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Manish Karamchandani April 14, 2015

Functional Analysis of a *De Novo* Missense *GRIN1* Mutation Associated with Intractable Seizures

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An abstract of
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Abstract

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By Manish Karamchandani

NMDA receptors (NMDARs) are a subset of ligand gated ion channel glutamate receptors highly involved in normal neuronal function. Dysfunction of NMDARs has been associated with many neurological disorders such as epilepsy, schizophrenia, and developmental delay. In this paper we characterize the effect of a *de novo* missense mutation in the gene encoding the GluN1 subunit (*GRIN1*) on NMDAR function and regulation. This mutation (GluN1-A652T) was identified in a 7-year-old patient suffering from medically refractory epilepsy and severe epileptic encephalopathy. The amino acid residue affected by the mutation is located in transmembrane domain 3 (M3), a region of the receptor critical for channel gating. Using electrophysiological recordings, we have determined that the mutation increases potency for the agonists glutamate and glycine when co-expressed with GluN2B, -2C, and -2D. These data suggest that the GluN1-A652T mutation may cause hyper-excitation of neurons through NMDAR overactivity, and thus the patient's epileptic condition. This characterization presents the opportunity for pharmacological rescue through the off label use of FDA-approved NMDAR antagonists. Four FDA-approved compounds were evaluated for their ability to inhibit the GluN1-A652T containing NMDAR as a possible therapy for the patient's phenotype.

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Introduction

Glutamate Receptors

Glutamate receptors are an essential part of normal central nervous system (CNS) function. Glutamate, an endogenous, small amino acid neurotransmitter, is the primary excitatory neurotransmitter within the CNS, making glutamate receptors an important part of neural circuitry (Petroff, 2002). Glutamate receptors are highly involved in learning and memory, in processes such as long-term potentiation (LTP) and long-term depression (LTD) (Hunt and Castillo, 2011). While known for their involvement in learning and memory, glutamate receptors are also involved in motor and sensory function, and CNS development (Rossi *et al.*, 2013; Vilar *et al.*, 2013; Mattison *et al.*, 2014). Given the high degree of involvement of glutamate receptors in normal function, any alteration to receptor expression or function could drastically alter CNS function and result in a diseased phenotype. Diseases associated with receptor dysfunction include: excitotoxicity and neurodegeneration, traumatic brain injury and ischemia, seizures, addiction, ADHD, autism, Parkinson's disease, and Huntington's disease, among others (Beal *et al.*, 1992; Bitanihirwe *et al.*, 2009; Chapman, 2000; Cuomo *et al.*, 2009; Diguet *et al.*, 2004; Elia *et al.*, 2011; Schmeisser *et al.*, 2012; Vadasz *et al.*, 2007). Glutamate receptors also exist outside of the CNS, serving a wide range of functions, but the context of this paper will be on the CNS function.

Glutamate receptors can be divided into two classes, ionotropic and metabotropic (Palmada and Centelles, 1998). Ionotropic receptors are involved in mediating ion flow through the neuronal membrane. Ionotropic glutamate receptors are ligand gated ion channels, regulating flow of sodium, potassium, and calcium ions (Traynelis *et al.*, 2010).

Metabotropic receptors mediate intracellular G-protein coupled signaling (Palmada and Centelles, 1998). Metabotropic glutamate receptors modulate ionotropic glutamate receptor expression and function, as well as the risk of excitotoxicity of the neuron (Skeberdis *et al.* 2001; Ambrosini *et al.*, 1995).

Within the class of ionotropic, there are three major types of receptors: AMPA, Kainate, and NMDA (Traynelis *et al.*, 2010). All three types of ionotropic receptors are integral membrane proteins with a central pore formed by four subunits. AMPA receptors (AMPARs) are named for α-amino-3-hydrox-5-methyl-4-isoxazolepropionic acid (AMPA), an artificial glutamate analog that is a selective agonist of the receptor. AMPARs play a critical role in synaptic plasticity, helping depolarize the post-synaptic membrane to induce LTP and LTD, as well as being a part of the potentiated or depressed response (Maren *et al.*, 1993). Kainate receptors, named for the selective agonist kainate, are not as well understood as AMPA and NMDA receptors, but are thought to play a poorly understood role in synaptic plasticity, with expression on both the pre- and post-synaptic membranes (Huettener, 2003; Contractor *et al.*, 2000). NMDA receptors (NMDARs) are named for the selective agonist *N*-methyl-D-aspartate (NMDA), an artificial glutamate analog. NMDARs are critical for processes involved in synaptic plasticity, namely LTP and LTD. NMDARs are expressed throughout the CNS, and are primarily found on the postsynaptic membrane. NMDARs are the primary focus of this paper.

The mechanisms by which glutamate receptors become activated are as follows. First, an action potential propagated down an axon enters the presynaptic terminal. The action potential will depolarize the synaptic terminal and activate voltage gated calcium channels (Nicholls and Attwell, 1990). The influx of calcium will bind proteins to trigger the

release of glutamate containing vesicles and fusing of the vesicles to the presynaptic membrane, releasing glutamate into the synaptic cleft (Figure 1) (Nicholls and Attwell, 1990; Südhof, 2013). Glutamate is then free to bind AMPARs, NMDARs, and kainate receptors, inducing a conformational change of the protein that leads to opening of the ion channels. The subsequent ion flow will allow for continued transmission of the action potential.

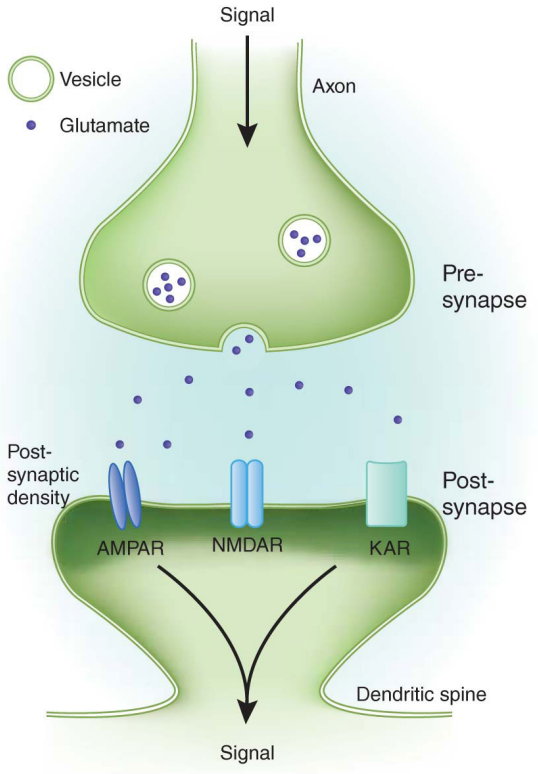

Figure 1: Cartoon of glutamate release (Gécz, 2010).

NMDA receptors

NMDARs are transmembrane, tetrameric assemblies that act as non-selective ion channels that allow for the flow of sodium, potassium, and calcium ions (Traynelis *et al.*, 2010). NMDARs are unique among glutamate receptors in that they require simultaneous

binding of glutamate and glycine, another endogenous amino acid. At resting membrane potentials, NMDAR channel pores are blocked by magnesium ions. In addition to activation by glutamate and glycine, depolarization of the post-synaptic membrane is required in order to reduce the block and conduct ion flow (Traynelis *et al*., 2010).

NMDARs are chiefly responsible for synaptic plasticity through the processes of LTP and LTD (Hunt and Castillo, 2011). Synaptic plasticity, which is the ability of the synapse to strengthen or weaken the connection between the pre-and post- synaptic neurons, is related to LTP and LTD through a calcium dependent signaling pathway. In the case of LTP, once calcium enters through NMDAR channels it will bind protein kinases that then phosphorylate AMPARs, increasing the conductance of those channels, as well as recruiting more AMPARs to the synaptic membrane (Figure 2) (Song and Huganir, 2002). The calcium also serves to activate a second messenger cascade that increases the activity of protein kinase A (PKAII) as well as Ca^{2+}/calmodulin dependent protein kinase A II (CaMKII), which increases the degree of phosphorylation of AMPARs (Sweatt, 1999; Hunt and Castillo, 2011). Conversely, low calcium will have the opposite effect, activating protein phosphatases that dephosphorylate AMPARs and lead to internalization of AMPARs (Beattie *et al.*, 2000).

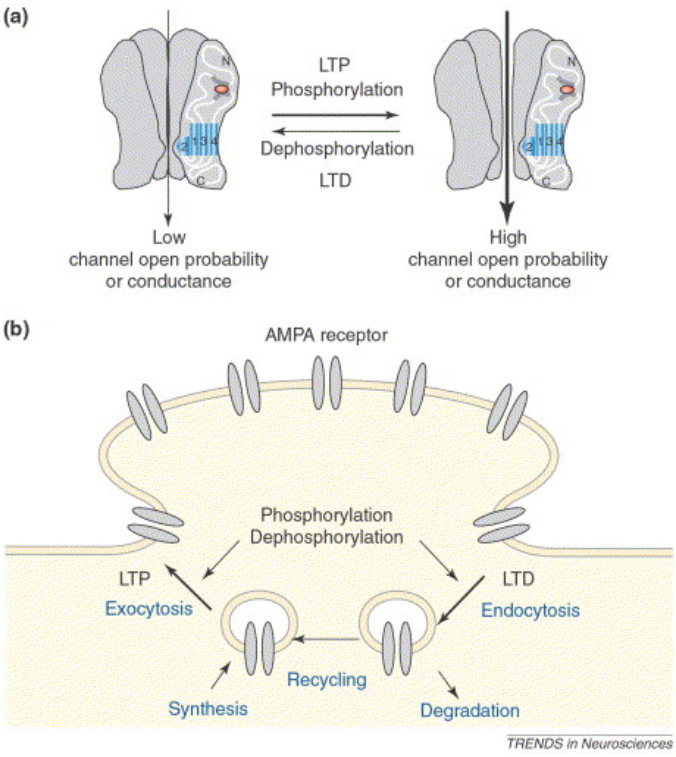

Figure 2: Cartoon model depicting AMPAR regulation during LTP and LTD (Song and Huganir, 2002).

As NMDAR subunits are proteins, they contain several domains that affect receptor function. NMDAR subunits consist of the amino terminal domain (ATD), the ligand binding domain (LBD), the transmembrane domain (TMD), and the carboxyl terminal domain (CTD) (Figure 3) (Traynelis *et al.*, 2010). The ATD and LBD are both extracellular and bind a host of agonists, antagonists, and modulators. The TMD spans the cellular membrane and is critical for channel gating. The TMD consists of four domains: M1, M2, M3 and M4. The CTD is intracellular and is responsible for regulation and trafficking of the receptor (Traynelis *et al.*, 2010).

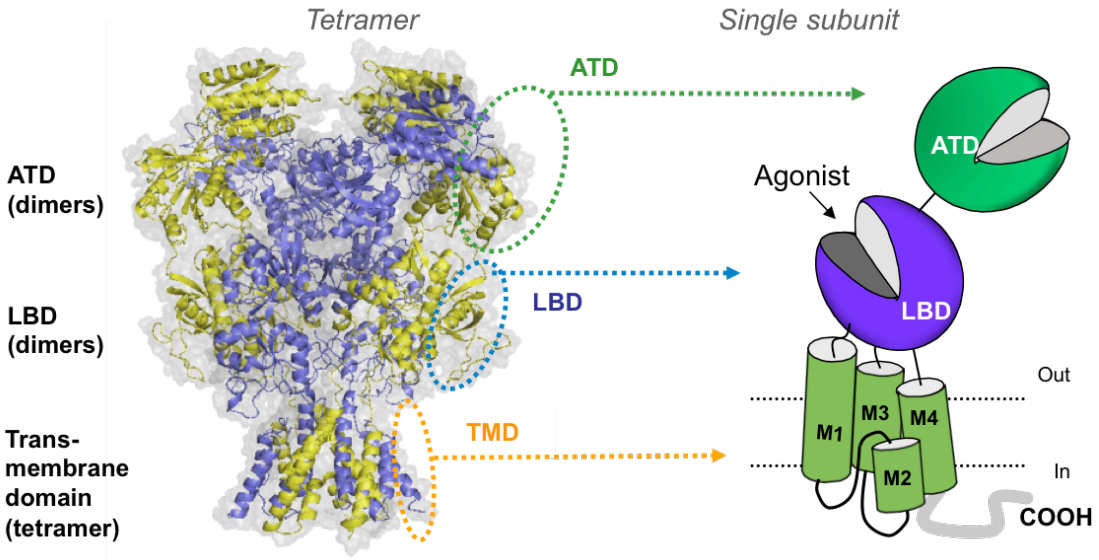

Figure 3: Model and cartoon depicting quaternary structure of NMDAR as well as tertiary structure of a single subunit. Courtesy of Traynelis laboratory and Dr. Pieter Burger.

The structure of NMDARs has been described as a dimer of dimers. The tetramer is composed of two GluN1 subunits and two GluN2 subunits that arrange to form an ion pore (Figures 3, 4) (Traynelis *et al.*, 2010). GluN1 subunits exist as eight splice variants of the *GRIN1* gene, while GluN2 subunits are encoded by four genes known as *GRIN2A*, *GRIN2B*, *GRIN2C*, and *GRIN2D*. The GluN1 subunit is always expressed and present in the tetramer assembly. GluN1 subunits control phosphorylation, deactivation time course, and modulator sensitivity. GluN2 subunits are differently expressed both temporally and spatially, and control functional properties of the receptor (Traynelis *et al.*, 2010). The different GluN2 subunits are known as GluN2A, GluN2B, GluN2C, and GluN2D. Typically, the GluN2 subunits within a receptor are of the same type (e.g. two GluN2A subunits paired with two GluN1 subunits), however in some regions of the brain the receptor can be

triheteromeric, with the GluN2 subunits being of different types (e.g. one GluN2A subunit and one GluN2B subunit paired with two GluN1 subunits) (Traynelis *et al.*, 2010).

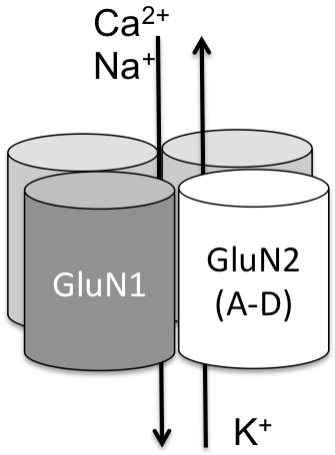

Figure 4: Cartoon model depicting subunit pairing and ion pore. Courtesy of Traynelis laboratory.

While GluN2 subunits are similar in structure, their differences can control channel function, particularly the deactivation time course. GluN2A channels have a faster deactivation time course than other subunits (Vicini *et al.*, 1998). GluN2B subunits exhibit slower recovery from desensitization than GluN2A subunits, and exhibit the second fastest deactivation time course. GluN2C subunits have the third fastest deactivation time course. Finally, GluN2D subunits have the slowest deactivation time course (Vicini *et al.*, 1998). Expression of the subunits is also different, both temporally and spatially (Figure 5). Using a rat model, mRNAs for GluN2B and GluN2D have been detected prenatally, pointing to their involvement in embryonic CNS development, while mRNAs for GluN2A and GluN2D have been first detected near birth (Monyer *et al.*, 1994). In adult rats, mRNAs for GluN2A were detected primarily in the cerebral cortex while GluN2B mRNAs were detected in the cerebrum and the thalamus. For GluN2C, mRNAs were primarily detected in the

cerebellum. GluN2D mRNAs were detected in the brainstem and diencephalon (Figure 5) (Akazawa *et al.*, 1994).

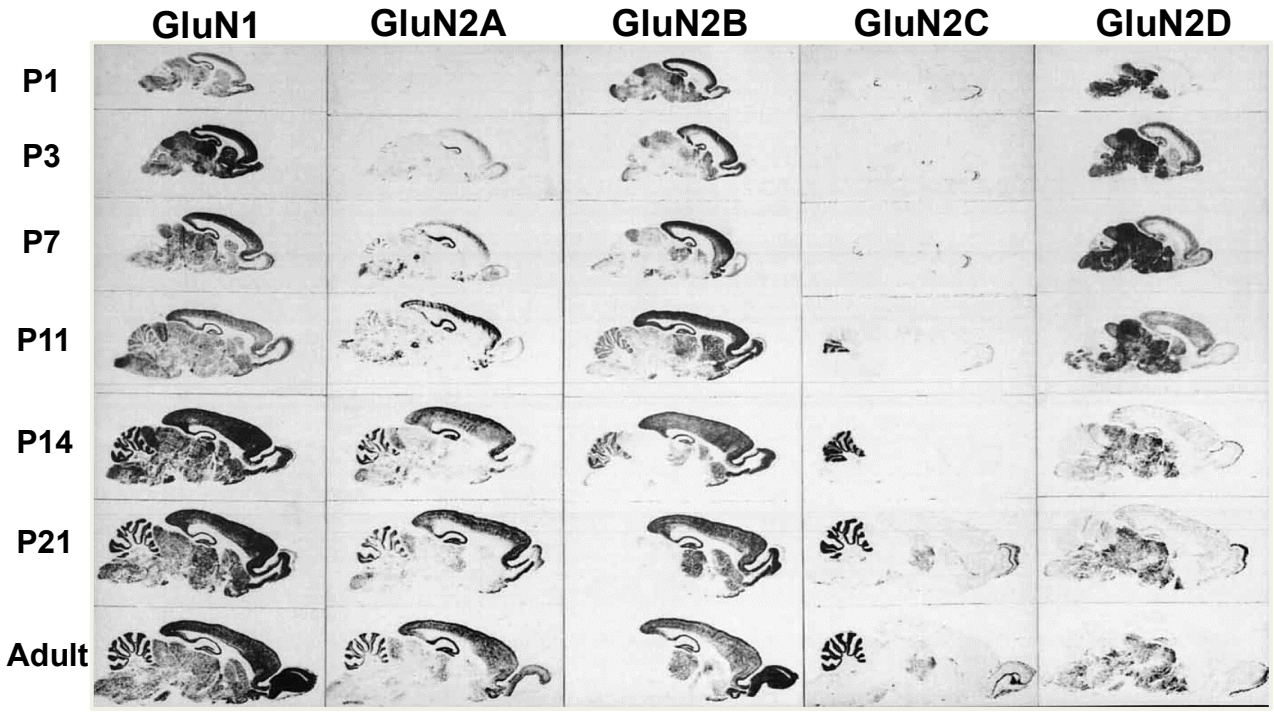

Figure 5: Negative film images of rat brain *in situ* hybridization. (Akazawa *et al.*, 1994).

NMDAR modulators act on different domains to change the protein conformation or block the channel in order to exert their effect. The two most common endogenous ligands are glutamate and glycine (Table 1). Glutamate binds the LBD of GluN2 subunits, while glycine binds the LBD of GluN1 subunits (Traynelis *et al.*, 2010). The major negative modulators that are of interest for this paper are zinc ions, protons, and magnesium ions (Table 1). Zinc ions inhibit NMDARs through two mechanisms (Rachline *et al.*, 2005; Amico-Ruvio *et al.*, 2011). The first mechanism is high affinity binding to the ATD of GluN2A that reduces channel open probability, and the second is a low affinity voltage dependent binding to amino acid residues lining the pore that blocks the channel (Paoletti

et al., 1997; Amico-Ruvio *et al.*, 2011). Magnesium ions inhibit the channel in a similar manner as the low affinity zinc ion block. The amino acid residues lining the pore are strongly negative, attracting the positively charged ion into the pore and blocking it (Ruppersberg *et al.*, 2002). As the synaptic membrane becomes depolarized, the positive charge will repel the magnesium ion, allowing for ion flow. It is important to note that this block exists at resting membrane potentials, but can be applied at a higher concentration in vitro to study channel function. Protons inhibit NMDARs by interacting with charged residues on GluN1 to change the conformation of the receptor and gate the receptor (Low *et al.* 2003). The residues are believed to be a part of the linker between M3 and a portion of the LBD.

Some common NMDAR antagonists used to treat a variety of disorders and diseases include memantine, amantadine, and dextromethorphan/dextrorphan (Table 1). Each of these drugs will be tested in this paper. Memantine, a drug used for the treatment of Alzheimer's disease, is a channel blocker that acts non-competitively (Chen *et al.*, 1997). Amantadine is used for the treatment of Parkinson's disease, and is also a channel blocker. Additionally, amantadine stabilizes the closed state of the channel, accelerating closure of the channel (Blanpied *et al.*, 2005). Dextromethorphan is an antitussive, with dextrorphan being its major metabolite (Wong *et al.*, 1987). Dextromethorphan inhibits the receptor by blocking the ion channel (Netzer *et al.*, 1993). Dextrorphan, like its parent drug, is a noncompetitive channel blocker (Finnegan *et al.*, 1989).

Table 1: Modulators listed with the associated subunit, binding site, and effect.

Modulator	Subunit	Binding site	Effect
Glutamate	GluN2	LBD	Agonist
Glycine	GluN1	LBD	Agonist
Zinc ion	GluN2A	ATD/Channel	Antagonist
Magnesium ion	---------	Channel	Antagonist
Proton	GluN1	TMD/LBD	Antagonist
Memantine	---------	Channel	Antagonist
Amantadine	---------	Channel	Antagonist
Dextromethorphan	---------	Channel	Antagonist
Dextrorphan	---------	Channel	Antagonist

NMDAR Mutations

As previously mentioned, the high degree of involvement of glutamate receptors in normal functioning of the CNS points to a possible role in neurological disorders. As of the writing of this paper, there have been 169 identified NMDAR mutations associated with various neurological disorders such as ADHD, autism spectrum disorder, developmental delay/mental retardation, epilepsy, intellectual disability, and schizophrenia (Table 2). Many more mutations continue to be identified. For the mutations identified, there does not seem to be a domain that is more responsible for disease than the others, as 42 were associated with the ATD, 38 with the LBD, 32 with the TMD, and 57 with the CTD (Table 3). However when analyzing the mutations among subunits 81 were associated with GluN2A, 36 with GluN2B, 31 with GluN2C, 12 with GluN2D, and 9 with GluN1, pointing to diseases that have a higher prevalence with certain subunits. The high number associated with GluN2A could point to a higher penetrance of disease with mutations in this subunit. The low number associated with GluN1 probably relates to the necessity of a functional copy of

the subunit. As GluN1 is expressed with every receptor assembly, a single mutation could disrupt function globally resulting in altered CNS development and have severe consequences for the fetus.

Table 2: Human NMDAR mutations in developmental disorders. Courtesy of Dr. Hongjie Yuan.

Subunit	Total	ADHD	ASD	DD/MR	Epi	ID	SZ
GluN1	9	0	1	2	4	4	2
GluN2A	81	8	4	39	60	22	7
GluN2B	36	2	10	3	5	15	8
GluN2C	31	0	8	0	0	14	10
GluN2D	12	0	3	1	1	1	9
Total	**169**	10	26	45	70	56	36

ADHD: Attention Deficit Hyperactivity Disorder; ASD: Autism Spectrum Disorder; DD: Developmental Delay; Epi: Epilepsy; ID: intellectual disability; MR: Mental Retardation; SCZ: Schizophrenia. *many mutations have more than one phenotype. Combination of published and unpublished data (see text for references).

Table 3: Locations of human NMDAR mutations. Courtesy of Dr. Hongjie Yuan.

Domain	GluN1	GluN2A	GluN2B	GluN2C	GluN2D	Total
ATD	2	24	8	7	1	42
LBD	1	22	10	3	2	38
TMs + linker	6	12	9	3	2	32
CTD	0	23	9	18	7	57
Total	9	81	36	31	12	169

ATD is the amino terminal domain, LBD is the ligand binding domain, TMs are the membrane associated elements, linkers are short regions of the polypeptide chain between the various domains, and CTD is the intracellular C-terminal domain.

The disorder of note in Table 2 is epilepsy, which will be the focus of this paper. Epilepsy has been associated with 70 NMDAR mutations, mostly with the GluN2A subunit.

In studies of common childhood epilepsy syndromes, mutations in the *GRIN2A* gene were found and linked to the syndromes (Lesca *et al.*, 2013; Lemke *et al.*, 2013; Carvill *et al.*, 2013). While epilepsy is highly associated with GluN2A, it is important to note that epilepsy is also associated with 4 GluN1 mutations, as this is of relevance to the patient described later in this paper.

Mutations in the subunits can affect surface trafficking of the receptor, thus contributing to the diseased phenotype. Glutamate binding is necessary for GluN2B subunit surface trafficking, with mutations in the LBD of the subunits decreasing surface trafficking (She *et al.*, 2012). It has also been determined that there is a motif in the M4 in GluN2 subunits responsible for release of NMDARs from the endoplasmic reticulum (ER) (Stephenson *et al.*, 2009). Mutations in the motif resulted in retention of NMDARS in the ER. While GluN2 subunits can control release of NMDARS from the ER, GluN1 subunits can control the rate of release (Stephenson *et al.*, 2009). Additionally, the CTD interacts with scaffold proteins that assist NMDARs in trafficking (Stephenson *et al.*, 2009). While channel function is important for normal functioning, it is not the only factor that can cause disease. If such mutations that affect intracellular receptor trafficking were to occur in a human, this could lead to under activation of the neuron and circuitry, potentially causing a neurological disorder.

Patient's Information

The focus of this paper is on a female pediatric patient suffering from epilepsy. Epilepsy is defined as a condition of abnormal and increased neural activity that leads to seizures (Fisher *et al.*, 2005). Aged 7, she has medically refractory epilepsy (unresponsive

to medication) as well as severe epileptic encephalopathy, meaning that her neuronal tissue is degenerating, likely due to her epilepsy. As a result of her condition, she is non-ambulatory (unable to walk) and non-communicative. Her DNA as well as that of her family was sent to Baylor College of Medicine, where her exome was sequenced. It was determined that she has a heterozygous, *de novo*, missense mutation on chromosome 9, a switch from guanine to adenine. This base pair switch results in an amino acid change, from alanine to threonine, on the *GRIN1* gene. The mutation, GRIN1-A652T, affects the 652nd amino acid residue of GluN1, which is located on M3 (Figures 6, 7), a domain critical for channel gating. This position is conserved down to *C. elegans*, highlighting the importance of the sequence to normal function and life. Using software to predict the consequence of this mutation, SIFT predicts “damaging”, PolyPhen2 predicts “probably damaging”, and Mutation taster predicts “disease causing”.

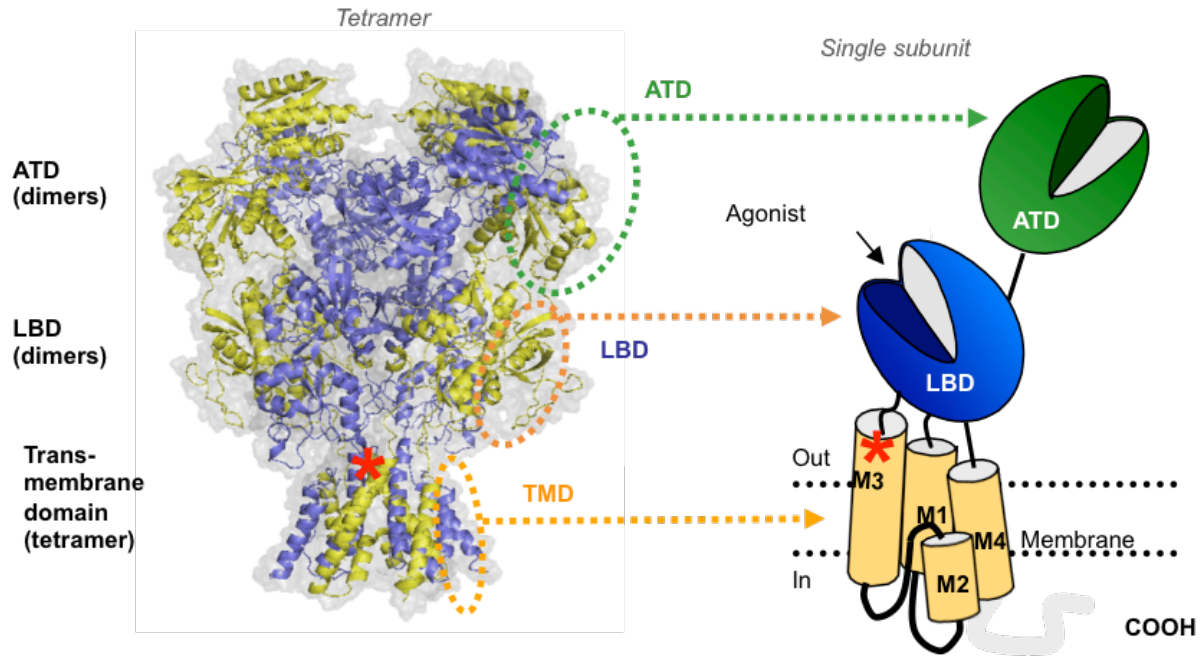

Figure 6: Location of mutation on NMDAR (marked with red asterisk). Courtesy of Traynelis laboratory and Dr. Pieter Burger.

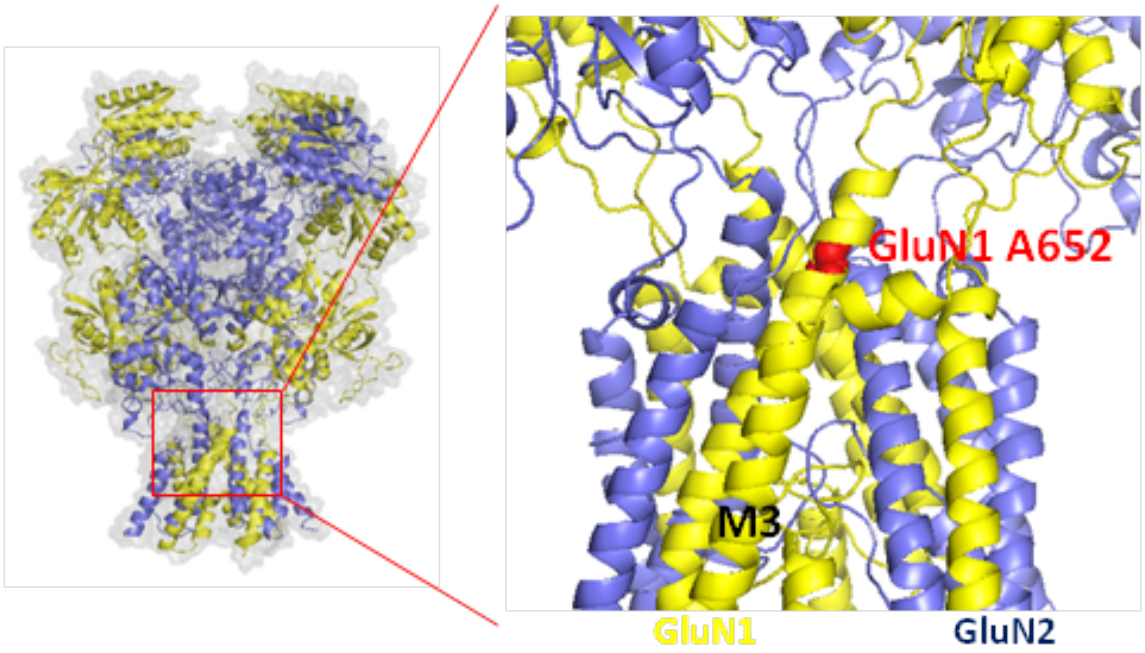

Figure 7: Location of the residue, Ala652. Courtesy of Dr. Hongjie Yuan and Dr. Stephen Traynelis.

As mentioned previously, the GluN1 subunit has a low frequency of identified mutations, especially in association with epilepsy. Given the global expression of GluN1, her condition is presumably due to the fact that she has a heterozygous mutation rather than a homozygous one. As she is able to produce some normal GluN1 subunits, her NMDAR function is not completely impaired. If she were to have a homozygous mutation, it is questionable that she would be alive, as she would have global dysfunction of her NMDARs.

Research Aims

This project has two aims. First, to characterize the impact of the mutation on NMDAR channel function, and second, to identify compounds that can potentially provide therapeutic effect to the patient. While it is ideal to determine a therapy that would completely reverse her condition, it is not realistic to do so given resources and current capabilities. Rather, the goal is to have a reduction in the frequency of her epilepsy episodes and improve her quality of life. Previously, work by Drs. Stephen Traynelis and Hongjie Yuan and their NIH/UDP collaborators has provided molecular mechanisms for therapeutic relief of epilepsy. One such example is a 6-year-old epileptic patient who had a *GRIN2A* mutation. Similar procedures as those done in this paper produced a potential personalized therapy, which in fact resulted in the patient having 3.3 seizures per week, down from the 11.1 seizures per week he was having prior to treatment (Yuan *et al.*, 2014; Pierson *et al.*, 2014).

Materials and Methods

Site directed mutagenesis

The GRIN1-A652T mutation was constructed using the *Quikchange* protocol. Polymerase chain reaction was performed using GRIN1 template DNA, primers designed to introduce the mutation, and the remaining necessary reagents. The reaction was run overnight using a thermocycler protocol optimized for GC content. To degrade the methylated parent DNA template, *Dpn*1 enzyme was added to the completed PCR reaction and incubated for 3 hours at 37°C. Using the heat shock method, the mutant cDNA was then transformed into competent *E. coli* cells, and then plated onto LB agarose media with ampicillin and grown at 37°C for 16-18 hours to ensure successful transformation. Individual colonies were further grown in 2xYT media with ampicillin at 37°C for 16-18 hours to replicate the plasmid further.

Following replication of the plasmid, the cDNA was isolated using the Qiagen (Venlo, Netherlands) QIAprep Spin Miniprep kit and protocol. The sequence of the cDNA was then verified via third party sequencing from Eurofins mwg Operon (Huntsville, Alabama). The isolated cDNA was then linearized using a restriction digest. The restriction digest was incubated at 37°C for 3 hours, and the cut DNA was verified using gel electrophoresis. The cut DNA was then purified using ethanol precipitation. Sodium acetate pH 5.2 and ethanol were used to pellet the DNA, which was then resuspended in water.

RNA synthesis

RNA was synthesized using an Ambion (Austin, Texas) mMessage mMachine T7 transcription kit and the manufacturer's protocol. Gel electrophoresis was used to confirm product formation.

Two Electrode Voltage Clamp (TEVC)

The GRIN1-A652T RNA was then mixed with RNA coding for GluN2A, GluN2B, GluN2C, or GluN2D subunits. Then, using a Drummond (Broomal, Pennsylvania) nano-injector, the RNA was injected into *Xenopus laevis* oocytes (approximately 15 oocytes for each RNA mixture). Oocytes were obtained from Ecocyte (Austin, Texas). The oocytes were then allowed to incubate at 15°C for 2-3 days in Barth's solution composed of (in mM): 88 NaCl, 1 KCl, 24 $NaHCO_3$, 10 HEPES, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.91 $CaCl_2$, as well as 100 µg/mL gentamycin, 40 µg/mL streptomycin and 50 µg/mL penicillin. This procedure produced oocytes that contained GluN1-A652T/GluN2A, GluN1-A652T/GluN2B, GluN1-A652T/GluN2C, or GluN1-A652T/GluN2D receptors. Wild type GluN1/GluN2(A-D) receptors were also produced using a similar protocol.

Two-electrode voltage clamp was then performed using a series of protocols testing the response with different ligands. Oocytes were tested 2-5 days post-injection. Oocytes were placed in a recording chamber designed with a dual-track, with a single perfusion line splitting into two in order to perfuse the two oocytes. Recordings were made with two-electrode voltage clamps (Warner model OC725B, Hamden, CT). Recording solution (the wash solution), with the exception of the zinc and magnesium recording solutions, was composed of (in mM): 90 NaCl, 1 KCl, 10 HEPES, 0.5 $BaCl_2$, 0.01 EDTA, at pH 7.4. The zinc

and magnesium solution was composed of (in mM): 90 NaCl, 1 KCl, 10 HEPES, and 1.0 $BaCl_2$, at pH 7.3.

For the glutamate solutions, recording solution was aliquoted into a separate container to which glycine (100 mM) was added. The solution was then aliquoted into separate vials to which different concentrations of glutamate were added (0.1-100 μM). This creates solutions with constant glycine concentrations and differing glutamate concentrations, allowing the effects of glutamate concentration to be tested. The same protocol was used for the glycine recording solutions. Glutamate (100 mM) was added to a stock aliquot of the recording solution (wash solution), and the mixture was aliquoted to different vials where different concentrations of glycine were added (0.1-100 μM).

To create the zinc, magnesium, memantine, amantadine, dextromethorphan, and dextrorphan solutions, the appropriate recording solution (wash solution) was aliquoted into a separate container, to which glutamate and glycine were added (100 mM each). The glutamate/glycine solution was then aliquoted into separate vials, to which different concentrations of agonist/antagonist was added (0.3-300 μM). To create the proton solution, the glutamate/glycine solution was aliquoted into two vials, one made to pH 6.8, and the other to pH 7.6.

The holding potential for all experiments was -40 mV, except for zinc (-20 mV) and magnesium (-60 mV). After clamping the oocytes, solutions were perfused over the oocytes using a timed protocol dependent on the ligand used. To obtain EC_{50} values, the following equation was used:

$$\text{Response} = 100\% / (1 + (EC_{50}/[\text{agonist}])^N)$$

where N is the Hill slope.

Responses for the negative modulators and FDA-approved compounds were fitted with the following equation:

Response (%) = $[(100\text{-minimum})/(1-([\text{concentration}]/IC_{50})^{N}) + \text{minimum}]$

where N is the Hills slope, and minimum is the degree of residual inhibition at a saturating concentration of drug.

All reagents were purchased from Sigma (St. Louis, Missouri). All experiments were performed on two or more separate oocyte preparations (injected oocytes from different frogs). Data are analyzed statistically using unpaired *t* test and are expressed as mean ± SEM. Significance for all tests was set at $p < 0.05$. Error bars presented in all figures are SEM.

Results

To evaluate if the mutant GluN1-A652T affects NMDAR function, TEVC recordings were performed on the wild type GluN1- or the mutant GluN1-A652T-injected oocytes co-expressed with GluN2A, GluN2B, GluN2C, or GluN2D, respectively. Sets of concentration-effect curves were generated and the potency of agonists and antagonists were evaluated. For experiments with agonist, the data of interest is the EC_{50} values, which is the concentration at which there is half the maximal excitatory response. For experiments with antagonists, the data of interest is the IC_{50} values, which is the concentration at which there is half the maximal inhibitory response.

Each curve represents an average of the data produced from a particular construct. Curves with white boxes represent wild type GluN1 subunits co-expressed with one of the

GluN2(A-D) subunits, the wild type receptor. Curves with red circles represent the GluN1-A652T subunit co-expressed with one of the GluN2(A-D) subunits, the mutant receptor.

Effects of GluN1-A652T on Agonist Potency

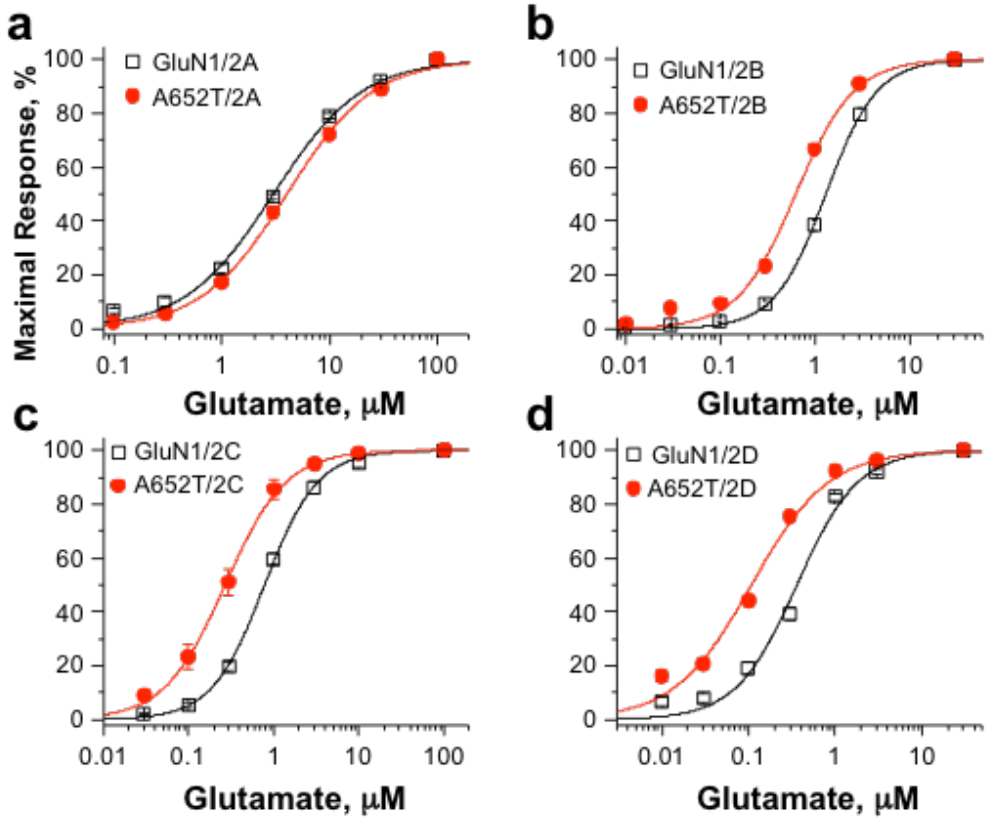

Figure 8: Concentration effect curves for glutamate.

Table 4: Summary of EC_{50} values for glutamate.

	Construct	EC_{50} μM (n)
GluN2A	WT GluN1	3.1 ± 0.2 (14)
	GluN1-A652T	4.3 ± 0.3* (24)
GluN2B	WT GluN1	1.4 ± 0.1 (11)
	GluN1-A652T	0.64 ± 0.06* (12)
GluN2C	WT GluN1	0.81 ± 0.06 (11)
	GluN1-A652T	0.29 ± 0.05* (6)
GluN2D	WT GluN1	0.37 ± 0.03 (20)
	GluN1-A652T	0.11 ± 0.01* (19)

Statistically significant values are marked with an asterisk (Unpaired *t*-test, $p < 0.05$). n represents the number of oocytes tested with the particular construct.

At the maximal concentration of glycine, when co-expressed with wild type GluN2A, the mutant (GluN1-A652T/GluN2A) showed a small, but significant, decrease in glutamate potency (increased EC_{50}): 4.3 μM vs 3.1 μM of WT GluN1/GluN2A (Figure 8a; Table 4). Conversely, when co-expressed with GluN2B, GluN2C, or GluN2D, the mutation caused an increase in potency (decreased EC_{50}) (0.64 μM vs 1.4 μM for GluN2B; 0.29 μM vs. 0.81 μM for GluN2C; 0.11 μM vs. 0.37 μM for GluN2D) (Figures 8b-d; Table 4). These data indicate that GluN1-A652T co-expressed with GluN2B, 2C, or 2D, can be activated by lower levels of glutamate.

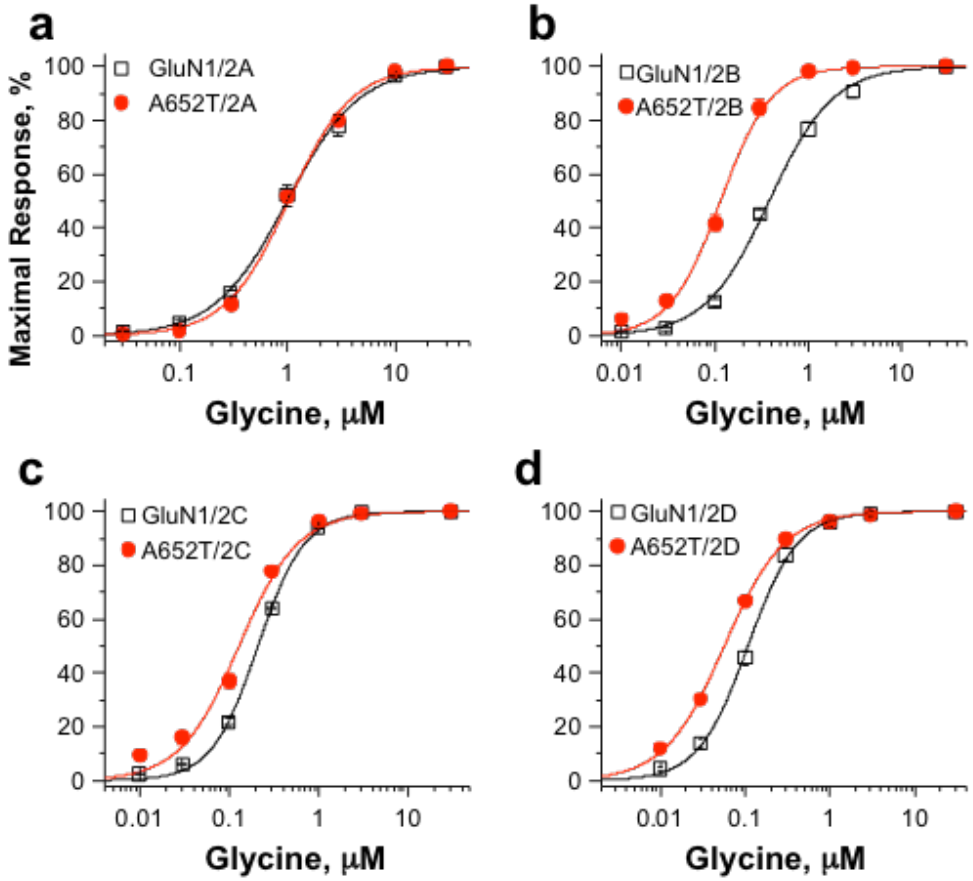

Figure 9: Concentration effect curves for glycine.

Table 5: Summary of EC_{50} values for glycine.

	Construct	EC_{50} µM (n)
GluN2A	WT GluN1	1.1 ± 0.1 (6)
	GluN1-A652T	1.1 ± 0.1 (14)
GluN2B	WT GluN1	0.39 ± 0.02 (8)
	GluN1-A652T	0.12 ± 0.01* (8)
GluN2C	WT GluN1	0.21 ± 0.01 (8)
	GluN1-A652T	0.13 ± 0.01* (8)
GluN2D	WT GluN1	0.12 ± 0.01 (12)
	GluN1-A652T	0.06 ± 0.005* (16)

Statistically significant values are marked with an asterisk (Unpaired *t*-test, $p < 0.05$). n represents the number of oocytes tested with the particular construct.

I next tested the effect of the mutation on glycine potency. The mutation had the same effect on glycine as it did on glutamate, an increase in potency when co-expressed with GluN2B, GluN2C, or GluN2D (0.12 μM vs 0.39 μM for GluN2B; 0.13 μM vs 0.21 μM for GluN2C; 0.06 μM vs 0.12 μM for GluN2C) (Figure 9b-d; Table 5).

These data suggest that the mutant GluN1-A652T, when co-expressed with GluN2B, GluN2C, and GluN2D, causes an increase in glutamate and glycine potency that may result in NMDAR overactivity.

Effects of GluN1-A652T on negative allosteric regulation

Endogenous zinc and protons inhibit NMDAR function in the brain (Traynelis *et al.*, 2010), likely serving as regulators to limit NMDAR function. Therefore, I next tested if the GluN1-A652T mutation can influence extracellular zinc or proton regulation of NMDAR function.

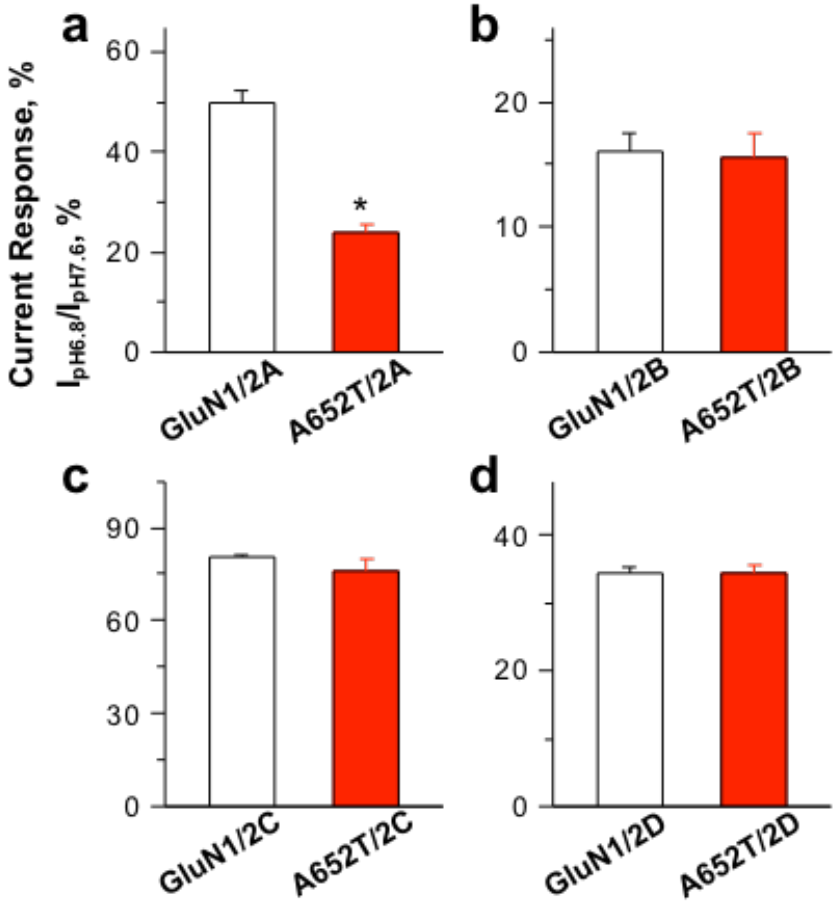

Figure 10: Effect of GluN1-A652T on proton sensitivity. Statistically significant values are marked with an asterisk (Unpaired *t*-test, p < 0.05).

Table 6: Percent change in current response for each construct.

	Construct	Current response $I_{pH6.8}/I_{pH7.6}$, % (n)
GluN2A	WT GluN1	50 ± 2.5 (11)
	GluN1-A652T	24 ± 1.7* (12)
GluN2B	WT GluN1	16 ± 1.5 (12)
	GluN1-A652T	16 ± 2.0 (12)
GluN2C	WT GluN1	81 ± 0.6 (6)
	GluN1-A652T	76 ± 3.6 (6)
GluN2D	WT GluN1	35 ± 0.9 (10)
	GluN1-A652T	34 ± 1.2 (12)

Statistically significant values are marked with an asterisk (Unpaired *t*-test, p < 0.05). n represents the number of oocytes tested with the particular construct.

For proton inhibition, the ratio percentage of current amplitudes were analyzed in two different pH values, pH 6.8 vs. pH 7.6. The mutant GluN1-A652T displays a decrease in percentage when co-expressed with GluN2A (24% vs 50%) (Figure 10a; Table 6), indicating that the mutation enhances proton inhibition for GluN2A. However, when co-expressed with GluN2B, GluN2C, or GluN2D, the mutation had no significant effect on the current ratio of pH 6.8 vs. pH 7.6 (Figure 10b-d; Table 6).

I next tested the effect of the GluN1-A652T mutation on high affinity voltage-independent zinc inhibition. As the zinc binding mechanism only involves GluN2A, the other GluN2 subunits (B-D) were not tested.

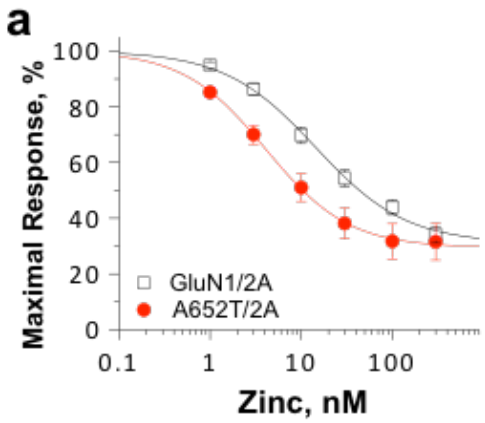

Figure 11: Concentration effect curve for zinc with GluN2 constructs.

Table 7: Summary of zinc inhibition.

	Construct	IC_{50} nM (n, max%[&])
GluN2A	WT GluN1	15 ± 2.8 (9, 66%)
	GluN1-A652T	5.1 ± 0.7* (9, 68%)

Statistically significant values are marked with an asterisk (Unpaired *t*-test, $p < 0.05$). n represents the number of oocytes tested with the particular construct. [&]maximal inhibition at 300 nM.

The mutation increased potency for zinc by decreasing the IC_{50} to 5.1 nM from the wild type value of 15 nM (Figure 11; Table 7). These data suggest that the GluN1-A652T mutation can enhance high affinity voltage-independent zinc inhibition when co-expressed with the GluN2A subunit.

Effect of GluN1-A652T on magnesium inhibition

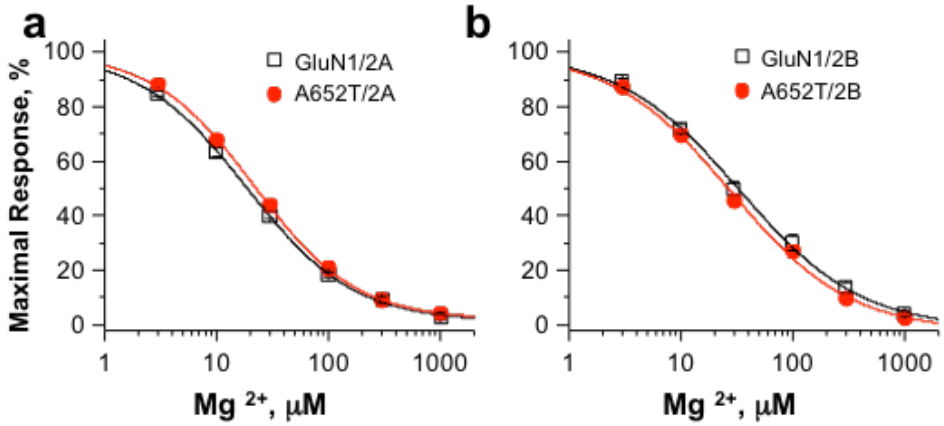

Figure 12: Concentration effect curves for magnesium.

Table 8: Summary of magnesium inhibition.

	Construct	Magnesium IC_{50} µM (n)
GluN2A	WT GluN1	19 ± 1.2 (8)
	GluN1-A652T	24 ± 2.4 (6)
GluN2B	WT GluN1	42 ± 5.6 (8)
	GluN1-A652T	38 ± 5.7 (8)

Statistically significant values are marked with an asterisk (Unpaired *t*-test, $p < 0.05$). n represents the number of oocytes tested with the particular construct.

One of the most important features of NMDARs is the voltage-dependent magnesium block (Nowak *et al.*, 1984; Mayer *et al.*, 1984). For this experiment, we only tested the effect of magnesium on GluN2A and GluN2B, as the magnesium sensitivity is very

low for GluN2C and GluN2D, meaning that the IC_{50} values would be higher than the concentration of magnesium present in the brain. For magnesium, there was not found to be a statistically significant effect on inhibition. For GluN2A and GluN2B, there was no significant change in the IC_{50}, indicating that the mutation has no significant effect on magnesium inhibition (Figure 12; Table 8).

To summarize the data collected and analyzed up to this point, the GRIN1-A652T mutation increased glutamate and glycine potency for the GluN2B, GluN2C, and GluN2D subunit receptors. The mutation also increased potency for zinc and proton inhibition, and had no significant effect on magnesium inhibition.

Effects of FDA approved antagonists on GluN1-A652T containing NMDAR function

As the patient's epileptic condition was unresponsive to conventional anti-epileptic drugs, a number of FDA approved antagonists were evaluated for their ability to inhibit GluN1-A652T containing NMDAR function. The drugs tested were memantine, dextromethorphan, dextrorphan, and amantadine. TEVC recordings on oocytes were performed and concentration-effect curves for both the mutant and wild type receptors were produced. IC_{50} values for both the wild type and mutant NMDARs were compared to determine the effect on the function of the mutant receptor.

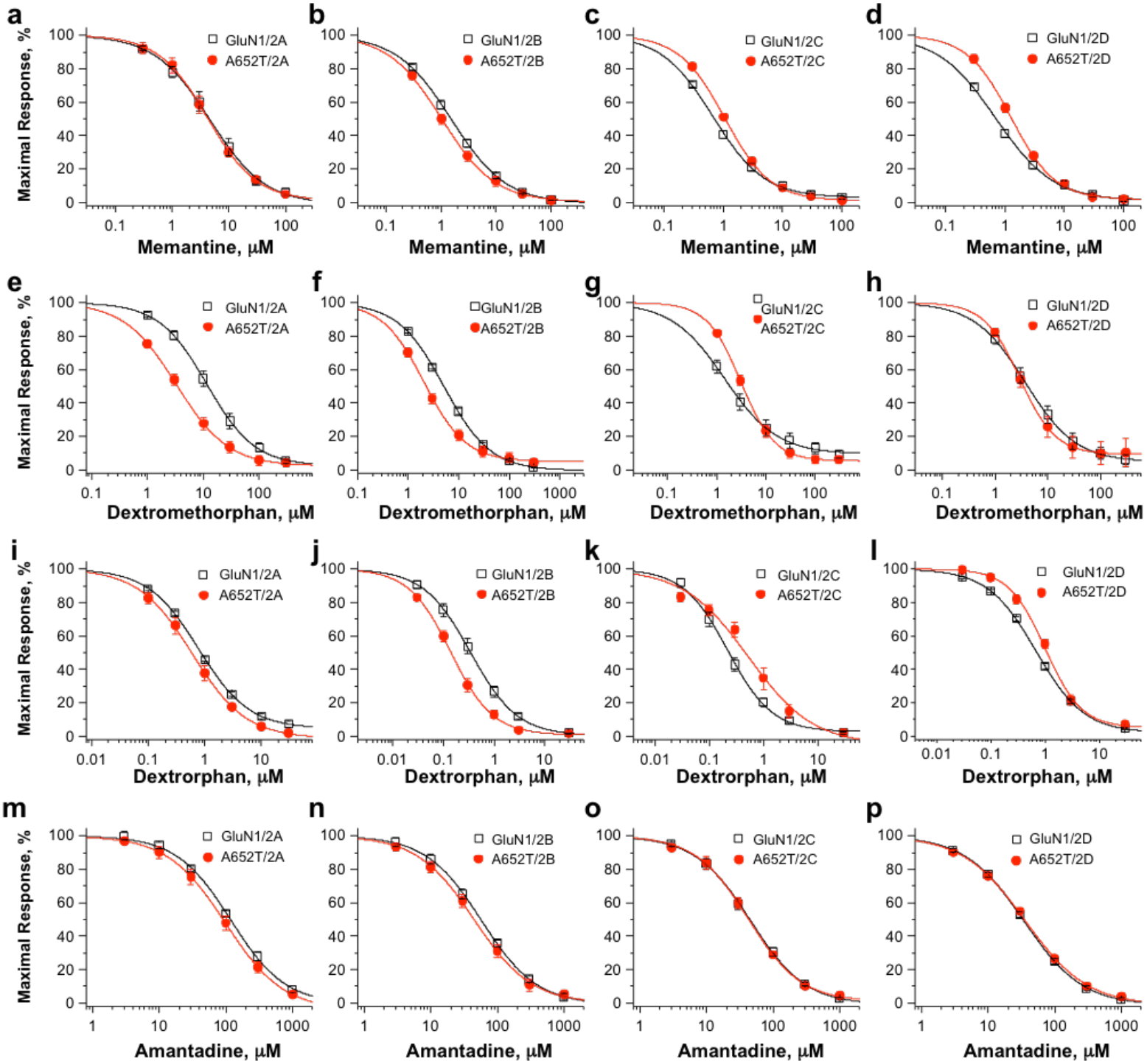

Figure 13: Concentration-effect curves for memantine, dextromethorphan, dextrorphan, and amantadine.

Table 9: Summary of IC_{50} values for FDA approved compounds.

	Construct	Memantine IC_{50} µM (n)	Dextromethorphan IC_{50} µM (n)	Dextrorphan IC_{50} µM (n)	Amantadine IC_{50} µM (n)
GluN2A	WT GluN1	5.4 ± 1.3 (6)	13 ± 3.4 (6)	0.8 ± 0.06 (6)	127 ± 12 (5)
	GluN1-A652T	4.3 ± 1.7 (6)	3.6 ± 0.60* (6)	0.66 ± 0.11 (6)	109 ± 16 (5)
GluN2B	WT GluN1	1.6 ± 0.2 (6)	5.2 ± 0.35 (5)	0.36 ± 0.07 (6)	61 ± 4.7 (4)
	GluN1-A652T	1.1 ± 0.2 (6)	2.3 ± 0.22* (6)	0.23 ± 0.03 (6)	54 ± 10 (6)
GluN2C	WT GluN1	0.7 ± 0.06 (6)	1.7 ± 0.39 (6)	0.23 ± 0.03 (6)	47 ± 6.2 (8)
	GluN1-A652T	1.1 ± 0.06* (6)	3.1 ± 0.30* (6)	0.62 ± 0.13* (6)	44 ± 4.1 (6)
GluN2D	WT GluN1	0.75 ± 0.113 (8)	4.3 ± 0.66 (6)	0.74 ± 0.06 (6)	36 ± 1.9 (7)
	GluN1-A652T	1.5 ± 0.07* (6)	4.0 ± 0.43 (6)	1.1 ± 0.12* (5)	37 ± 1.7 (7)

Statistically significant values are marked with an asterisk (Unpaired *t*-test, $p < 0.05$). n represents the number of oocytes tested with the particular construct.

Each of the four FDA approved antagonists evaluated showed similar potency (similar IC_{50} values) between the mutant and wild type receptor for all GluN2 subunits (A-D). These data indicate that all four antagonists can inhibit GluN1-A652T containing NMDAR function.

Discussion

Analysis of the mutant receptor data identifies two interesting characteristics of the receptor that could contribute to the patient's pathology. First, the mutant receptor has increased potency for glutamate and glycine when co-expressed with the GluN2B, GluN2C, and GluN2D subunits that leads to NMDAR hyper-excitability. From the data it appears that

the patient's NMDARs are responding at lower concentrations of agonist, suggesting that her receptors may be overactive at normal physiological levels of agonist in her brain. As previously mentioned, epilepsy is a condition of increased and abnormal neural activity. The determination that her NMDARs are overactive fits in this model as it suggests that her NMDAR overactivity may contribute to, or may be the cause of, neural overactivity that results in an epileptic condition. This characteristic is especially important as it also identifies a potential target to alleviate her condition.

The second important characteristic is that her mutant receptor has increased potency for zinc as well as increased inhibition due to protons with the GluN2A subunit. The GluN2A subunit was the only subunit that did not have increased potency to glutamate and glycine. This characteristic is interesting in that increased inhibition of an excitatory synapse does not match the characteristics of her condition, which is neural overactivity. How this characteristic plays a role in her condition is unknown. However, when considering neural circuitry, it is possible that this effect may influence a part of a circuit that then leads to over-activity and her epileptic condition. A way this characteristic, as well as the previous one, could contribute to the patient's phenotype is through a change in the balance of NMDAR activity in principal cells of the CNS. Principal cells express both GluN2A and GluN2B subunit containing NMDARs. Increased inhibition of GluN2A subunit containing NMDARs by endogenous zinc, and enhancement of GluN2B subunit containing NMDARs by glutamate and glycine will change the balance of NMDAR activity and thus the neural circuitry and activity.

Memantine has been shown to have anti-convulsive effects in multiple animal models of epilepsy (Ghasemi and Schachter, 2011), and has been used in children without

apparent toxicity (Chez *et al.*, 2007; Erickson *et al.*, 2011). Our *in vitro* TEVC recording analysis indicated that memantine inhibited GluN1-A652T containing NMDARs with a similar IC_{50} value as the wild type for all four GluN2 subunits (A-D) (Figure 13a-d; Table 9). This suggests that memantine can effectively reduce GluN1-A652T containing NMDAR hyperactivity. More importantly, memantine was previously used in a pediatric patient with intractable seizures and it brought relief to the patient's seizures (Pierson *et al.*, 2014). Therefore, memantine might be a reasonable candidate for testing, followed by dextromethorphan.

To further examine the effects of the GluN1-A652T mutation on NMDAR function, several additional experiments could be performed. The first would be to perform concentration-jump experiments in order to measure the effects of the mutation on the deactivation time course following removal of glutamate. The deactivation time course sets the time course of the NMDAR-mediated component of the synaptic current. Examining the change in response rise time during a rapid concentration jump experiment would reveal how the mutation affects channel gating, as the time it takes for the channel to transition from the closed state to the open state influences the response rise time. These data would also validate the results of the TEVC experiments. The EC_{50} value used to determine a change in agonist sensitivity is influenced by the association and dissociation rates of the agonist to its binding pocket, as well as the efficacy of the agonist to produce channel opening. If this mutation causes the channel pore to be open longer, which it likely does given its location in M3, it thus influences the efficacy of the agonist. The efficacy can influence the EC_{50} (Colquhoun, 1998). The EC_{50} measured would be lower as there would be a larger response (more current) at a lower concentration of agonist.

Another experiment would be to perform MTSEA oocyte recording experiments to explore changes in the open probability. This experiment would allow us to examine how the mutation affects the likelihood of channel opening. In MTSEA oocyte recording, cysteine residues replace native residues in the amino acid sequence of the mutant NMDAR, and agonist is perfused over the receptor, followed by MTSEA (a cysteine modifier). MTSEA will bind exposed cysteine residues and influence the conformation of the receptor, and often alter the kinetics of channel opening. When the cysteine residues are placed at certain positions in the M3 region, modification by MTSEA locks the channel open, increasing efficacy to 1.0 (Jones *et al.*, 2002; Yuan *et al.*, 2005). By measuring the degree of potentiation, we can determine the open probability prior to cysteine modification. Evaluating the differences in channel opening between the mutant and wild type receptor for the selected residues will provide insight as to how the mutation affects the relationship between the receptor structure and the probability of channel opening.

Single channel recording is another useful experiment to perform as it would reveal the properties of a single GluN1-A652T-containing NMDARs. Evaluation of channel conductance, mean open time, and the closed periods between openings, when patches contain a single receptor, would provide insight on which steps involved in channel activation, if any, are perturbed by mutants. Analysis of these data would allow us to verify or refute our hypotheses derived from our TEVC results, and help determine if changes in receptor function observed in mutant receptors were due to a change in receptor trafficking or a change in receptor function. Finally, a mouse model in which the GluN1-A652T mutation has been inserted using homologous recombination could be produced.

Doing so would allow us to determine if the mutation does in fact result in the epileptic phenotype.

While this study was designed to evaluate potential treatments that could provide relief to a patient suffering from a debilitating condition, it is also important for other reasons. This study has identified and characterized a rare mutation that results in NMDAR hyperactivity, while also identifying compounds that can inhibit the dysfunction. This mutation has unexpected GluN2 selectivity even though it is a GluN1 mutation, providing insight about the possible interactions between the two distinct classes of NMDAR subunits in functional receptors. The results also showed altered zinc binding to the GluN2A subunit due to a mutant GluN1 subunit, again highlighting potential subunit interactions. This study has also verified that several FDA-approved drugs are candidates for off-label use to treat NMDAR-mutation associated epilepsy in this patient. Finally, the study also provides insight on the target therapies of NMDAR mutation related diseases, especially in the use of medically refractory epilepsy.

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