate the slow component of excitatory synaptic transmission in the brain (Traynelis et al., 2010). NMDARs are heterotetramers consisting of two GluN1 subunits and two GluN2 subunits, which contain the binding pocket for glycine and glutamate, respectively. The GluN1 subunits arise from a single gene (GRIN1) that can be alternatively spliced into eight variants. There are four genes (GRIN2A–D) that encode the GluN2 subunits (GluN2A–2D). GluN2 subunits showed varying expression throughout the brain both spatially and temporally (Akazawa et al., 1994; Monyer et al., 1994). Both GluN2A and GluN2C subunit expression increases after birth, whereas GluN2B and GluN2D subunit expression decreases with age in most brain regions. Often, there is an increase in GluN2A expression in the same regions that show a decrease in GluN2B levels, which is referred to as a GluN2A/2B developmental switch. This change in GluN2 receptor subtype is accompanied by important developmental changes in behavior and synaptic function (Liu et al., 2004; Groc et al., 2006; Dalton et al., 2012).

ABBREVIATIONS: ABD, agonist-binding domain; ATD, amino-terminal domain; CTD, cytosolic carboxyl terminal domain; EPSC, excitatory postsynaptic current; FDA, Food and Drug Administration; HEK, human embryonic kidney; MD, molecular dynamics; MTSEA, 2-aminoethyl methane thiosulfonate hydrobromide; NMDAR, N-methyl-D-aspartate receptor; POPEN, open probability; RMSF, root-mean-square fluctuation; TCN-201, N-[4-(benzamidocarbamoyl)[phenyl][methyl]-3-chloro-4-fluorobenzensulfonamide; TEVC, two-electrode voltage clamp; TMD, transmembrane domain; WT, wild type.
Each subunit in the NMDAR complex contains four semi-autonomous domains: the amino-terminal domain (ATD), the agonist-binding domain (ABD), the transmembrane domain (TMD), and a cytosolic carboxyl terminal domain (CTD). The ABDs are arranged into bilobed clamshell-shaped structures, with an upper (D1) and lower lobe (D2) creating a binding pocket for agonists. Activation of the NMDAR requires simultaneous binding of glycine to GluN1 and glutamate to GluN2 subunits.

Multiple studies, including elucidation of crystal structures of isolated ABDs of glutamate receptor ion channels, revealed that agonist binding promotes a closed-cleft conformation of the bilobed ABD, which is translated into rearrangement of short linkers connected to the transmembrane helices that enable the cation-selective pore to open (Armstrong et al., 1998; Sun et al., 2002; Jin et al., 2003; Furukawa et al., 2005; Inanobe et al., 2005; Talukder et al., 2010; Mayer et al., 2011; Vance et al., 2011; Hansen et al., 2013; Karakas and Furukawa, 2014; Kazi et al., 2014; Lee et al., 2014; also see Traynelis et al., 2010). Opening of NMDAR channels leads to an increase in the intracellular Ca\(^{2+}\) concentration as well as neuronal membrane depolarization (Traynelis et al., 2010). NMDARs play important roles in learning, motor and sensory function, and nervous system development as well as in a wide range of pathologic conditions such as stroke, Parkinson’s disease, Huntington’s disease, schizophrenia, and epilepsy (Mony et al., 2009; Traynelis et al., 2010; Parsons and Raymond, 2014).

Recent advances in next-generation whole exome sequencing technologies have identified a large number of de novo mutations and rare variants in the GRIN family of genes in patients with neurologic conditions, including severe intellectual disability, developmental delay, and intellectual disability (Burnashev and Sheng, 2002). The lack of systematic evaluation of the effects of these variants on channel function precludes an understanding of the mechanism by which de novo mutations and rare variants impact clinical phenotype and disease progression, and prevents a mechanism-based exploration of new therapeutic strategies.

We performed functional and molecular studies on a reported GRIN2A missense mutation (p.Met817Val, hereafter M817V) identified in a female patient with profound developmental delay and refractory epilepsy (evaluated at 4 years old; Venkateswaran et al., 2014). The patient displayed multiple seizure types (partial complex with secondary generalization, tonic, myoclonic, and atypical absence), which did not fit within a specific epileptic syndrome (Venkateswaran et al., 2014). Electroencephalography at 14 months demonstrated diffuse slowing, with background posterior dominant rhythm and diffuse background slowing at 3–4 Hz (Venkateswaran et al., 2014). The patient also showed a history of delayed development and low vision, and had not developed fine motor skills. The patient’s development progressed minimally without any episodes of regression. Cranial magnetic resonance imaging demonstrated prominence of extraaxial cerebrospinal fluid spaces with normal myelination. Additionally, the corpus callosum appeared thin and slightly elongated (Venkateswaran et al., 2014).

In this study, we provide in vitro electrophysiologic data showing that NMDARs containing GluN2A(M817V) display enhanced agonist potency, prolonged synaptic-like response time course, reduced sensitivity to endogenous negative modulators, and increased channel mean open time and single-channel open probability. The location of this residue, which resides within five residues of the de novo gain-of-function mutation GluN2A(L812M) (Pierson et al., 2014; Yuan et al., 2014), further implicates the M4 linker/transmembrane helix as a critical participant in channel gating (Kazi et al., 2013). Furthermore, the functional alterations described here will lead to profound hyperactivation of NMDARs, which is almost certainly pathogenic at some level and may likely contribute to the phenotype of seizures.

Because the seizures are refractory to conventional antiepileptic drugs, we also evaluated a number of NMDAR-targeted compounds, including U.S. Food and Drug Administration (FDA)–approved NMDAR antagonists, for their ability to inhibit NMDARs containing GluN2A(M817V). Our results indicate that functional evaluation is a necessary first step toward elucidation of the molecular mechanism underlying the GRIN mutation-associated neurologic conditions. Functional data provide additional insight into GRIN phenotype-genotype correlations, therapeutically relevant information, and structural elements that control NMDAR gating.

**Materials and Methods**

**Molecular Biology.** The plasmids used were human wild-type (WT) GluN1-1a (GenBank accession numbers NP_015566), GluN2A (NP_000824), GluN2B (NP_000825), GluN2D (NP_000827.1), and rat GluN1-1a (U11418 and U69261), GluN2A (D15211), and GluN2C (M91563). All cDNAs were cloned into the mammalian expression vector pcI-neo (U47120) (Hedegaard et al., 2012). Rat cDNA were provided by Drs. S. Heinemann (Salk Institute), S. Nakanishi (Kyoto University), and P. Seeburg (University of Heidelberg). Mutagenesis was performed using the QuikChange protocol from Stratagene (San Diego, CA) (Low et al., 2000). Pfu polymerase, dNTPs, and buffer were purchased from BioAgricult (Osaka, Japan). Methylated DNA was digested with Dpn I from Takara (Mountain View, CA) for 3 hours at 37°C and the nicked double-stranded mutant DNA was transformed into Stellar Competent Cells from Clontech (Mountain View, CA). The DNA was prepared using the Qiaprep Spin Miniprep kit from Qiagen (Valencia, CA).

Sequences were verified through the mutagen region using dyeoxy sequencing from Eurofins MWG Operon (Huntsville, AL). The cDNA was linearized by enzyme Not I and cRNA was synthesized according to manufacturer instructions (Ambion/Life Technologies, Austin, TX). The cRNA (5–10 ng total) in RNase-free water was microinjected into oocytes using a Drummond Nanosept H (Broomall, PA). The constructs of triheteromeric receptors were generated using rat GluN1 and GluN2A with modified C-terminal peptide tags, as described by Hansen et al. (2014). Two peptide tags (C1 and C2) were generated from the leucine zipper motifs found in GABA\(_{A_1}\) and GABA\(_{B_2}\) immediately followed by dicyline KRTN endoplasmic reticulum retention signals (Jackson et al., 1990, 1993; Zerangue et al., 2001). The leucine zipper motifs allow the two domains C1 and C2 to form a coiled-coil interaction that masks the dicyline KRTN retention motif. These C-terminal retention signals were fused to the WT and mutant GluN2A receptors to yield GluN2A-C1, GluN2A-C2, GluN2A-C1-M817V, GluN2A-C2-M817V. Only receptors with one copy of a C1 tag and one copy of a C2 tag will mask the endoplasmic reticulum retention signal and reach the cell surface. Coexpressing with rat GluN1-1a (hereafter GluN1) with C1- and C2-tagged GluN2A generated receptors with the following subunit combinations: GluN1/GluN2A-C1/GluN2A-C2 (referred to 2A/2A), GluN1/GluN2A-C1/M817V/GluN2A-C2 (referred to M817V/2A), and GluN1/GluN2A-C1/M817V/GluN2A-C2/M817V (referred to M817V/M817V).

**Homology Modeling, Molecular Dynamics, and Computational Studies.** Amino acids were numbered with the initiating methionine set to 1. A protein family alignment was generated for the NMDA (GluN1/GluN2A–D), AMPA (GluA1–4), and kainate (GluA5–7) receptor families using T-Coffee (Notredame et al., 2000). The alignments were used to generate multiple sequence alignments to include all known members of the GRIN superfamily using MAFFT (Katoh and Standley, 2013) and MUSCLE (Edgar, 2004) algorithms. Multiple sequence alignments were combined using Clustal W 2.0 (Larkin et al., 2007). The protein sequences were used to generate a phylogenetic tree using the neighbor-joining method (Saitou and Nei, 1987) with the Jukes–Cantor correction (Jukes and Cantor, 1969). The tree was compiled using the Phylip package (Felsenstein, 1992).
(GluK1–5) receptors using the program Muscle (Edgar, 2004). Homology models were generated for a diheteromeric GluN2A receptor from two template structures (PDB entries; 5FXH and 4P5E) using modeler 9v14 (Sali and Overtoning, 1994). The models were subjected to quality analysis using the PDBsum Generator (www.ebi.ac.uk/ pdbsum; Laskowski, 2009). The model (Supplemental_GluN2A-WT-270.pdb) was prepared for analysis using the protein preparation wizard in which protonation states were assigned followed by an energy minimization to relieve unfavorable constraints (Schrödinger Release 2016-3; Protein Preparation Wizard, Epik version 3.7, Impact version 7.2. Prime version 4.5; Schrödinger, LLC, New York, NY). The ATDzs were removed to reduce computational time.

The diheteromeric GluN1/GluN2A model was prepared for molecular dynamics (MD) simulation using the program Desmond (Schrödinger Release 2016-3; Desmond Molecular Dynamics System; version 4.7, D.E. Shaw Research; Maestro-Desmond Interoperability Tools, version 4.7; Schrödinger, LLC). A POPC (300K) membrane was added to the model and solvated within an orthorhombic box shape with a buffer distance of 10 Å using the simple point charge water model. The overall system was neutralized at pH 7.0 using an ion concentration of 0.15 NaCl. The system was relaxed using the Desmond relaxation model, followed by a production run of 10 ns under NPT conditions using the Nose-Hoover thermostat (300K) and particle mesh Ewald electrostatics (Essmann et al., 1995) with a cutoff of 9 Å. Time step calculations were performed every 2 femtoseconds. We saved 1000 frames from the MD simulations. Simulations were performed on the WT M817V (GluN2A) and M813V (GluN1) mutations. The structures captured during the MD simulation was aligned based on the M1, pre-M1, and M2 helices of GluN1 subunit and the M4 helix of the GluN2 subunit before measuring the root-mean-square fluctuation (RMSF). The RMSF of pre-M1 linker region (all C,S,N,O atoms) were compared between the WT GluN2 and the mutant GluN2A(M817V) for the duration of the production run (10 ns). Similarly, the equivalent pre-M1 regions of GluN1(M813V) were compared with WT GluN1. Visual Molecular Dynamics and Pymol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC.) were used to perform the RMSF calculation ( Humphrey et al., 1996).

The distance between the Co of residues on the pre-M1, M3, and M4 regions of the WT M817V (GluN2A) and M813V (GluN1) were monitored during the simulations to check for stability of the region after the introduction of the mutation (Supplemental Fig. 1). The change in stability (Δ stability) and affinity (Δ affinity) for GluN2A (M817V; frame 270) and GluN1 (M813V; frame 1) were calculated using the BioLuminate module of the Schrödinger Suite (Schrödinger Release 2016-3). Calculations were performed using the implicit (continuum) solvation model of Prime MM-GBSA (Schrödinger Release 2016-3; Desmond Molecular Dynamics System; version 4.7, Prime version 4.5; Schrödinger, LLC). The resulting mutations were refined by allowing side-chain and backbone minimization with a 5 Å cutoff.

**Two-Electrode Voltage Clamp Current Recordings.** Two-electrode voltage clamp (TEVC) current recordings were performed on unfertilized Xenopus laevis oocytes (Ecocyst, Austin, TX), which were injected with 5–10 ng cRNA in 20–50 nl of water (1:2 for diheteromeric GluN1:GluN2A or GluN1:GluN2A(M817V); 1:66 for triheteromeric GluN1:GluN2A-C1:GluN2A-C2, or GluN1:GluN2A-C1(M817V)GluN2A-C2, or GluN1:GluN2A-C1(M817V)GluN2A-C2(M817V), and were kept at 19°C in Barth’s solution for 2 to 4 days before recording. Barth’s solution contained (in mM) 88 NaCl, 2.4 NaHCO3, 1.0 KCl, 0.33 Ca(NO3)2, 0.41 CaCl2, 0.82 MgSO4, and 5 Tris/HCl (pH 7.4). The oocyte recording solution contains (in mM) 90 NaCl, 1 KCl, 10 HEPES, 0.5 BaCl2, and 0.01 EDTA (pH 7.4).

The solution for Za+ dose–response experiments was made fresh daily from ZnCl2 powder and 10 mM tricine at pH 7.3 (Tyuyama et al., 1998). Solution exchange was achieved through a computer-controlled eight-modal valve positioner (Digital MVP Valve, Hamilton, CT).

Current responses were recorded at a holding potential of −40 mV (unless otherwise stated) with voltage and current electrodes (filled with 0.3M and 3.0M KCl, respectively). The electrodes were prepared from thin-wall glass micropipettes (TW150F-4; World Precision Instruments, Sarasota, FL) by a dual-stage glass micropipette puller (PC-10;
Narishige, Tokyo, Japan) and filled with internal solution that contained (in mM) 150 NaCl, 10 HEPES, 3 KCl, 0.5 CaCl₂, 2 MgCl₂, 5 BAPTA, 2 NaATP, and 0.3 NaGTP, pH 7.35. Transfected HEK 293 cells were perfused with external recording solution that contained (in mM) 150 NaCl, 10 HEPES, 30 mM-nanotill, 3 KCl, 1 CaCl₂, and 0.01 EDTA (pH 7.4, 23°C).

The current response was recorded with an Axopatch 200B amplifier (Molecular Devices, Union City, CA) at holding potential of −60 mV at room temperature (23°C). A two-barreled theta-glass micropipette was used for rapid solution exchange controlled by a piezo-electric translator (Burleigh Instruments, Newton, NJ). The current response time course was fitted using ChanneLab (Synaptosoft, Decatur, GA) by eq. 5:

\[
\text{Response} = \text{Amplitude}_{\text{FAST}}(\exp(-\text{Time}/\tau_{\text{FAST}})) + \text{Amplitude}_{\text{SLOW}}(\exp(-\text{Time}/\tau_{\text{SLOW}}))
\]

Single-channel recordings of excised outside-out patches from HEK 293 cells transiently transfected with GluN1:GluN2A or GluN1:GluN2A(M817V) were performed at a holding potential of −80 mV with an Axopatch 200B (23°C). The electrodes (7–9 MΩ) were prepared from filamented thick-wall glass pipettes (150F-4; Warner Instruments, Hamden, CT), pulled by a dual-stage glass micropipette puller (PC-10; Narishige, Tokyo, Japan), and coated with Sylgard (Dow Corning, Midland, MI); the tip was fire-polished. The same puller (PC-10; Narishige, Tokyo, Japan), and coated with Sylgard (Dow Corning, Midland, MI), was used to activate NMDARs in whole-cell recordings was used. We added 0.01 EDTA (pH 7.4), and maximum likelihood fitting of open and shut intervals was performed using ChanneLab (Synaptosoft, Decatur, GA). Because outside-out patches in this experiment likely contained more than a single active channel, we only analyzed stretches of the data record that contained a single active channel to determine chord conductance, mean open time, and mean shut time. The open probability (P(open)) during stretches with a single active channel was estimated from the total open time divided by the total length analyzed, determined from the mean open time, mean shut time, and number of analyzed events; this analysis will underestimate P(open) since periods with two or more channels open are omitted from analysis. All reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. Data were expressed as mean ± S.E.M. and analyzed statistically using unpaired t test or ANOVA. P < 0.05 was considered statistically significant unless otherwise noted. Error bars in all figures are S.E.M. The number of samples was calculated to obtain a power to detect a 50% change greater than 0.8 for all experiments.

### Results

#### Structural Features of GluN2A(M817V)

We evaluated the functional effects of a de novo GRIN2A missense mutation (c.2449A>G, p.Met817Val, hereafter referred to as M817V, NCBI nucleotide accession number NM_001134407.1), which is a remarkably deleterious variant predicted by several algorithms, including POLYPHEN2 (as “pathogenic”) (Polyphen) version 2; see http://genetics.bwh.harvard.edu/pph2/), PHAST (score 616) (Phylogenetic Analysis with Space/Time models; see http://compgen.cshl.edu/phast/), and GERP (score 4.2) (Genomic Evolutionary Rate Profiling; see Cooper et al., 2005; Venkateswaran et al., 2014). This methionine residue is conserved in several vertebrate species and across GluN1 and all GluN2 subunits (Fig. 1A), suggesting a potentially conserved role in NMDAR channel function. Met817 is located at the beginning portion of the extracellular end of the M4 transmembrane helix (Fig. 1, A and B), which resides downstream from a linker region connecting the M4 transmembrane helix to the S2 portion of the agonist-binding domain. This linker has been proposed to be involved in channel gating (Taluudker and Wollmuth, 2011; Yuan et al., 2014). The region of S2-M4 linker and the beginning part of M4 are in van der Waals contact with the M3 helix and pre-M1 region (Fig. 1C), both suggested to be involved in channel gating (Thomas et al., 2006; Traynelis et al., 2010; Kazi et al., 2013; Ogden et al., 2017). The proximity to the ABD that moves with agonist binding may allow the pre-M4 and M4 region to influence the movement and position of the M3 helices that form the channel gate (Kazi et al., 2013).

To better understand the effects of the GluN2A(M817V) mutation, a homology model of a diheteromeric GluN1/GluN2A receptor was generated, optimized, hydrated, and equilibrated using molecular dynamics, resulting in a biologically relevant structure (see Supplemental_GluN2A-WT.pdb). This structure was used to study the effects of the M817V mutation on protein stability, and the change of affinity between the different subunits (chains) using energy calculations (Biologies Suite 2016-3: BioLuminate, version 2.4; Schrödinger, LLC, New York, NY).

The change in stability (Δ stability) is defined as the difference in free energy between the folded state and the unfolded state of the receptor, whereas the change in affinity (Δ affinity) is defined as the difference in binding affinity between the WT side chain or mutated side chain and the rest of the protein (other chains). The Δ stability after introducing the M817V mutation into GluN2A was 2.79 and 3.53 kcal/mol for the two GluN2 polypeptide chains in the tetramer (referred to as B and D; see Karakas and Furukawa, 2014; Lee et al., 2014), suggesting that the mutation decreases the stability in the region of interest (positive values suggest that the mutant is less stable than the native protein). The Δ affinity changed by 10.02 and 10.80 kcal/mol for chains B and D, respectively. This positive value suggests that the mutant side chain had weaker interactions with the rest of the protein than the WT side chain would.

The modeling showed that Met817 protrudes toward and interacts with both the M1 and M3 transmembrane helices of the GluN1 subunit (Fig. 1D), and thus the Δ affinity reflects decreased affinity of the side chain for the GluN1 transmembrane helices. The mutation is also in close proximity to the pre-M1 helix (Fig. 1D), which is under strong selection and harbors an excess of disease-associated mutations with no variants in the Exome Aggregation Consortium browser (ExAC), a database of allele frequencies in the healthy population that serves as a reference set for severe disease studies (Lek et al., 2016).

**Failure to find variants in this server may suggest M817V is a relevant structure (see Supplemental_GluN2A-WT.pdb).** This structure was used to study the effects of the M817V mutation on protein stability, and the change of affinity between the different subunits (chains) using energy calculations (Biologies Suite 2016-3: BioLuminate, version 2.4; Schrödinger, LLC, New York, NY).

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2.25–4.99 Å and 2.47–3.45 Å WT chains A and C, respectively) (Fig. 2, B and C). We also monitored the distance of Ca atoms of residues located on the GluN2A-M4, GluN1-M3, and pre-M1 helices and found the region to be stable during our simulations (Supplemental Fig. 1).

Our hypothesis is that the change of the conserved gating residue Met817 to Val disrupts the interaction between the M4 helix of GluN2A and the pre-M1, M1, and M3 helices of GluN1, allowing increasing thermal motion of these regions of interest. We interpret the perturbation of the interactions between GluN2 M4 with GluN1 M3 as capable of influencing channel pore opening by reducing the energy for receptor activation. To confirm this hypothesis, the functional properties of GluN2A (M817V)-containing NMDARs were evaluated and compared with WT GluN2A-containing receptors.

**GluN2A(M817V) Enhances Agonist Potency.** We first evaluated the effects of GluN2A(M817V) on agonist potency using two-electrode voltage clamp current recordings from *Xenopus* oocytes. Analysis of the glutamate concentration–response relationship to determine the half-maximally effective concentration (EC50) of agonists showed that GluN2A (M817V) increased the glutamate potency by 9.5-fold, with EC50 values of 0.39 μM compared with 3.7 μM for mutant and WT receptors, respectively (Fig. 3A; Table 1). Similarly, the potency of glycine for GluN2A(M817V) was increased 7.3-fold compared with the WT GluN2A, with the EC50 value...
decreasing to 0.15 μM from 1.1 μM for WT receptors (Fig. 3B; Table 1). These data suggest that the GluN2A(M817V)-containing NMDARs can be activated by a lower concentration of agonists.

Because the mutation in this patient is heterozygous and the functional NMDAR complex contains two copies of the GluN2 subunit, some NMDARs in this individual should have a single copy of mutant GluN2A(M817V). We therefore employed a strategy to control receptor trafficking and subunit composition on the cell surface. By engineering a pair of modified GluN2A subunits that contain complementary sets of coiled-coil domains followed by an endoplasmic reticulum retention signal (Hansen et al., 2014; Yuan et al., 2014), we can generate receptors containing 0, 1, and 2 copies of the GluN2A(M817V) on the cell surface. We repeated the experiments that establish the concentration–response relationship to investigate the effects of a single copy of GluN2A(M817V) on agonist potency. The data showed that a single copy of GluN2A(M817V) produced an intermediate, but significant increase in both glutamate potency (EC50 values for 2A/2A 3.6 ± 0.1 μM, n = 8; M817V/2A# 1.1 ± 0.02 μM, n = 6; M817V/M817V# 0.47 ± 0.05 μM, n = 6) and glycine potency (EC50 values for 2A/2A 1.0 ± 0.04 μM, n = 8; M817V/2A# 0.34 ± 0.02 μM, n = 7; M817V/M817V# 0.14 ± 0.01 μM, n = 8, *P < 0.05, one way analysis of variance for log(EC50), compared with WT 2A/2A; Fig. 3, C and D). These results confirm that a single copy of mutant GluN2A can significantly alter receptor function.

GluN2A(M817V) Reduces Inhibition by Endogenous Negative Modulators. One important feature of NMDARs is negative regulation by a number of endogenous extracellular ions, including Mg2++, protons, and Zn2+ (Traynelis et al., 2010; Paoletti et al., 2013). Two experiments were performed to evaluate the Mg2++ inhibition on GluN2A(M817V). The concentration–response curves (Fig. 4A; Table 1) showed a reduced potency for Mg2++ inhibition for GluN2A(M817V) with an increased IC50 value of 80 μM compared with 24 μM of WT receptors at a holding potential of −60 mV. The current-voltage...
curves (Fig. 4B; Table 1) revealed nearly 2-fold more current in the mutant compared with WT receptors at a holding potential of −60 mV. Fitting the current-voltage curves obtained in the presence of 300 μM Mg2+ to the Woodhull equation revealed that the affinity of Mg2+ in the absence of an electric field, $K_{D,0}$, was decreased from 8.5 mM in WT GluN2A to 19.7 mM in GluN2A(M817V); there was virtually no change in the electric field. Does not alter the apparent depth of the binding site in the response curves, and.

TABLE 1
Summary of pharmacologic data for M817V
The data were expressed as mean ± S.E.M. (n); n is the number of oocytes.

<table>
<thead>
<tr>
<th></th>
<th>WT 2A</th>
<th>M817V</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate, EC50, μM (n)</td>
<td>3.7 ± 0.2 (10)</td>
<td>0.39 ± 0.05 (14)a</td>
<td>1.74E−13</td>
</tr>
<tr>
<td>Glycine, EC50, μM (n)</td>
<td>1.1 ± 0.07 (10)</td>
<td>0.15 ± 0.02 (13)a</td>
<td>2.07E−07</td>
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<tr>
<td>Mg2+, IC50, μM (n)b</td>
<td>24 ± 2.5 (16)</td>
<td>80 ± 16 (15)b</td>
<td>9.94E−06</td>
</tr>
<tr>
<td>Mg2+ IV (n)c</td>
<td>13 ± 1.3% (7)</td>
<td>25 ± 2.8% (6)c</td>
<td>0.00322</td>
</tr>
<tr>
<td>Proton, % (n)</td>
<td>41 ± 1.5 (9)</td>
<td>89 ± 1.7 (8)c</td>
<td>2.37E−13</td>
</tr>
<tr>
<td>Proton, IC50, pH (n)</td>
<td>7.0 (6)</td>
<td>6.2 (7)</td>
<td></td>
</tr>
<tr>
<td>Zinc, IC50, nM (n)</td>
<td>8.6 ± 1.4 (8)</td>
<td>31 ± 4.8% (8)c</td>
<td>0.00019</td>
</tr>
<tr>
<td>% Inhibition by zinc</td>
<td>60 ± 3.3% (8)</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

ND, not determined (the concentration–response curve could not be fitted, most likely because of the similar potency for voltage–independent and voltage-dependent inhibition by Zn2+ in mutant receptors).

We subsequently evaluated the effects of extracellular protons and zinc on the GluN2A(M817V)-containing NMDARs. Determination of the concentration–response relationship of protons revealed a reduced proton sensitivity in GluN1/ GluN2A(M817V), with an IC50 value corresponding to pH 6.2 compared with pH 7.0 for the WT GluN1/GluN2A (Fig. 4C; Table 1). The reduced proton sensitivity of GluN2A(M817V) is predicted to enhance current responses to approximately 140% of that for WT GluN1/GluN2A for recordings at physiologic pH 7.3 by reducing the level of tonic protonation from 33% to 7%. GluN2A(M817V) also diminished approximately 2-fold the extent of high-affinity zinc inhibition observed at 300 nM zinc (Fig. 4D; Table 1). Taken together, these data suggested that GluN2A(M817V) may enhance excitatory drive as a result of the increased activation at low concentrations of agonists and the reduced inhibition by endogenous magnesium, protons, and zinc.

GluN2A(M817V) Prolongs Synaptic-Like Response Time Course. The deactivation time course after rapid removal of glutamate from NMDARs has been suggested to control the time-course of the NMDAR component of the excitatory postsynaptic current (EPSC) (Lester et al., 1990). To assess the effects of GluN2A(M817V) on the deactivation time course, we measured current responses after glutamate removal using a rapid solution exchange system in whole-cell voltage clamp current recordings from transiently transfected HEK 293 cells expressing WT GluN1/GluN2A or GluN1/GluN2A(M817V). GluN2A(M817V) significantly prolonged the glutamate deactivation time course fitted by two exponential components, increasing the weighted $\tau_w$ to 632 milliseconds compared with 43 milliseconds for WT GluN1/GluN2A (P < 0.0071, unpaired t test, controlled family-wise error rate by using the Holm-Bonferroni correction; Fig. 5A and Table 2). To mimic synaptic events, we also measured current responses by briefly moving the cell into the agonist solution for 3–5 milliseconds (brief application). As observed for the responses to prolonged (1.5 seconds) application of glutamate, GluN2A(M817V) activated by brief application of glutamate also had a slower deactivation time course, with a $\tau_w$ of 381 ± 52 milliseconds compared with 40 ± 3.0 milliseconds for WT GluN2A (P = 6.5E-05, unpaired t test; Fig. 5B). These data suggest NMDARs that contained GluN2A (M817V) have a prolonged deactivation response time course,
and thus a prolonged time course of the NMDAR component of the EPSC at synapses that use GluN2A.

**GluN2A(M817V) Alters Single-Channel Properties.**

To assess the effects of this mutant on single-channel properties, we recorded steady-state single-channel unitary currents in outside-out patches excised from HEK 293 cells transiently expressing GluN1/GluN2A or GluN1/GluN2A(M817V) (Fig. 6). Analysis of the pooled data for WT GluN1/GluN2A showed one predominant chord conductance state (75 ± 2.4 pS, n = 7 patches), assuming a reversal potential of 0 mV (Fig. 6A; Table 2).

NMDARs that contained GluN2A(M817V) had a similar chord conductance level (71 ± 3.7 pS, n = 5 patches; *P* = 0.43, unpaired *t* test; Fig. 6B; Table 2), suggesting that this mutation did not change the ion permeation properties. However, the mutant receptor showed an over 2-fold increase in mean channel open time (4.3 milliseconds versus 2.1 milliseconds for WT). In addition, there was a 3.5-fold decrease in mean shut time (0.8 milliseconds versus 2.8 milliseconds for WT) for these multichannel patches, suggesting both an increase in single-channel open probability within a burst (*P*<sub>OPEN</sub>: 0.85 versus 0.43 for WT) and in opening frequency (Fig. 6, C and D; Table 2).

To further evaluate the effects of this mutation on single-channel open probability, we measured the degree of MTSEA (2-aminoethyl methane thiosulfonate hydrobromide) potentiation on a GluN1/GluN2A receptor with a mutation in the GluN1 SYTANLAAF gating region, allowing subsequent

**TABLE 2.** Summary of biophysical properties for M817V

<table>
<thead>
<tr>
<th>Property</th>
<th>WT 2A</th>
<th>M817V</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude (peak, pA/pF)</td>
<td>125 ± 33</td>
<td>267 ± 91</td>
<td>0.239</td>
</tr>
<tr>
<td>Amplitude (SS, pA/pF)</td>
<td>73 ± 15</td>
<td>196 ± 64</td>
<td>0.145</td>
</tr>
<tr>
<td><em>I</em>_OFF/PEAK</td>
<td>0.65 ± 0.09</td>
<td>0.78 ± 0.05</td>
<td>0.218</td>
</tr>
<tr>
<td>10%–90% Rise time (ms)</td>
<td>7.8 ± 0.5</td>
<td>7.7 ± 0.7</td>
<td>0.913</td>
</tr>
<tr>
<td>τ&lt;sub&gt;FAST&lt;/sub&gt; deactivation (ms)</td>
<td>31 ± 3.0</td>
<td>416 ± 100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.006</td>
</tr>
<tr>
<td>τ&lt;sub&gt;SLOW&lt;/sub&gt; deactivation (ms)</td>
<td>429 ± 138</td>
<td>1207 ± 259</td>
<td>0.079</td>
</tr>
<tr>
<td>%τ&lt;sub&gt;FAST&lt;/sub&gt; deactivation</td>
<td>97%</td>
<td>71%</td>
<td>–</td>
</tr>
<tr>
<td>τ&lt;sub&gt;W&lt;/sub&gt; deactivation (ms)</td>
<td>43 ± 3.2</td>
<td>632 ± 103&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.00036</td>
</tr>
<tr>
<td>Charge transfer, pA x ms/pF</td>
<td>5,520</td>
<td>179,043</td>
<td>–</td>
</tr>
<tr>
<td>Number of cells</td>
<td>6</td>
<td>8</td>
<td>–</td>
</tr>
<tr>
<td><em>P</em>&lt;sub&gt;OPEN&lt;/sub&gt; (from channels)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.43 ± 0.03</td>
<td>0.85 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.2E-07</td>
</tr>
<tr>
<td>Mean open time, ms&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.1 ± 0.1</td>
<td>4.3 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.5E-06</td>
</tr>
<tr>
<td>τ&lt;sub&gt;1&lt;/sub&gt;, ms (% area)</td>
<td>0.1 ± 0.01 (23)</td>
<td>0.2 ± 0.02 (6)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.00655</td>
</tr>
<tr>
<td>τ&lt;sub&gt;2&lt;/sub&gt;, ms (% area)</td>
<td>2.6 ± 0.1 (77)</td>
<td>4.5 ± 0.3 (94)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3.4E-05</td>
</tr>
<tr>
<td>Mean shut time, ms&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.8 ± 0.4</td>
<td>0.8 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.00107</td>
</tr>
<tr>
<td>τ&lt;sub&gt;1&lt;/sub&gt;, ms (% area)</td>
<td>0.2 ± 0.03 (26)</td>
<td>0.1 ± 0.02 (30)</td>
<td>0.157</td>
</tr>
<tr>
<td>τ&lt;sub&gt;2&lt;/sub&gt;, ms (% area)</td>
<td>0.7 ± 0.1 (30)</td>
<td>0.6 ± 0.08 (35)</td>
<td>0.274</td>
</tr>
<tr>
<td>τ&lt;sub&gt;3&lt;/sub&gt;, ms (% area)</td>
<td>4.2 ± 0.5 (35)</td>
<td>1.5 ± 0.1 (34)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.00043</td>
</tr>
<tr>
<td>Major amplitude (pA)</td>
<td>6.0 ± 0.2</td>
<td>5.7 ± 0.3</td>
<td>–</td>
</tr>
<tr>
<td>γ, pS</td>
<td>75 ± 2.4</td>
<td>71 ± 3.7</td>
<td>0.4285</td>
</tr>
<tr>
<td>Number of patches</td>
<td>7</td>
<td>5</td>
<td>–</td>
</tr>
<tr>
<td>% Potentiation by MTSEA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>439 ± 34</td>
<td>78 ± 6.1</td>
<td>–</td>
</tr>
<tr>
<td><em>P</em>&lt;sub&gt;OPEN&lt;/sub&gt; (from MTSEA)</td>
<td>0.16 ± 0.01</td>
<td>0.93 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.4E-08</td>
</tr>
<tr>
<td>Number of oocytes</td>
<td>10</td>
<td>16</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup> *P* < 0.0071–0.0083 compared with the WT (unpaired *t* test); FWER (family-wise error rate) was controlled using the Holm-Bonferroni correction.

<sup>b</sup>Channel open probability (*P*<sub>OPEN</sub>) was calculated for portions of the data record with a single active channel, and reflects the product of the number of channels and open probability for WT receptor; mutant channels spent so much time open that there was little chance that the activity without double openings reflected that of two channels.

<sup>c</sup>*P* < 0.0056–0.0125 compared with the WT (unpaired *t* test); FWER (family-wise error rate) was controlled using the Holm-Bonferroni correction.

<sup>d</sup>Statistical tests were not run on the slowest shut time components given the limited number of periods underlying this component.

<sup>e</sup>Evaluated by TEVC recordings on *Xenopus* oocytes, see Materials and Methods and Supplemental Fig. 2.

<sup>f</sup>*P* < 0.05 compared with the WT (unpaired *t* test). For all comparisons, power to detect a 50% difference was 0.8.
covalent modification by MTSEA to lock the receptors open (Jones et al., 2002; Yuan et al., 2005). We calculated the open probability based on the degree of MTSEA potentiation of the NMDAR response to maximally active agonist in TEVC recordings on *Xenopus* oocytes. The MTSEA-mediated increase in current response is reciprocally related to the open probability before MTSEA treatment (see Materials and Methods). The MTSEA-derived estimates of open probability showed a similar result to that of single-channel recording in excised outside-out patches from mutant receptors and confirmed a significant increase of calculated channel open probability by GluN2A(M817V) (Table 2 and Supplemental Fig. 2).

**Function of GluN2A(Met817) Is Conserved for All GluN2 Subunits.** Because the methionine at position 817 is conserved across all GluN subunits, we investigated whether the function of this residue is also conserved. We generated Met-Val mutations at the analogous position in GluN1, GluN2B, GluN2C, and GluN2D subunits (M813V in GluN1, M818V in GluN2B, M815V in GluN2C, and M845V in GluN2D) and evaluated the potency (EC$_{50}$ values) of glutamate and glycine at each using TEVC from *Xenopus* oocytes.

Similar to GluN2A(M817V), the Met-Val mutation in other GluN2 subunits increased glutamate potency compared with WT receptors by 3.4-fold for GluN2B(M818V), 7.3-fold for GluN2C(M815V), and 4.1-fold for GluN2D(M845V). Glycine potency was also increased compared with the corresponding WT GluN2 by 4.5-fold for GluN2B(M818V), 2.3-fold for GluN2C(M815V), and 1.6-fold for GluN2D(M845V) (Fig. 7). These data suggest that the function of the Met817 residue, which interacts with GluN1 transmembrane domains, is conserved across all GluN2 NMDAR subunits. By contrast, the equivalent GluN1(M813V) mutation, when coexpressed with WT GluN2A subunit showed no significant change in the glutamate EC$_{50}$ value (5.8 μM versus 4.5 μM for the WT) and only a modest 1.8-fold increase in glycine EC$_{50}$ value (2.4 μM versus 1.3 μM for the WT) (Fig. 7). These findings suggest a different role in gating for this region for GluN1 compared with GluN2.

Given that the actions of Met-Val mutation were not conserved in GluN1, we calculated the Δ stability and Δ
affinity of introducing the Met-Val mutation into the corresponding position in GluN1 subunit. The Δ stability was 5.58 and 2.04 kcal/mol for the two GluN1 polypeptide chains in the tetramer (referred to as A and C; see Karakas and Furukawa, 2014; Lee et al., 2014), suggesting that the mutation modestly decreases the stability in the region of interest (positive values suggest that the mutant is less stable than the native protein). The Δ affinity changed by 12.48 and 13.25 kcal/mol for chains A and C, respectively. This positive value suggests that the mutant side chain had weaker interactions with the rest of the protein than the side chain in the WT receptor. In the heterodimeric GluN1(M813V) mutant receptor MD simulations, the pre-M1 region of GluN2A subunit (residues 543–550) showed enhanced RMSF values compared with the WT receptors (3.39–4.78 Å and 1.27–2.43 Å for mutant versus 1.15–4.07 Å and 1.69–3.28 Å for the WT chains B and D, respectively). These results support a more subtle observed effect on the receptor when introducing the M813V mutation in GluN1 compared with M817V in GluN2A.

Pharmacology on GluN2A(M817V) Mutant Receptors.
Because the patient’s seizures were not fully controlled by conventional antiepileptic drugs, we evaluated the potency at NMDARs containing GluN2A(M817V) of several NMDAR inhibitors, including FDA-approved NMDAR blockers memantine, dextromethorphan and its metabolite dextrorphan, amantadine, ketamine, tomoxetine, and an uncompetitive GluN2A-selective antagonist TCN-201 (N-[4-(benzamidocarbamoyl)phenyl]methyl-3-chloro-4-fluorobenzenesulfonamide), which binds to a site within the agonist binding domain dimer interface to decrease glycine potency.

In vitro analysis in Xenopus oocytes revealed a wide range of effects of GluN2A(M817V) on channel blocker potency. For example, memantine inhibited GluN2A(M817V)-containing NMDARs with a 6-fold lower potency, with an IC50 of 29 μM for mutant compared with 5.3 μM for WT GluN2A recorded on the same day (Fig. 8). Similarly, the anesthetic ketamine inhibited the mutant receptors with a 5-fold lower potency than WT NMDARs, with an IC50 of 43 μM for GluN2A(M817V) compared with 8.4 μM for WT GluN2A (Fig. 8). TCN-201 was 9-fold less potent on the mutant receptors (2.7 μM versus 0.3 μM for WT; Fig. 8), which is consistent with the 7-fold increase in glycine potency for GluN2A(M817V).

By contrast, both dextromethorphan and its metabolite dextrorphan had a mildly reduced potency on GluN2A(M817V)-containing NMDARs compared with the WT receptors (1.8-fold and 1.9-fold, respectively; Fig. 8). Amantadine potency was modestly reduced 2.4-fold for mutant compared with WT receptors. Tomoxetine, an antidepressant, had an IC50 value of 13 μM on the mutant NMDARs compared with 7.1 μM of the WT.

These data indicated that some FDA-approved NMDAR channel blockers can reduce GluN2A(M817V)-mediated NMDAR hyperactivity with similar or modestly reduced potency compared with WT receptors, and thus these compounds might show some effect on mutant NMDARs in vivo, depending on drug exposure levels. These results are consistent with pharmacology at other pore mutations, which showed differential sensitivity to different channel blockers (Pierson et al., 2014).

Discussion
Genetic alterations in GRIN2A have been suggested to be involved in pediatric epileptic syndromes, such as early-onset epileptic encephalopathy, acquired epileptic aphasia...
Landau-Kleffner syndrome), Rolandic epilepsy, the continuous spike-and-waves during slow-wave sleep syndrome, and benign epilepsy with centrotemporal spikes (Endele et al., 2010; de Ligt et al., 2012; Carvill et al., 2013; Lesca et al., 2013; Pierson et al., 2014; Yuan et al., 2014; Gao et al., 2017; also see Yuan et al., 2015). Despite a large number of de novo mutations and disease-associated rare variants that have been identified, there remains little functional information about the effect of these variations. For example, the patient with a GluN2A(M817V) de novo mutation studied here had refractory epilepsy that did not fit with a specific epileptic syndrome (Venkateswaran et al., 2014). However, no information regarding the effect of this mutation was available. Indeed, it was unclear if the mutant receptor was even functional. This lack of functional data prevents the formulation of mechanistic hypotheses about how GRIN2A variation might contribute to these conditions.

We have evaluated the functional consequences of this mutation, and the results suggest that the GluN2A(M817V) produces a gain-of-function, with enhanced agonist potency and channel function. NMDARs containing GluN2A(M817V) are 3- to 7-fold more sensitive to agonist (Table 1) than WT receptors, indicating the activation of GluN2A(M817V)-containing NMDARs may occur with low agonist concentrations than might be insufficient to activate WT NMDARs. The enhanced potency increases the deactivation time course, which is predicted to increase the time course of the EPSC. It seems possible that the gain-of-function GluN2A(M817V) mutation can induce overactivation of NMDARs, which may provide excessive excitatory drive that could contribute to the generation of epileptiform activity or establishment of a seizure focus. The excessive activity of GluN2A(M817V) could possibly lead to excitotoxic neuronal injury along with neuronal loss (Choi 1994; Rothman and Olney, 1995). Other GRIN2 de novo mutations that produce a profound gain of function have been shown to promote neurotoxicity in vitro (Li et al., 2016; Ogden et al., 2017), which may explain the thinned corpus callosum observed in patient magnetic resonance imaging scans as well as the cognitive impairment, motor deficit, and other neurologic dysfunction (Venkateswaran et al., 2014).

The region of GluN2A harboring the M817V mutation has been suggested to influence NMDAR channel gating by a number of studies (Talukder et al., 2010; Salussolia et al., 2011; Talukder and Wollmuth 2011; Yuan et al., 2014). The functional changes of GluN2A(M817V) are similar with a previously reported GluN2A mutation (L812M) (Fig. 9A), which enhanced agonist potency, reduced sensitivity to endogenous inhibitors, prolonged deactivation rate, and increased channel open probability (Yuan et al., 2014). The methionine residue at position 817 in the closed NMDAR structures (Karakas and Furukawa 2014; Lee et al., 2014; see also Sobolevsky et al., 2009) is nearly within van der Waals contact (∼5Å) with the GluN1 pre-M1 helix and the nine-amino acid motif SYTANLAAF in transmembrane domain M3 of GluN1, both regions that are intolerant to change and under strong selection (Swanger et al., 2016; Ogden et al., 2017). The “cuff” helix pre-M1 is a linker region between the agonist-binding domain and transmembrane domain M1 and has been
shown to be involved in channel gating, allosteric modulation, and receptor desensitization (Beck et al., 1999; Kashiwagi et al., 2002; Thomas et al., 2006; Sobolevsky et al., 2007; Chang and Kuo 2008; Mullasseril et al., 2010; Talukder et al., 2010; Ogden and Traynelis 2013; Alsaloum et al., 2016; Ogden et al., 2017). The highly conserved gating motif SYTANLAAF has been suggested to form the helical bundle crossing that occludes cation flow through the channel pore, and the transmembrane domain M3 is hypothesized to be a transduction element that couples agonist binding and channel opening (Jones et al., 2002; Yuan et al., 2005; Sobolevsky et al., 2009; Karakas and Furukawa, 2014; Lee et al., 2014; Karakas et al., 2015). We hypothesize this trio of interactions plays a central role in gating, as well as mediating intersubunit interactions that are relevant for the stability of the closed state. In addition, the selective action of GluN1(M813V) on glycine potency (although modest) raises the possibility that this mutation may perturb the position or degrees of freedom of the GluN1 S2-M4 linker.

Computationally the GluN2(M817V) mutation results in a decrease in the affinity between the M4 helix and the rest of the protein, which can be explained by the reduction of the length and size of valine compared with the WT methionine sidechain, which protrudes toward and interacts with the M3 and M1 helices of the GluN1 subunit (Fig. 9B). These findings are supported by an increase thermal motion captured during the MD simulation of the residues surrounding the M817V mutation, in particular the pre-M1 helix of the GluN1 subunits (Fig. 2). This suggests that this mutation destabilizes the closed conformation through perturbation of a GluN1-GluN2 pore interaction. We therefore hypothesize that the methionine to valine mutation alters these intersubunit interactions and both reduces the energy threshold of channel opening and reduces the energy of the open pore.

We also show that the effects of the Met-Val mutation on glutamate and glycine potency are conserved across all GluN2 subunits but not the GluN1 subunit, consistent with an asymmetric contribution of this residue to NMDAR function between the GluN1 and GluN2 subunits (Banke and Traynelis, 2003; Kazi et al., 2013; Tajima et al., 2016; Ogden et al., 2017). The conservation of function among the GluN2 subunits is consistent with an important role for this residue in gating. These findings highlight the importance of this region in channel opening, suggest this residue is a key site for GluN1-GluN2 interactions, and provide the foundation for future work aimed at fully understanding the effects of mutations on the interactions among GluN2-M4, GluN1-M1, GluN1-M3, and GluN1-preM1 helices on channel function.

The seizures of this patient could not be controlled by classic antiepileptic drugs, including levetiracetam, clonazepam, and valproic acid (Venkateswaran et al., 2014). Although the addition of antiepileptic medications targeting the glutamatergic pathway and g-aminobutyric acid (GABA) receptor (topiramate) significantly decreased the seizure frequency, the effect was sustained only for 10 months (Venkateswaran et al., 2014), suggesting a need for new treatment strategies. A different patient with a previously reported gain-of-function GluN2A mutation (L812M) has a history of early-onset epileptic encephalopathy, developmental delay, and intractable seizures (Pierson et al., 2014; Yuan et al., 2014). Moreover, in vitro experiments showed that memantine reduced receptor function and provided a sustained reduction in seizure burden
Mechanism of a Refractory Epilepsy-Related GRIN2A Mutation

(Pierson et al., 2014). In this study, several NMDAR-targeted drugs, including FDA-approved drugs, were evaluated for actions on GluN2A(M817V) containing NMDAR-mediated current responses. In vitro functional analysis showed that the mutant receptor is less sensitive to several trapping channel blockers, including memantine and ketamine, than the nearby GluN2A(L812M) mutation (Pierson et al., 2014). Interestingly, other channel blockers are less sensitive to the effects of this mutation, suggesting broad assessment of FDA-approved drugs that can alter mutant receptors may help identify agents that retain activity at specific mutations in NMDARs.

Functional evaluation of FDA-approved NMDAR inhibitors on the mutant NMDAR-mediated responses provides a path to better understand the role of NMDARs in patient symptoms and suggests new therapeutic strategies to consider for patients harboring this or similar mutations in NMDARs that are associated with NMDAR hyperactivity. However, a great deal of mechanistic study and clinical research remains to be completed before a rationale strategy can be devised to potentially treat these patients.

Authorship Contributions

Participated in research design: Burger, Traynelis, Yuan.
Conducted experiments: Chen, Tankovic, Kusumoto, Yuan.
Contributed new reagents or analytic tools: Burger.
Performed data analysis: Chen, Tankovic, Burger, Traynelis, Yuan.
Wrote or contributed to the writing of the manuscript: Chen, Tankovic, Burger, Kusumoto, Traynelis, Yuan.

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Supplemental information

Functional Evaluation of a *De Novo* GRIN2A Mutation Identified in a Patient with Profound Global Developmental Delay and Refractory Epilepsy

Wenjuan Chen, Anel Tankovic, Pieter B. Burger, Hirofumi Kusumoto, Stephen F. Traynelis, and Hongjie Yuan
Supplemental Figure S1. (A) The distance measured over the 10 ns MD run between the Cα atoms of GluN2A M817/V817 (Helix-M4 GluN1) and Ser646 (Helix GluN1-M3) and Gln559 (Helix Pre-M1 GluN1). Results for the WT simulations is given in blue and the M817V is given in magenta. (B) The distance measured over the 10 ns MD run between the Cα atoms of GluN1 M813/V813 (Helix-M4 GluN2A) and Ser644 (Helix GluN2A-M3) and Gln554 (Helix Pre-M1 GluN2A). Results for the WT simulations is given in blue and the M813V is given in red. All distances are reported in angstroms.
**Supplemental Figure S2.** We evaluated the channel open probability by measuring the degree of MTSEA (200 µM) potentiation using TEVC recordings from *Xenopus* oocytes expressing the WT GluN2A (A) or the mutant GluN2(M817V) (B) coexpressed with GluN1(A652C) at holding potential of -40 mV in presence of 100 µM glutamate and 100 µM glycine (open bar) and 0.2 mM MTSEA (closed bar). Open probability is inversely correlated with the degree of potentiation (see Methods). We interpret the reduction in the current response by MTSEA for GluN1(A652C)/GluN2(M817V) receptors to reflect near maximal open probability of the mutant receptors, with the current reduction by MTSEA resulting from MTSEA reduction of single channel conductance (Yuan et al., 2005).