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

Department of Pharmacology, Emory University School of Medicine, Rollins Research Center, Atlanta, GA, 30322 (W.C., A.T., H.K., S.F.T., H.Y.)
Department of Neurology, Xiangya Hospital, Central South University, Changsha, 410013, China (W.C.)
Department of Chemistry, Emory University, Atlanta, GA, 30322 (P.B.B.)
Center for Functional Evaluation of Rare Variants (CFERV), Emory University School of Medicine, Rollins Research Center, Atlanta, GA, 30322 (S.F.T., H.Y.)
Running Title: (60 char max): *Mechanism of a refractory epilepsy-related GRIN2A mutation*

Address correspondence to: Stephen F. Traynelis, or Hongjie Yuan, Department of Pharmacology, Center for Functional Evaluation of Rare Variants (CFERV), Emory University School of Medicine, Rollins Research Center, Atlanta, GA, 30322. E-mail: strayne@emory.edu, or hyuan@emory.edu

The number of text pages: 43
The number of tables: 2
The number of figures: 9
The number of references: 74
The number of words in the Abstract: 242
The number of words in the Introduction: 858
The number of words in the Discussion: 1271
The number of supplemental figures: 2
The number of supplemental pdb files: 1

**Abbreviations:**

ABD, agonist-binding domain; ATD, amino-terminal domain; CTD, cytosolic carboxyl terminal domain; EPSC, excitatory postsynaptic current; FWER, family wise error rate; GFP, green fluorescent protein; MD, molecular dynamics; MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide; NMDAR, N-methyl-D-aspartate receptor; P_{OPEN}, open probability; RMSF, root-mean-square fluctuation; TEVC, two-electrode voltage clamp; TMD, transmembrane domain; WT, wild type
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 neurological 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 (Mg$^{2+}$, 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 over-activation of NMDAR and drives neuronal hyperexcitability, which may contribute to the patient's observed epileptic phenotype. Molecular modelling 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 FDA-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 8 variants. There are 4 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).

Each subunit in the NMDAR complex contains four semiautonomous 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 bi-lobed 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 bi-lobed 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; Furukawa et al., 2005; Hansen et al., 2013; Inanobe et al., 2005; Jin et al., 2003; Karakas and Furukawa, 2014; Kazi et al., 2014; Lee et al., 2014; Mayer 2011; Talukder et al., 2010; Vance et al., 2011; see Traynelis et al., 2010). Opening of NMDAR channels leads to an increase in the intracellular Ca\textsuperscript{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 pathological 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 \textit{de novo} mutations and rare variants in the \textit{GRIN} family of genes in patients with neurological conditions, including seizure syndromes, developmental delay, and intellectual disability (Burnashev and Szepetowski, 2015; Yuan et al., 2015; Hu et al., 2016). However, the lack of systematic evaluation of the effects of these variants on channel function precludes an understanding of the mechanism by which \textit{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 \textit{GRIN2A} missense mutation (p.Met817Val, hereafter M817V) identified in a female patient with profound developmental delay and refractory epilepsy (evaluated at 4-year 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 (EEG) at 14 months,
demonstrated diffuse slowing with background activity at 4–5 Hz with no electroclinical correlation. At 24 months of age, EEG demonstrated absence of the 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 (MRI) demonstrated prominence of extraaxial cerebrospinal fluid (CSF) spaces with normal myelination. Additionally, the corpus callosum appeared thin and slightly elongated (Venkateswaran et al., 2014).

In this study, we provide in vitro electrophysiological 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 5 residues of the de novo gain-of-function mutation GluN2A(L812M) (Yuan et al., 2014; Pierson 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 hyper-activation of NMDARs, which is almost certainly pathogenic at some level and may likely contribute to the phenotype of seizures. Since the seizures are refractory to conventional anti-epileptic drugs, we also evaluated a number of NMDAR-targeted compounds, including 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 neurological conditions. Functional data provides 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 U08261), GluN2A (D13211) and GluN2C (M91563). All cDNAs were subcloned 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, USA) (Low et al., 2000). Pfu polymerase, dNTPs, and buffer were purchased from BioAcademia (Osaka, Japan). Methylated DNA was digested with Dpn I from Takara (Mountain View, CA, USA) for 3 hours at 37°C and the nicked double-stranded mutant DNA was transformed into Stellar Competent Cells from Clontech (Mountain View, CA, USA). The DNA was prepared using the Qiaprep Spin Miniprep kit from Qiagen (Valencia, CA, USA). Sequences were verified through the mutated region using dideoxy sequencing from Eurofins MWG Operon (Huntsville, AL, USA). cDNA was linearized by enzyme Not I and cRNA was synthesized according to manufacturer instructions (Ambion). cRNA (5-10 ng total) in RNase-free water was microinjected into oocytes using a Drummond Nanoject II (Broomall, PA, USA). 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_β1 and GABA_β2 immediately followed by dilycine KKTN 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 dilycine KKTN 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. Co-expressing 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 Modelling, 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 (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 4PE5) using modeler 9v14 (Sali and Overington, 1993). The models were subjected to quality analysis using the *PDBsum generator* (http://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,
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, New York, NY, 2016). A POPC (300K) membrane was added to the model and solvated within an orthorhombic box shape with a buffer distance of 10Å using the SPC 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 PME electrostatics (Essman et al., 1995) with a cutoff of 9Å. Time step calculations were performed every 2 fs. 1000 frames were saved from the MD simulations. Simulations were performed on the wild-type, M817V (GluN2A) and M813V (GluN1) mutations. The structures captured during the MD simulation was aligned based on the M1, pre-M1 and M3 helices of GluN1 subunit and the M4 helix of the GluN2 subunit prior to 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 GluN2A 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 to 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 Ca of residues on the pre-M1, M3 and M4 regions of the wild-type, M817V (GluN2A) and M813V (GluN1) were monitored during the simulations to check for stability of the region following the introduction of the mutation (Supplemental Fig S1). 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: Prime, version 4.5, Schrödinger, LLC, New York, NY, 2016.). The resulting mutations were refined by allowing side-chain and backbone minimization with a 5Å cutoff.

Two-Electrode Voltage Clamp Current Recordings

Two-electrode voltage clamp (TEVC) current recordings were performed on unfertilized *Xenopus laevis* oocytes (Ecocyte, Austin, TX, USA), which were injected with 5-10 ng cRNA in 20-50 nl of water (1:2 for di-heteromeric GluN1:GluN2A or GluN1:GluN2A(M817V); 1:6:6 for tri-heteromeric GluN1:GluN2A-C1:GluN2A-C2, or GluN1:GluN2A-C1(M817V):GluN2A-C2, or GluN1:GluN2A-C1(M817V):GluN2A-C2(M817V), and were kept in 19°C in Barth’s solution for 2-4 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 mM EDTA (pH 7.4). The solution for Zn²⁺ dose-response experiments was made freshly daily from ZnCl₂ powder and 10 mM tricine at pH 7.3 (Traynelis et al., 1998). Solution exchange was achieved through a computer-controlled 8-modular valve positioner (Digital MVP Valve, Hamilton, CT, USA). 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 capillaries (TW150F-4, World Precision Instruments, Sarasota, FL, USA) by a dual-stage glass micropipette puller (PC-10, Narishige, Tokyo, Japan). Data was acquired by a two-electrode voltage-clamp amplifier (OC725, Warner Instrument,
Hamden, CT, USA) at room temperature (23°C). 100 μM glutamate and 100 μM glycine were used in all oocyte experiments unless otherwise stated. The agonist concentration-response data were fitted with

\[ \text{Response}(\%) = \frac{100}{1 + \left( \frac{\text{EC}_{50}}{[\text{agonist}]} \right)^{nH}} \]

where \( nH \) is the Hill slope and \( \text{EC}_{50} \) is the agonist concentration that elicited a half maximal response. \( \text{IC}_{50} \) values for the negative modulators and NMDAR-targeted inhibitors were obtained by fitting the concentration-response curves with the following equation:

\[ \text{Response}(\%) = \frac{(100 - \text{minimum})}{1 + \left( \frac{[\text{modulator}]}{\text{IC}_{50}} \right)^{nH}} + \text{minimum} \]

where \( nH \) is the Hill slope, \( \text{IC}_{50} \) is the concentration that produces a half-maximal effect, and \( \text{minimum} \) is the degree of residual inhibition at a saturating concentration of the evaluated modulators or inhibitors. The channel open probability (\( P_{\text{OPEN}} \)) was calculated (Yuan et al., 2005) from the degree of MTSEA-induced (2-aminoethyl methanethiosulfonate hydrobromide) (Toronto Research Chemicals, Toronto, Ontario, Canada) potentiation as follows:

\[ P_{\text{OPEN}} = \left( \frac{\gamma_{\text{MTSEA}}}{\gamma_{\text{CONTROL}}} \right) \times \left( \frac{1}{\text{Potentiation}} \right) \]

where \( \gamma \) is the chord conductance for channels before and after MTSEA modification (Yuan et al., 2005). The voltage dependence and \( K_{\text{D,0 mV}} \) were estimated by fitting the Woodhull equation (Woodhull, 1973) to the data obtained in the absence and presence of \( \text{Mg}^{2+} \) using the equation:

\[ I_{\text{UNBLOCKED}}(V) = I(V) / \left( 1 + [\text{Mg}^{2+}]_o / K_{\text{D,0 mV}} \exp(z\delta EF/RT) \right) \]

where \( I_{\text{UNBLOCKED}}(V) \) is the unblocked current at a given voltage in the presence of \( \text{Mg}^{2+} \), \( I(V) \) is the current in the absence of \( \text{Mg}^{2+} \) at a given voltage, \([\text{Mg}^{2+}]_o \) is the extracellular \( \text{Mg}^{2+} \) concentration (300 μM), \( K_{\text{D,0 mV}} \) is the \( K_D \) in the absence of an applied electric field, \( z \) is the valence (2 for \( \text{Mg}^{2+} \)), \( \delta \) is the effective fraction of the electric field at the binding site, and \( E, F, R, \) and \( T \) have their usual meanings.
Cell Culture and Transfection

HEK 293 cells (CRL 1573, ATCC, Manasas, VA, USA) for di-heteromeric receptor experiments were maintained in standard media (DMEM/Glutha-Max media with 10% dialyzed fetal bovine serum and 10 µg/ml streptomycin) at 37°C and 5% CO2. The calcium phosphate method was used to transiently transfect the cells with plasmid cDNA encoding green fluorescent protein (GFP) and NMDAR subunits in pCI-neo (5:1:1 for GFP: GluN1: GluN2A or GluN2A(M817V)), as previously described (Hansen et al., 2013). Each well was transfected with 12.5 mM CaCl₂, 2.5 mM BES solution (N,N-Bis(2-hydroxyethyl)-2-aminethanesulfonic acid, N,N-Bis(2-hydroxyethyl)taurine; 14 mM NaCl; 75 µM Na₂HPO₄, pH 6.95) and 1 µg/ml DNA mixture for 4 hours at 37°C. The cells were then incubated overnight in standard media with NMDAR antagonists AP-5 (D,L-2-amino-5-phosphonovalerate, 200 µM) and 7-CKA (7-chlorokynurenic acid, 200 µM).

Whole Cell Voltage Clamp and Single Channel Recordings

Whole cell and single channel voltage clamp recordings were performed on transiently transfected HEK 293 cells 12-72 hours post transfection (Yuan et al., 2009). The patch electrodes (resistance 3-5MΩ) for whole cell voltage clamp current recordings were pulled from thin-walled glass micropipettes (TW150F-4, World Precision Instruments, Sarasota, FL, USA) by a dual-stage glass micropipette puller (PC-10, Narishige, Tokyo, Japan) and filled with internal solution that contained (in mM) 110 D-gluconate, 110 CsOH, 30 CsCl, 5 HEPES, 4 NaCl, 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 D-mannitol, 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, USA) 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 piezoelectric translator (Burleigh Instruments, Newton, NJ, USA). The current response time course was fitted using ChanneLab (Synaptosoft, Decatur, GA, USA) by the equation below:

\[
Response = Amplitude_{FAST} \exp(-\text{time}/\tau_{FAST}) + Amplitude_{SLOW} \exp(-\text{time}/\tau_{SLOW})
\]

Equation 5

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 (G150F-4, Warner Instruments Inc., Hamden, CT, USA), pulled by a dual-stage glass micropipette puller (PC-10, Narishige, Tokyo, Japan), and coated with Sylgard (Dow Corning, Midland, MI, USA); the tip was fire-polished. The same internal pipette solution in whole-cell recordings was used. 1 mM glutamate and 50 μM glycine were added to the external recording solution that contained (in mM) 150 NaCl, 10 HEPES, 3 KCl, 0.5 CaCl₂, and 0.01 EDTA (pH 7.4), and this solution used to activate NMDARs in outside-out patch recordings. Currents were digitized at 40 kHz and anti-aliased low pass filtered at 8 kHz (-3 dB Bessel 8-pole; Frequency Devices, Ottawa, IL, USA). Unitary currents in excised outside-out patches were idealized in QUB (http://www.qub.buffalo.edu; Auerbach and Zhou, 2005; Qin, 2004), and maximum likelihood fitting of open and shut intervals was accomplished with ChanneLab (Synaptosoft, Decatur, GA, USA). 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 ($P_{\text{OPEN}}$) 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 $P_{\text{OPEN}}$ since periods with two or more channels open are omitted from analysis.

All reagents were purchased from Sigma (unless otherwise stated). Data were expressed as mean ± SEM and analyzed statistically using unpaired $t$ test. Significance for all tests was set at $p < 0.05$ unless otherwise noted. Error bars in all figures are SEM. 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 $\text{GRIN2A}$ 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”) (Polymorphism Phenotyping v2; 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. 1A,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 (Talukder et al., 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-270.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 (Biologics Suite 2016-3: BioLuminate, version 2.4, Schrödinger, LLC, New York, NY, 2016). The change in stability (\( \Delta \) 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 (\( \Delta \) 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 \( \Delta \) 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 & 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 \( \Delta \) 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 modelling showed that Met817 protrudes towards 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 likely a pathogenic or disease-associated variant (see http://exac.broadinstitute.org/about; Ogden et al, 2017). We performed molecular dynamics simulations to calculate and compare changes in the residue movement of the tetrameric GluN1/GluN2A subunits harboring the GluN2A(M817V) mutant to WT receptors, using root-mean-square fluctuation (RMSF) as a metric (Fig. 2). In the heterodimeric GluN2A(M817V) mutant receptor, the pre-M1 region of GluN1 subunit (residues 552-559) showed enhanced RMSF values compared with the WT receptors (3.45-6.77 Å and 2.16-4.99 Å for mutant vs. 2.25-4.99 Å and 2.47-3.45 Å WT chains A and C, respectively) (Fig. 2B,C). We also monitored the distance of Cα 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 S1). 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 to 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 (EC$_{50}$) of agonists showed that GluN2A(M817V) increased the glutamate potency by 9.5-fold, with EC$_{50}$ values of 0.39 µM compared to 3.7 µM for mutant and WT receptors, respectively (*Fig. 3A* and *Table 1*). Similarly, the potency of glycine for GluN2A(M817V) was increased 7.3-fold compared to the WT GluN2A, with the EC$_{50}$ value decreasing to 0.15 µM from 1.1 µM for WT receptors (*Fig. 3B* and *Table 1*). These data suggest that the GluN2A(M817V)-containing NMDARs can be activated by a lower concentration of agonists.

Since 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 (EC$_{50}$ 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 (EC$_{50}$ values for 2A/2A 1.0 ± 0.04 µM, n = 8; M817V/2A 0.8 ± 0.05 µM, n = 6; M817V/M817V 0.47 ± 0.05 µM, n = 6).
M817V/2A $\pm$ 0.34 ± 0.02 µM, n = 7; M817V/M817V $\pm$ 0.14 ± 0.01 µM, n = 8, *p<0.05, one way ANOVA, compared with WT 2A/2A; **Fig. 3C,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 Mg$^{2+}$, protons, and Zn$^{2+}$ (Traynelis et al., 2010; Paoletti et al., 2013). Two experiments were performed to evaluate the Mg$^{2+}$ inhibition on GluN2A(M817V). The concentration-response curves (**Fig. 4A; Table 1**) showed a reduced potency for Mg$^{2+}$ inhibition for GluN2A(M817V) with an increased IC$_{50}$ value of 80 µM compared to 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 to WT receptors at a holding potential of -60 mV. Fitting the current-voltage curves obtained in the presence of 300 µM Mg$^{2+}$ to the Woodhull equation revealed that the affinity of Mg$^{2+}$ in the absence of an electric field, K$_{D,0 \text{mV}}$, was decreased from 8.5 mM in WT GluN2A to 19.7 mM in GluN2A(M817V); there was virtually no change in the product z$\delta$ (WT 2.32, M817V 2.35), suggesting the mutations does not alter the apparent depth of the binding site in the electric field.

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 IC$_{50}$ value corresponding to pH 6.2 compared to 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 physiological
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 following 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 following 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 ms compared to 43 ms for WT GluN1/GluN2A ($p < 0.0071$, unpaired t-test, controlled FWER 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 responses to prolonged (1.5 sec) 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 ms compared to 40 ± 3.0 ms for WT GluN2A ($p = 6.5E-05$, unpaired t-test). 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 utilize 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 and 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 and 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 ms vs. 2.1 ms for WT). In addition, there was a 3.5-fold decrease in mean shut time (4.3 ms vs 2.1 ms for WT) for these multi-channel patches, suggesting both an increase in single channel open probability within a burst (POPEN: 0.85 vs. 0.43 for WT) and in opening frequency (Fig. 6C,D and Table 2). To further evaluate the effects of this mutation on single channel open probability, we measured the degree of MTSEA (2-aminoethyl methanethiosulfonate hydrobromide) potentiation on a GluN1/GluN2A receptor with a mutation in the GluN1 SYTANLAFF gating region, allowing subsequent 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 prior to 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. S2).

The Function of GluN2A(Met817) is Conserved for all GluN2 Subunits

Since 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 (EC50 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 to 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 to 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 co-expressed with WT GluN2A subunit showed no significant change in the glutamate EC50 value (5.8 µM vs. 4.5 µM for the WT) and only a modest 1.8-fold increase in glycine EC50 value (2.4 µM vs. 1.3 µM for the WT) (Fig. 7). These findings suggest a different role in gating for this region for GluN1 compared to 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 -0.24 kcal/mol for the two GluN1 polypeptide chains in the tetramer (referred to as A and C, see Karakas & 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 vs. 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 to M817V in GluN2A.

**Pharmacology on GluN2A(M817V) Mutant Receptors**

Since 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, 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 IC$_{50}$ of
29 μM for mutant compared to 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 to 8.4 μM for WT GluN2A (Fig. 8). TCN-201 was 9-fold less potent on the mutant receptors (2.7 μM vs 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 to the WT receptors (1.8-fold and 1.9-fold, respectively; Fig. 8). Amantadine potency was modestly reduced 2.4-fold for mutant compared to WT receptors. Tomoxetine, an anti-depressant, had an IC50 value of 13 μM on the mutant NMDARs compared to 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 to 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, LKS), Rolandic epilepsy (RE), the continuous spike-and-waves during slow-wave sleep syndrome (CSWSS), and benign epilepsy with centrotemporal spikes (BECTS) (Endele et al., 2010; de Ligt et al., 2012; Lesca et al., 2013; Carvill et al., 2013; Lemke et al.,
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 3-7-fold more sensitive to agonist (Table 1) than WT receptors, indicating the activation of GluN2A(M817V)-containing NMDARs may occur with low agonist concentrations that 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. The mutant also showed significantly reduced inhibition by endogenous extracellular magnesium, protons and zinc. Together these factors are predicted to increase the charge transfer occurring during synaptic transmission by over 10-fold (Table 2). 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 MRI scans, as well as the cognitive impairment, motor deficit, and other neurological 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 recent studies (Talukder et al., 2010, 2011; Salussolia et al., 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 position of residue methionine at position 817 in the closed AMPAR and NMDAR structures (Karakas and Furukawa 2014; Lee et al., 2014; 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 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 SYTANLAFF 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 (Sobolevsky et al., 2009; Jones et al., 2002; Yuan et al., 2005; Karakas and Furukawa, 2014; Karakas et al., 2015). We hypothesize this trio of interactions plays a central role in gating, as well as mediating inter-
subunit 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 to the WT methionine sidechain, which protrudes towards and interacts with the M3 and M1 helices of the GluN1 subunit (Fig. 9B). These finding 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 inter-subunit 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 classical antiepileptic drugs including levetiracetam, clonazepam and valproic acid (Venkateswaran et al., 2014). Although the addition of antiepileptic medications targeting the glutaminergic pathway and γ-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 (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, and Yuan.

*Conducted experiments*: Chen, Tankovic, Kusumoto, and Yuan.

*Performed data analysis*: Chen, Tankovic, Burger, Traynelis, and Yuan.

*Performed molecular modelling*: Burger.

*Wrote or contributed to the writing of the manuscript*: Chen, Tankovic, Burger, Kusumoto, Traynelis, and Yuan.
REFERENCES


Footnotes

Chen W. and Tankovic A. contributed to this work equally

**Funding:** This work was supported by NIH-the Eunice Kennedy Shriver National Institute of Child Health & Human Development (NICHD) [grant R01HD082373], the Xiangya-Emory Medical Schools Visiting Student Program; the Emory+Children’s Pediatric Center Seed Grant Program, the National Center for Advancing Translational Sciences of the National Institutes of Health [under Award Number UL1TR000454], NIH- National Institute of Neurologic Disorders and Strok (NINDS) [grants R01NS036654, R01NS065371, and R24NS092989]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies.

**Competing Financial Interests:** S.F.T. is a consultant of Janssen Pharmaceuticals, Inc., Pfizer Inc, Boehringer Ingelheim Pharma GmbH & Co. KG, and co-founder of NeurOp Inc.
FIGURE LEGENDS

Figure 1. Location of a *do novo* missense mutation GluN2A(M817V).

(A) A schematic topology showing domain architecture of *GRIN2A*/GluN2A and protein sequence alignment showing transmembrane domain M4 across vertebral species and all NMDAR GluN subunits. The residue methionine at position 817 harboring the mutation is located at the beginning of transmembrane domain M4 and is conserved in vertebral species and among all GluN subunits. ATD, amino terminal domain; S1 and S2, first and second polypeptide sequences comprising the agonist binding domain (ABD); M1, M3, and M4, transmembrane domains; M2: re-entrant pore loop; CTD, carboxy terminal domain.

(B,C,D) A homology model of a tetrameric GluN1/GluN2A receptor built from GluN1/GluN2B (Karakas and Furukawa, 2014; Lee et al., 2014). The GluN1 is orange and the GluN2A is blue. The residue harboring the M817V mutation is highlighted in magenta (B). The expanded side view (C) shows the location of Met817 (cyan), superimposed mutant Val817 (magenta), and possible interactions with transmembrane domain M1 and M3 of GluN1 subunit.

(D) The GluN1/GluN2A diheteromeric transmembrane region (ATD and LBD removed) viewed from the top down through the pore. *Left panel*, GluN1/GluN2A transmembrane region of GluN2A(M817V). *Right panel*, an expanded view of the M817V mutation (M colored in cyan and V colored in magenta) and its location among the GluN1 transmembrane helices; GluN2A-M817 protrudes and interacts with the M1 and M3 helices of GluN1. Met817 is also in close proximity to the pre-M1 helix.

Figure 2. Introducing the M817V into GluN2A increased movement of the GluN1 Pre-M1
(A) The root mean square fluctuation (RMSF) of the MD simulations were evaluated from molecular dynamics simulations in a homology model of a GluN1/GluN2A tetrameric receptor built from GluN1/GluN2B (Karakas and Furukawa, 2014; Lee et al., 2014; see Materials and Methods). The box highlights a region of interest shown in panels B (expanded side view) and C (expanded top view). The thickness of the ribbon denotes the magnitude of the root mean squared fluctuations in atomic position, which identified regions with more thermal motion and presumably higher energy. Introducing GluN2A(M817V) into the tetrameric complex markedly increased fluctuation of the GluN1 pre-M1 region, as can be seen by the increase diameter of the ribbon. The ribbon thickness was selected to illustrate the difference within this polypeptide chains in this simulation, and is not shown at comparable scale to atomic distances.

Figure 3. GluN2A(M817V) increases the agonist potency.

(A,B) Composite concentration-response curves for glutamate and glycine on di-heteromeric GluN1/GluN2A (WT 2A) and GluN1/GluN2A(M817V). Fitted parameters are shown in Table 1. (C,D) Composite concentration-response curves for glutamate and glycine at tri-heteromeric receptors GluN1/GluN2A/GluN2A (labelled 2A/2A), GluN1/GluN2A(M817V)/GluN2A (labelled M817V/2A) and GluN1/GluN2A(M817V)/GluN2A(M817V) (labelled M817V/M817V). The potency of glutamate (in the presence of 100 µM glycine) and of glycine (in the presence of 100 µM glutamate) were evaluated by TEVC recordings on *Xenopus* oocytes at holding potential of -40 mV (See Materials and Methods).

Figure 4. GluN2A(M817V) reduces sensitivity to endogenous negative modulators.
(A,B) Composite Mg\(^{2+}\) concentration-response curves and Mg\(^{2+}\) current-voltage (I-V) curves for di-heteromeric receptors indicate a decreased Mg\(^{2+}\) inhibition of GluN2A(M817V)-containing receptors. For the I-V curve, all responses were normalized to those recorded at +30 mV.

(C,D) Composite inhibitory concentration-response curves for proton and zinc show decreased proton and zinc inhibition in GluN2A(M817V)-containing receptors. The data were generated by TEVC recordings on Xenopus oocytes at a holding potential of -60 mV for Mg\(^{2+}\) concentration-response curves, -40 mV for proton concentration-response curves, and -20 mV for zinc concentration-response curves. Fitted parameters are given in Table 1.

**Figure 5. GluN2A(M817V) prolongs synaptic-like response time course.**

The representative current response time course from a whole cell voltage clamp recording (V\(_{\text{HOLD}}\) -60 mV) of GluN1/GluN2A (WT 2A) and GluN1/GluN2A(M817V) (M817V) receptors to rapid application of long (1.5 sec) (A) and brief (5 ms) (B) duration of 1mM glutamate. Responses are shown normalized to the WT response at the moment glutamate was removed. Saturating glycine (50 µM) was present in all of solutions. Fitted parameters are given in Table 2.

**Figure 6. GluN2A(M817V) alters the single channel properties.**

(A,B) Representative steady-state recordings from an outside-out patch containing GluN1/GluN2A (WT 2A) (A) or GluN1/GluN2A(M817V) (M817V) NMDARs (B) excised from transfected HEK 293 cells. Unitary currents were activated in these excised outside-out patches by saturating concentrations of co-agonists (1 mM glutamate and 50 µM glycine) at a holding
potential of -80 mV. Data are shown on two different time scales. “c” indicates the closed state and “o” indicates the open state.

(C,D) The pooled open and shut time duration histograms are shown for all WT GluN2A and the GluN2A(M817V) single channel recordings. Open time duration histograms were made from 129,255 open periods for the WT GluN2A and 234,470 open periods for the GluN2A(M817V) containing NMDARs. Closed duration histograms were made from 128,466 closed periods for the WT GluN2A and 221,877 closed periods for the Glu2A(M817V) mutation. Data are from 7 patches that contained WT GluN1/GluN2A and 5 patches that contained GluN1/GluN2A(M817V); fitted parameters are given in Table 2.

Figure 7. Conserved effects of GluN2A(M817V) on agonist potency across GluN2 subunits.

Composite concentration-response curves were determined by TEVC recordings ($V_{\text{HOLD}}$ -40 mV) from *Xenopus* oocytes expressing different GluN-containing receptors harboring Met-Val mutation at the same position as 817 in GluN2A: GluN1/GluN2A and GluN1(M813V)/GluN2A; GluN1/GluN2B and GluN1/GluN2B(M818V); GluN1/GluN2C and GluN1/GluN2C(M815V); GluN1/GluN2D and GluN1/GluN2D(M845V). Upper panels (A) show the composite glutamate concentration-response curves determined in the presence of 100 µM glycine. Lower panels (B) show the composite glycine concentration-response curves determined in the presence of 100 µM glutamate. Mean ± SEM fitted EC$_{50}$ values in µM and numbers are given.

Figure 8. The effects of NMDAR-targeted inhibitors including FDA-approved drugs on GluN1/GluN2A(M817V) receptors.
Composite concentration-response curves of FDA-approved NMDAR antagonists (A, memantine; B, dextromethorphan; C, dextrophan; D, amantadine; E, ketamine; and, F, tomoxetine) were obtained from TEVC recordings from Xenopus oocytes expressing GluN1/GluN2A (WT 2A) and GluN1/GluN2A(M817V) in the presence of 100 µM glutamate and 100 µM glycine at holding potential of -40 mV. The concentration-response curve for the non-competitive GluN2A-selective inhibitor (G, TCN 201) was determined in 3 µM glycine. Mean ± SEM fitted IC50 values in µM and maximal percentage inhibition are given.

Figure 9. Potential interaction of methionine at 817 with pre-M1 helix and SYTANLAADF motif.

(A) The GluN1/GluN2A transmembrane region viewed from the top down through the pore. Right panel: an expanded view of the residues leucine at 812 (colored in magenta) and methionine at 817 (colored in cyan) and their locations relative to the GluN1 pre-M1 helix (colored in red) and GluN1 M3 SYTANLAADF motif (colored in grey).

(B) GluN2A-M817 (colored in cyan) is close enough to interact with the GluN1 pre-M1 helix (colored in red) and M3 SYTANLAADF motif (colored in grey) of GluN1: side view (left panel) and top view (right panel). Only GluN2A-M4, GluN1-M1/pre-M1, and GluN1-M3 are shown. The position of GluN2A(L812M) is show for comparison.

Supplemental_GluN2A-WT-270.pdb. A GluN2A wild-type structure used for the MD simulations. The pdb structure (excluding the amino terminal domains) represents the 270th frame of the GluN2A wild-type structure captured during a 10 ns molecular dynamics simulation. This structure was used to calculate the effect on the Δ stability and Δ affinity after introducing
the M817V mutation.
Table 1. Summary of pharmacological data for M817V

<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)*</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)*</td>
<td>2.07E-07</td>
</tr>
<tr>
<td>Mg2+, IC50, μM (n)</td>
<td>24 ± 2.5 (16)</td>
<td>80 ± 16 (15)*</td>
<td>9.94E-06</td>
</tr>
<tr>
<td>Mg2+ IV (n)</td>
<td>13 ± 1.3% (7)</td>
<td>25 ± 2.8% (6)*</td>
<td>0.00322</td>
</tr>
<tr>
<td>Proton, % (n)</td>
<td>41 ± 1.5 (9)</td>
<td>89 ± 1.7 (8)*</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>ND</td>
<td>—</td>
</tr>
<tr>
<td>% inhibition by zinc</td>
<td>60 ± 3.3% (8)</td>
<td>31 ± 4.8% (8)*</td>
<td>0.00019</td>
</tr>
</tbody>
</table>

The data were expressed as mean ± SEM (n); n is the number of oocytes.
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.
*p < 0.05 compared to WT 2A, unpaired t-test. For all comparisons, power to detect a 50% change was 0.8.
× holding potential was -60 mV.
% percentage current remaining in the presence of 0.3 mM MgCl2 at -60 mV.
\[\text{percentage of the current at pH 6.8 compared to that at pH 7.6.}\]
\[\text{at 300 nM of zinc.}\]
<table>
<thead>
<tr>
<th></th>
<th>WT 2A</th>
<th>M817V</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amplitude (peak, pA/pF)</strong></td>
<td>125 ± 33</td>
<td>267 ± 91</td>
<td>0.239</td>
</tr>
<tr>
<td><strong>Amplitude (SS, pA/pF)</strong></td>
<td>73 ± 15</td>
<td>196 ± 64</td>
<td>0.145</td>
</tr>
<tr>
<td>$I_{ss}/I_{peak}$</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>$\tau_{fast}$ deactivation (ms)</td>
<td>31 ± 3.0</td>
<td>416 ± 100*</td>
<td>0.006</td>
</tr>
<tr>
<td>$\tau_{slow}$ deactivation (ms)</td>
<td>429 ± 138</td>
<td>1207 ± 259</td>
<td>0.033</td>
</tr>
<tr>
<td>%$\tau_{fast}$ deactivation</td>
<td>97%</td>
<td>71%</td>
<td>—</td>
</tr>
<tr>
<td>$\tau_{w}$ deactivation (ms)</td>
<td>43 ± 3.2</td>
<td>632 ± 103*</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>N</td>
<td>6</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>$P_{open}$ (from channels)$^a$</td>
<td>0.43 ± 0.03</td>
<td>0.85 ± 0.02**</td>
<td>6.2E-07</td>
</tr>
<tr>
<td>Mean open time, ms</td>
<td>2.1 ± 0.1</td>
<td>4.3 ± 0.3**</td>
<td>6.5E-06</td>
</tr>
<tr>
<td>$\tau_1$, ms (% area)</td>
<td>0.1 ± 0.01 (23)</td>
<td>0.2 ± 0.02 (6)**</td>
<td>0.00055</td>
</tr>
<tr>
<td>$\tau_2$, ms (% area)</td>
<td>2.6 ± 0.1 (77)</td>
<td>4.5 ± 0.3 (94)**</td>
<td>3.4E-05</td>
</tr>
<tr>
<td>Mean shut time, ms$^b$</td>
<td>2.8 ± 0.4</td>
<td>0.8 ± 0.08**</td>
<td>0.00107</td>
</tr>
<tr>
<td>$\tau_1$, ms (% area)</td>
<td>0.2 ± 0.03 (26)</td>
<td>0.1 ± 0.02 (30)</td>
<td>0.157</td>
</tr>
<tr>
<td>$\tau_2$, ms (% area)</td>
<td>0.7 ± 0.1 (30)</td>
<td>0.6 ± 0.08 (35)</td>
<td>0.274</td>
</tr>
<tr>
<td>$\tau_3$, ms (% area)</td>
<td>4.2 ± 0.5 (35)</td>
<td>1.3 ± 0.1 (34)**</td>
<td>0.00043</td>
</tr>
<tr>
<td>$\tau_4$, ms (% area)$^c$</td>
<td>12 ± 2 (10)</td>
<td>5.6 ± 1.0 (14)</td>
<td>—</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>$\gamma$, 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$^d$</td>
<td>439 ± 34</td>
<td>78 ± 6.1</td>
<td>—</td>
</tr>
<tr>
<td>$P_{open}$ (from MTSEA)</td>
<td>0.16 ± 0.01</td>
<td>0.93 ± 0.07$^e$</td>
<td>1.4E-08</td>
</tr>
<tr>
<td>N</td>
<td>10</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

The data were from three separate and different experiments (bounded by horizontal lines) and expressed as mean ± SEM.

$^a$ Channel open probability ($P_{open}$) 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 receptors; mutant channels spent so much time open that there was little chance that the activity without double openings reflected that of 2 channels.

$^b$ Statistical tests were not run on the slowest shut time components given the limited number of periods underlying this component.

$^c$ Evaluated by TEVC recordings on Xenopus oocytes, see Materials and Methods and Supplemental Fig. S2.
Figure-1

A

**GRIN2A**

ATD  S1  M1  M2  M3  S2  M4  CTD

**M4**

- Human: WLTGICHNEKNEVMSSQLDIDNMGVYMLAAAMALSLITFIWEHLFYWKLRFCFTG
- Dog: WLTGICHNEKNEVMSSQLDIDNMGVYMLAAAMALSLITFIWEHLFYWKLRFCFTG
- Rat: WLTGICHNEKNEVMSSQLDIDNMGVYMLAAAMALSLITFIWEHLFYWKLRFCFTG
- Mouse: WLTGICHNEKNEVMSSQLDIDNMGVYMLAAAMALSLITFIWEHLFYWKLRFCFTG
- Chicken: WLTGICHNEKNEVMSSQLDIDNMGVYMLAAAMALSLITFIWEHLFYWKLRFCFTG
- X-Tropicalis: WLTGICHNEKNEVMSSQLDIDNMGVYMLAAAMALSLITFIWEHLFYWKLRFCFTG
- Catfish: WLTGICHNEKNEVMSSQLDIDNMGVYMLAAAMALSLITFIWEHLFYWKLRFCFTG
- Human GluN1: WVRYQECDRS-NAPALTENPMGFPMLVAGGIVAG-IFLIFIEIAKYKRDARRK
- Human GluN2B: WLTGICHNEKNEVMSSQLDIDNMGVYMLGAAMALSLITFIWEHLFYQFRHCMEG
- Human GluN2C: WLTGICHNEKNEKSSQLDIDNMGVYMLLMVAMLALLVFAWHLVYKLRHSDPN
- Human GluN2D: WLTGICHNEKKEVMSSQLDIDNMGVYMLLMVAMLALLVFAWHLVYKLRHSDPN

**M817V**

- M817V pre-M1

**Membrane**

**Pore**

B

GluN1/GluN2A Tetramer

**ATD**

**ABD**

**TM**

C

D

M3

M4

M1

M3

M817V

Pore

pre-M1
Figure-2

WT GluN1/GluN2A tetramer

GluN1/GluN2A tetramer harboring GluN2A(M817V)

NMDAR without the ATD

side view
top view
Figure-3

A) Maximal Response, %

B) Maximal Response, %

C) Maximal Response, %

D) Maximal Response, %
Figure 5

A: Glutamate (WT 2A, M817V)

B: Glutamate (WT 2A, M817V)
Figure-7 NEW

A

WT GluN1/2A
4.5 ± 0.2 (13)
1-M813V/2A
5.8 ± 0.3 (15)

(WT GluN1/2A)
1-M813V/2A

0.01 0.1 1 10

Maximal Response, %

Glutamate, μM

WT 2B
1.3 ± 0.2 (7)
M818V
0.38 ± 0.05 (8)*

WT 2B
1.3 ± 0.2 (7)
M818V
0.38 ± 0.05 (8)*

0.01 0.1 1 10

Maximal Response, %

Glutamate, μM

WT 2C
1.1 ± 0.1 (6)
M815V
0.15 ± 0.01 (8)*

WT 2C
1.1 ± 0.1 (6)
M815V
0.15 ± 0.01 (8)*

0.01 0.1 1 10

Maximal Response, %

Glutamate, μM

WT 2D
0.49 ± 0.07 (9)
M845V
0.12 ± 0.02 (6)*

WT 2D
0.49 ± 0.07 (9)
M845V
0.12 ± 0.02 (6)*

0.01 0.1 1 10

Maximal Response, %

Glutamate, μM

B

WT GluN1/2A
1.3 ± 0.1 (12)
1-M813V/2A
2.4 ± 0.1 (17)*

(WT GluN1/2A)
1-M813V/2A

0.01 0.1 1 10

Maximal Response, %

Glycine, μM

WT 2B
0.45 ± 0.05 (10)
M818V
0.10 ± 0.02 (8)*

WT 2B
0.45 ± 0.05 (10)
M818V
0.10 ± 0.02 (8)*

0.01 0.1 1 10

Maximal Response, %

Glycine, μM

WT 2C
0.23 ± 0.03 (6)
M815V
0.10 ± 0.01 (6)*

WT 2C
0.23 ± 0.03 (6)
M815V
0.10 ± 0.01 (6)*

0.01 0.1 1 10

Maximal Response, %

Glycine, μM

WT 2D
0.18 ± 0.01 (7)
M845V
0.11 ± 0.02 (9)*

WT 2D
0.18 ± 0.01 (7)
M845V
0.11 ± 0.02 (9)*