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***GRIN2D*/GluN2D NMDA Receptor: Unique Features and Its Contribution to Pediatric Developmental and Epileptic Encephalopathy**

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Abstract

N-methyl-D-aspartate receptors (NMDARs), a subset of ligand-gated ionotropic glutamate receptors, are critical for learning, memory, and neuronal development. However, when NMDAR subunits are mutated, a host of neuropathological conditions can occur, including epilepsy. Recently, genetic variation within the *GRIN2D* gene, which encodes the GluN2D subunit of the NMDAR, has been associated with a set of early-onset neurological diseases, notably developmental and epileptic encephalopathy (DEE). Importantly, patients with *GRIN2D* variants are largely refractory to conventional anti-epileptic drug (AED) treatment, highlighting the need to further understand the distinctive characteristics of GluN2D in neurological and pathological functions. In this review, we first summarize GluN2D's unique spatial and temporal expression patterns, electrophysiological profiles, and contributions to both pre- and postsynaptic signaling. Next, we review thirteen unique case studies from DEE patients harboring ten different causal *GRIN2D* variants. These patients are highly heterogeneous, manifesting multiple seizure types, electroencephalographic recordings, and neurological and developmental outcomes. Lastly, this review concludes by highlighting the difficulty in treating patients with DEE-associated *GRIN2D* variants, and stresses the need for selective therapeutic agents delivered within a precise time window.

Keywords

Autism; Channelopathy; Developmental and epileptic encephalopathy; Developmental delay; Epilepsy; Glutamate receptor; Intellectual disability; Precision medicine; Translational study

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Conflict of interest

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1. Introduction

N-methyl-D-aspartate receptors (NMDARs) are ligand-gated ionotropic glutamate receptors which constitute a calcium-permeable component of fast excitatory neurotransmission. These receptors are heterotetramers (Figure 1A), comprised of two GluN1 subunits (one gene, *GRIN1*) and two GluN2 subunits (four genes, *GRIN2A-D*). NMDARs are unique in that they require both co-agonist binding (glycine/D-serine on GluN1 and glutamate on GluN2) and membrane depolarization in order to flux ions.^[1–3] At most resting membrane potentials, NMDARs are blocked by extracellular magnesium ions within the channel pore in a voltage dependent manner, which allows them to integrate neurotransmitter release with postsynaptic membrane depolarization.^[1] This ‘coincidence detection’ and significant calcium permeation endows NMDARs with the ability to modulate synaptic plasticity, making them key regulators of neuronal excitability.^[4] Moreover, NMDAR activity is critical for neurodevelopment, synaptogenesis, locomotion, spatial learning, memory formation, and general cognition.^[5–10]

Given their importance in normal brain activity, it is not surprising to discover that mutations within the *GRIN* genes are among some of the least tolerant in the genome.^[11–15] NMDAR mutations are associated with a host of neurological diseases, including schizophrenia, intellectual disabilities, autism, epilepsy, and attention-deficit/hyperactivity disorder (see XiangWei et al., 2018^[13]). However, the focus of this review will be on developmental and epileptic encephalopathy (DEE), which are a group of devastating epilepsies that combine severe seizures with the presence of one or more developmental abnormalities. Patients with DEE are largely resistant to standard anti-epileptic drug (AED) therapies and tend to show poor recovery from secondary neurodevelopmental symptoms even after seizure cessation. Given the severity of DEE and its poor prognosis, there is a pressing need to further our understanding of the molecular underpinnings of DEE, with a particular focus on epileptogenesis.

All of the *GRIN* genes are capable of generating DEE via rare or *de novo* genetic mutations. The *GRIN2A* and *GRIN2B* genes clearly demonstrate a strong correlation between genetic variation and an epileptic phenotype, however, the functions of these genes are very well understood and have been extensively researched. For example, PubMed articles containing ‘NR2A/GRIN2A/GluN2A’ generated 2,966 hits and a search for ‘NR2B/GRIN2B/GluN2B’ generated 4,397 (accessed on 8/19/2019). However, there is also a strong epilepsy-associated component seen within the *GRIN2D* gene, as 63% (10/16) of patients with *GRIN2D* variants display some form of epilepsy, and more specifically DEE. This gene encodes the GluN2D NMDAR subunit, and its function within the nervous system is largely understudied, with PubMed searches for ‘NR2D/GRIN2D/GluN2D’ yielding only 406 papers (accessed 8/19/19). GluN2D-containing NMDARs (GluN2D receptors) exhibit unique expression patterns, cellular specificity, and electrophysiological profiles, which are unparalleled amongst the *GRIN* gene family. The combination of these features make unraveling the precise epileptogenic role of *GRIN2D* mutations complex, yet highly intriguing. The goal of this review is to highlight some of these distinguishing physiological features and conclude by presenting several case studies of pediatric DEE patients with genetic variation within the

GRIN2D gene, with the hopes of putting epileptic mutations within this gene under the limelight.

2. Distinctive expression and functional features of GluN2D-containing NMDARs

2.1. GluN2D spatial and temporal expression patterns

The *GRIN2D* gene is located at 19q13.1-qter in the human genome, with its longest splice variant containing 1,336 amino acids.^[1] The GluN2D subunit shares >80% sequence homology with the GluN2C subunit, >70% sequence homology with the GluN2A and GluN2B subunits, and about 60% sequence homology with all other ionotropic glutamate receptors, including the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and the kainate receptor.^[1, 16] For a more detailed description of transcription, translation, trafficking, etc. of ionotropic glutamate receptors, readers are invited to examine Traynelis et al. 2010.^[1]

GluN2D mRNA expression begins in the latter portion of embryogenesis, not appearing until embryonic day (E)15–18 in rodents.^[17] Levels of GluN2D mRNA continue to increase during neurodevelopment, reaching peak expression level at postnatal day (P)7–10.^[17] After the neonatal phase, mRNA levels of GluN2D decrease and do not reach their relatively low steady-state expression level until late adolescence (P40–50).^[17, 18] Although not as extensively studied, GluN2D mRNA expression in human brain largely follows a similar spatial and temporal pattern as described both above and below.^[19, 20] However, most of this review in regards to functional data in native tissue will focus on rodent studies.

During the early phases of life, GluN2D has a ubiquitous expression pattern, appearing in the spinal cord (both dorsal and ventral horns)^[21], midbrain nuclei such as the periaqueductal gray and reticular formation^[21], all nuclei of the diencephalon (thalamus, hypothalamus, etc.)^[18, 22], select nuclei within the basal ganglia (striatum, substantia nigra, and subthalamic nucleus), retina^[23], cochlear/vestibular nerves^[24], olfactory bulb^[17], cerebellum^[17], and cerebral cortex including the hippocampal formation^[17, 18]. The widespread distribution of GluN2D during the embryonic and early neonatal phases of life give this subunit the opportunity to exert influence on circuit connectivity and function during a critical phase of neurodevelopment.

As the rodent ages, GluN2D expression remains in all of the regions listed above, but diminishes in transcript number and becomes more localized to particular cell types.^[17, 18, 25, 26] The most notable examples of GluN2D cell-type specificity occur in the hippocampus and cerebellum.^[27, 28] In juvenile hippocampus (P3–5), GluN2D receptors are believed to contribute to synaptic transmission in both glutamatergic pyramidal cells and GABAergic interneurons.^[28] However, throughout maturation, NMDAR-mediated currents in hippocampal pyramidal cells lose hallmarks of GluN2D receptors, such as a prolonged synaptic decay and insensitivity to extracellular magnesium (see section below), while these characteristics are retained in GABAergic interneurons.^[28] While the function of early GluN2D expression in excitatory pyramidal cells is not known, one plausible explanation is

that the GluN2D subunit is necessary for neuronal maturation. Further work using GluN2C/D-specific NMDAR modulators has confirmed the presence of GluN2D in GABAergic hippocampal interneurons of both the medial ganglionic eminence (MGE; parvalbumin-expressing basket cells, etc.) and caudal ganglionic eminence (CGE; cholecystokinin-expressing basket cells, etc.) embryonic origins.^[29–31] The sustained expression of GluN2D receptors on interneurons may indicate that this subunit is integral to maintain proper inhibitory drive to help control overall circuit function.

A similar change in GluN2D cell-type specificity occurs in the cerebellum. In early neurodevelopment, GluN2D has been found in Purkinje cells and in both stellate and Golgi cells.^[17, 27, 32] After adolescence (>P40), Purkinje cells no longer express GluN2D and it becomes restricted to just stellate and Golgi cells.^[17] This cell-type restriction during neonatal and preadolescent development may not be specialized to just the hippocampus and cerebellum, but could occur throughout all regions in which GluN2D is expressed during embryogenesis. Thus, a rare or *de novo* variant occurring within the *GRIN2D* gene, combined with the robust embryonic expression of GluN2D, would be able to impact nearly every facet of brain function. Empirical evidence for this hypothesis is presented later during this review, however, the neurological phenotypes of patients with *GRIN2D* variants are quite heterogeneous (see section 3.3).

2.2. Electrophysiological signatures of GluN2D-containing NMDARs

Glutamate ($EC_{50} = 0.2\text{--}0.4\ \mu\text{M}$; Figure 1B) and glycine ($EC_{50} = 0.1\text{--}0.2\ \mu\text{M}$) act with relatively high potency at GluN2D receptors^[1]. Glutamate shows over 5–6-fold higher potency at the GluN2D receptor than at GluN2A- or GluN2B-containing NMDARs (Figure 1B).^[1] As mentioned previously, NMDARs are unique in that they are blocked by extracellular magnesium at negative membrane potentials.^[1] This allows NMDARs to function as coincidence detectors, producing an inward current in response to presynaptic glutamate release when the postsynaptic membrane is depolarized.^[4] However, the potency of magnesium block on GluN2D receptors is 10-fold lower than that observed on GluN2A- and GluN2B-containing NMDARs.^[33, 34] This indicates that neurons expressing GluN2D receptors may be more sensitive synaptically-released glutamate than those expressing GluN2A- or GluN2B-containing NMDARs alone. Conversely, uncompetitive NMDAR pore blockers, such as memantine (5-fold more potent) and ketamine (2-fold more potent), are more potent at GluN2D receptors than on GluN2A and GluN2B receptors.^[1, 35]

Another unique electrophysiological characteristic of GluN2D receptors is their prolonged deactivation time. Using a rapid solution exchange system on transfected HEK cells, glutamate exposure can mimic a synaptic-like time course (1 ms exposure time).^[36] In this paradigm, GluN2D receptors exhibit a weighted tau, a measure used to describe receptor deactivation following agonist removal, of over 5,000 ms.^[18, 37, 38] This is over 10-fold slower than GluN2B and GluN2C receptors and ~100-fold slower than GluN2A receptors (Figure 1C).^[1] Additionally, unlike GluN2A and GluN2B receptors, GluN2D-mediated current responses are virtually void of desensitization following prolonged agonist exposure.^[18, 38–40] An exaggerated decay time with a near lack of desensitization makes GluN2D receptors capable of transferring a large quantity of ions across the plasma membrane,

sustaining depolarization for a sufficient amount of time to initiate burst-firing (see sections 2.3 and 2.5). However, GluN2D receptors also have a much lower open channel probability (Figure 1D), around 0.01 – 0.04%, and a slightly lower calcium permeability than GluN2A and GluN2B receptors.^[1, 41]

In addition to forming diheteromeric complexes, for example GluN1/GluN2D/GluN1/GluN2D, NMDARs can also be formed using two different GluN2 subunits. Multiple reports have demonstrated that both GluN1/GluN2A/GluN1/GluN2D and GluN1/GluN2B/GluN1/GluN2D triheteromeric assemblies are found in native tissue.^[1] The presence of two different GluN2 subunits will alter receptor characteristics, typically observed as a blending of the two diheteromeric forms.^[42] For example, the GluN2B/GluN2D triheteromeric receptor has a weighted tau of ~1,800 ms when transfected HEK cells are exposed to rapid synaptic-like glutamate exposure, whereas the weighted tau for diheteromeric GluN2B receptors is about 500 ms and the weighted tau for diheteromeric GluN2D receptors is >5,700 ms.^[43] Other receptor kinetic parameters for GluN2B/GluN2D triheteromeric complexes are also an intermediate of diheteromeric GluN2B and GluN2D receptors, such as open channel probability, magnesium sensitivity, and efficacy of modulators like ifenprodil, ketamine, and memantine.^[43] GluN2A/GluN2D triheteromeric assemblies have not been extensively evaluated for kinetic parameters, but it is plausible these receptors will also exhibit this unique intermediate blending phenotype.

2.3. Roles of GluN2D in postsynaptic neurotransmission

As mentioned previously, the GluN2D subunit is present in synaptic NMDARs in GABAergic interneurons in the hippocampus and cortex.^[29, 44] However, the ratio of true GluN2D diheteromeric receptors to triheteromeric assemblies with GluN2A and/or GluN2B is still poorly understood. Regardless of stoichiometry, the presence of GluN2D within the receptor complex may endow interneurons with the unique ability to generate dendritic calcium spikes, integrate long-range signals, and synchronize large groups of interneurons in coordinated firing via glutamate spillover.^[45–47] The prolonged synaptic decay, high glutamate potency, and modest magnesium block generated by the GluN2D subunit could allow interneurons to generate burst-firing, and thus could be partially responsible for their oscillatory and rhythmic nature.^[48] It should also be noted that although the GluN2D subunit is found in both MGE- and CGE-derived interneurons, the relative contribution of NMDAR-mediated current observed on CGE-derived interneurons is much higher.^[49] This suggests that NMDARs and the GluN2D subunit may not be the main driver for dendritic calcium spikes in MGE-derived interneurons. However, a recent study has shown that tonic activation of GluN2D receptors on developing cortical interneurons is necessary for proper intrinsic excitability, dendritic arborization, GABAergic synaptogenesis, and inhibitory tone onto excitatory pyramidal cells.^[44]

In addition to GABAergic interneurons, the GluN2D receptor plays a pivotal role in postsynaptic signaling within the basal ganglia. Subthalamic nuclei (STN) neurons have been shown to have a high expression of dendritic GluN2D receptors.^[50] The STN is the sole glutamatergic, excitatory nucleus within the basal ganglia, receiving excitatory drive from the thalamus and cortex, and providing an excitatory output to the globus pallidus

interna and substantia nigra pars reticulata.^[51] Thus, one important function of the STN is to modulate the strength of indirect pathway activity that reaches the thalamus and motor cortex. GluN2D receptors have also been reported on dopaminergic projection neurons in the substantia nigra pars compacta (SNc).^[52] Here, both GluN2B and GluN2D receptors provide the excitatory NMDAR-mediated current to facilitate the release of dopamine onto medium spiny neurons within the dorsal striatum. Both the STN and SNc are hypoactive in Parkinson's disease, and positive allosteric modulators specific for GluN2C/GluN2D receptors have shown some promise in rescuing motor symptoms in animal models.^[53] In addition, the central role of GluN2D receptors in modulating GABAergic interneuron activity and coordinating the balance of direct versus indirect pathway activation within the basal ganglia make mutations within this subunit directly poised to have a substantial impact on circuit excitability and coordinated motor function.

GluN2D receptors have also been confirmed to play a central role in postsynaptic signaling within the spinal cord.^[54] More specifically, GluN2D receptors have been found to mediate excitatory synaptic transmission in laminae I of the dorsal horn. The dorsal horn relays pain and temperature signals from sensory neurons in the periphery to the somatosensory cortex. The notion that the GluN2D receptors play a role in pain perception is supported by evidence using GluN2D-knockout mice, discussed more extensively below, as the GluN2D receptor was shown to be critical for the induction of AMPAR-mediated allodynia.^[55]

2.4. Roles of GluN2D in presynaptic neurotransmission

In addition to serving as postsynaptic dendritic receptors, GluN2D receptors have also been reported in presynaptic sites. Presynaptic GluN2D receptors have mainly been studied in the hippocampus and cerebellum, although within these regions they serve different roles in circuit function. In the hippocampus, presynaptic GluN2D receptors have been suggested to mediate the induction of long-term depression (LTD) between the canonical CA3-CA1 pyramidal cell synapse.^[56] More specifically, GluN2D receptors have been shown to be necessary for spike-time dependent LTD (t-LTD).^[56] This form of plasticity helps to marry the temporal sequence of postsynaptic signal generation with action potential firing within the same cell.^[57] In the context of t-LTD, if dendritic postsynaptic signals occur just after a cell fires an action potential, subsequent postsynaptic signaling strength will be diminished.^[57] Andrade-Talavera and colleagues report that t-LTD induction does not require any postsynaptic NMDAR activation, and that GluN2B and GluN2D receptors, along with postsynaptic metabotropic glutamate receptors, work in concert to generate this form of Hebbian synaptic depression.^[56]

In the cerebellum, presynaptic GluN2D receptors on stellate cells help modulate GABA release onto Purkinje cells.^[58] Here, researchers postulate that 'spilled over' glutamate from parallel fibers binds to presynaptic GluN2D receptors under times of rapid granule cell firing and increases stellate cell GABA release.^[58] Although the GluN2D subunit has been reported in multiple other brain regions, the remaining areas not discussed in this section have only been confirmed via localization studies (in situ hybridization, Western blots, or antibody binding). Thus, their direct role in circuit function is not fully understood. Regardless, GluN2D receptors play a critical role in modulating circuit function via their

direct involvement in plasticity induction and in the fine-tuning of presynaptic neurotransmitter release. It should also be noted that GluN2D receptors have not been reported on any glial cells.^[59]

2.5. Physiological role of the GluN2D subunit via knockout mice

GluN2D-knockout (KO) mice are viable, reproducible normally, and have no overt changes in neuronal histology.^[60] However, these mice do exhibit a unique locomotor phenotype. This locomotor phenotype is likely due to altered levels of monoamines, such as dopamine and norepinephrine, which were shown to be downregulated in KO mice in the hippocampus and striatum.^[61] Accordingly, KO mice have a diminished propensity for spontaneous motor movements via open field assays, but display no deficits in general locomotor abilities measured via the rotarod test.^[62] There were also no gross changes in mRNA levels of the other NMDAR subunits, however, expression within the synapse has not yet been described.^[60] On the other hand, GluN2D-KO mice have a reduced pharmacologically-induced hyperlocomotor phenotype to ketamine^[63] and phencyclidine^[62], both of which are pan-NMDAR pore blockers. This indicates a key role of GluN2D receptors in motor control, as would be postulated given their expression patterns in the spinal cord and basal ganglia. Finally, GluN2D-KO mice show deficits in spatial memory and prepulse inhibition.^[48]

GluN2D receptors are also partially responsible for the generation of high-frequency gamma oscillations (>60 Hz), as KO mice had a lower power threshold between 65–140 Hz.^[48] These oscillations are crucial for information processing, proper cortical functions, and generation of working memory.^[64–66] Gamma oscillations are generated via fast-spiking parvalbumin-positive GABAergic interneurons.^[64, 66] Sapkota and colleagues reported a decrease in PV-immunoreactivity of GluN2D-KO mice in the substantia nigra pars compacta and in the basolateral amygdala.^[48] Other brain regions, such as the hippocampus and frontal cortex, did not show changes in PV-immunoreactivity. However, the loss of GluN2D on PV-interneuron function as well as its impact on overall circuit function remain unexplored.^[48] Given the robust expression of GluN2D, especially during early neurodevelopment, it seems likely that perturbations to GluN2D function will impart a multitude of changes in cellular biology, synaptic function, and network output.

3. Rare and *de novo* variants in the *GRIN2D* gene contribute to developmental and epileptic encephalopathy

Genetic variations within the *GRIN2D* gene cause severe forms of developmental and epileptic encephalopathy. Epilepsy is one of the world's most prevalent neurological disorders, afflicting nearly 50 million people.^[67] Developmental and epileptic encephalopathy (DEE) represents a group of devastating neurological diseases, which are difficult to treat and involve multiple brain abnormalities in conjunction with severe convulsive seizures.^[68–70] DEE manifests early during infantile or adolescent development.^[71–73] Common comorbidities of DEE include slowed or incomplete neurological development, aberrant fine motor control, aphasia, and intellectual disabilities.^[68, 69] Children with DEE have a shortened life expectancy, and most patients find little to no relief from seizures with AEDs.^[74–78] Additionally, the prognosis for secondary symptoms in

DEE patients is poor, with some never developing proper intellectual, motor, and verbal function.^[79–82] This incomplete recovery from secondary symptoms suggests an underlying problem in neuronal circuitry that may become hardwired past a certain stage during development. Furthermore, children with DEE often require around-the-clock care, placing a heavy social and financial burden on primary caregivers, highlighting an urgent need to understand its disease etiology and progression.^[77, 83–86]

3.1 Overview and location of DEE-associated *GRIN2D* variants

DEE can be caused by a variety of factors, including both genetic and environmental. Recently, thanks to advances in whole exome sequencing, genetically-derived DEE has gained much-deserved attention and causative gene mutations have now been identified. To date, 16 unique missense *GRIN2D* variants, which are absent in the gnomAD database (Genome Aggregation Database, Cambridge, MA, <http://gnomad.broadinstitute.org/>, evaluated on 07–31-2019), have been reported.^[39, 87–89] Of these 16 variants in Table 1, 63% (10/16) are associated with DEE.^[39, 88, 89] These DEE-associated *GRIN2D* missense variants are scattered through various domains of the GluN2D subunit, except for the extracellular amino-terminal domain (ATD; Figure 2A). Of the 10 DEE *GRIN2D* variants, the agonist-binding domain (ABD) contains 1 variant, the transmembrane domain and linker regions (TMD) contains 7 variants, and the carboxyl-terminal domain (CTD) contains 2 variants (Figure 2B).

The ATD is furthest away from the plasma membrane and harbors sites for allosteric modulator binding and controls electrophysiological characteristics such as agonist potency and open channel probability.^[1, 90] However, some studies have shown that NMDAR complexes are still functional without the ATD, albeit they are not comparable to wild-type receptor function, potentially explaining why no variants have been found within this domain. The ABD is a highly conserved region of the NMDAR, and helps to convert agonist binding, such as glutamate at GluN2 subunits, into molecular motion for pore opening.^[1] The TMD of the receptor is also a critical region, harboring twelve transmembrane alpha helices (three per subunit) in addition to four reentrant loops arranged in a circle to form the receptor pore and ion selectivity filter.^[1] Thus, it is not surprising that the majority of disease-associated *GRIN2D* variants lie within the TMD. Recent studies have shown the pre-M1 and M1 regions of the TMD are intolerant to genetic variation in a healthy population.^[12] That is, if a mutation occurs in these regions, some form of neurological disease will likely manifest. Lastly, the CTD contains sites for protein-protein interactions, interacts with a host of intracellular proteins, and facilitates subunit trafficking from the endoplasmic reticulum to the plasma membrane.^[1] Although the majority of *GRIN2D* variants are centered around the TMD, similar mapping studies with *GRIN2A* variants have shown that epilepsy-associated mutations occur throughout all domains of the NMDAR, including the ATD.^[13, 14, 91]

3.2 Functional analysis of *GRIN2D* variants in heterologous systems

Of the 10 DEE-associated *GRIN2D* variants, 7 have undergone functional testing using heterologous expression systems.^[39, 89] The outcome of this testing has been summarized in Table 2, and includes data on: S573F, V667I, L670F, A675T, A678D, S1271L, and R1313W.

The use of these transient expression systems provides a multitude of electrophysiological and biological data, increasing our understanding of how each variant may be impacting synaptic transmission and neuronal viability. Using this data, these variants may be classified as gain-of-function (GoF, increased function compared to wild-type) or loss-of-function (LoF, diminished/decreased function compared to wild-type). There is no association between domain location along the receptor and functional outcome as both GoF and LoF variants are found throughout all domains of both *GRIN2A* and *GRIN2B*.^[13] Surprisingly, 57% (4/7) – S573F, A675T, S1271L, and R1313W – of DEE-associated *GRIN2D* variants might be considered LoF. GoF variants – V667I and L670F – make up 29% of those tested, while the A678D variant likely needs to undergo further testing before a proper GoF or LoF assignment can be made. Additionally, it should be noted that all of the GoF or LoF designations assigned above are only approximations based off previous work that involved more complete functional analyses and calculations.^[11]

The S573F mutation lies within the pre-M1 region, a critical linker within the TMD that has profound impact on receptor gating.^[1] The pre-M1 linker helps transfer molecular motion within the ABD to movement of the membrane-spanning helices within the TMD, allowing for receptor opening and ion flux across the membrane. Not surprisingly, the S573F variant impacts multiple aspects of receptor function including agonist potency, efficacy of magnesium block, current amplitude, and open channel probability.^[89] Although this variant shows a modest increase in agonist potency, this may be considered a LoF mutation as magnesium block is increased, while current amplitude, open channel probability, and surface expression are all diminished.^[89]

The V667I and L670F variants are both strong GoF mutations, found within the M3 helix of the TMD, and are just upstream of the highly conserved SYTANLAAF region which is also critical for receptor gating.^[1, 39] Both variants display an increased agonist potency and open channel probability, as would be expected for mutations within the region of the receptor.^[39, 89] Additionally, both V667I and L670F have prolonged deactivation times compared to wildtype GluN2D.^[39, 89] This feature makes these variants unique, as all other DEE-associated *GRIN2D* mutations show either no change in receptor deactivation or an accelerated (e.g. a shortened) deactivation time course, which is a measure of how long the receptor active open following agonist removal. Thus, both the V667I and L670F variants will flux more ions into the neuron, potentially leading to calcium-induced excitotoxicity and cell death.^[92] Indeed, cell viability assays with cultured cortical neurons transfected with a GluN2D-V667I plasmid revealed substantial neuronal swelling and extensive cell death.^[39]

The A675T and A678D variants also reside within the M3 helix of the TMD. The A675T variant shows an increased agonist potency together with a strong decrease in current amplitude, therefore this variant likely represents a LoF mutation.^[89] In fact, both the A675T and A678D variants had such small current amplitudes that measuring deactivation time was not possible. In both cases, the peak current amplitude measured in transfected HEK cells was less than 10% of a wildtype GluN2D-containing NMDA receptors.^[89] This intriguing finding suggests that both mutations severely impact the ability of the receptor to open in response to agonist binding. In addition, the A678D variant displayed an increase in

open channel probability, which makes classifying this variant more difficult. Either way, the profound decrease in current amplitude indicates that neurons harboring these variants will likely experience deficits in calcium influx. In agreement with these findings, transfected cultured neurons containing a GluN2D-A678D plasmid showed a significant decrease in viability.^[89]

The S1271L and R1313W variants are located within the CTD. This domain of the receptor has not been extensively studied but has been shown to be important for surface trafficking and a localization site for protein-protein interactions. Interestingly, both the S1271L and R1313W variants showed a decreased agonist potency, despite being localized far away from the ABD. However, this change in agonist potency was not robust enough to elicit changes in other aspects of receptor function as open channel probability, current amplitude, and deactivation time were all unchanged compared to wild-type. This combination of decreased agonist potency and decreased surface expression likely indicate that both S1271L and R1313W are LoF mutations.^[89] The finding that mutations within the CTD can generate DEE may be suggestive of the importance of protein-protein interactions facilitated by the GluN2D subunit in controlling circuit excitability.

In fact, unique to all variants tested for functional activity was a decrease in surface expression. Changes in surface expression have been reported previously with epilepsy-associated variants in the *GRIN2A* and *GRIN2B* genes, where most of which also showed a reduction in surface expression.^[11] The idea that any perturbation to the amino acid composition of the GluN2D subunit diminishes surface expression is interesting, perhaps suggesting that biology has precisely tuned surface expression for its maximal level. On the other hand, mutations that increase surface expression of GluN2D may be lethal and thus might not be represented within our current findings. Changes in surface expression may also impact the subunit composition and ratio of NMDAR assemblies. As neuronal circuits grow and develop, this alteration combined with the modification to receptor function, may invoke a multitude of maladaptive homeostatic plasticity mechanisms. Examples of this phenomenon can be shown in the vast range of neurological and developmental phenotypes seen in patients with *GRIN2D* variants.^[39, 87–89]

Overall, it seems that a loss of GluN2D function within the brain is sufficient to promote DEE, likely via hypofunction of GABAergic interneurons. The two GoF variants found, V667I and L670F, may also be indirectly diminishing interneuron function by initiating calcium-induced excitotoxicity. Future characterization of these variants using knock-in mouse models is needed to better understand the impact of these mutations on circuit function and interneuron viability.

3.3 Clinical symptoms and electroencephalographic signatures of patients with *GRIN2D* variants

Although DEE-associated *GRIN2D* variants may share some of the same functional characteristics, the seizure types, electroencephalographic (EEG) patterns, and developmental phenotypes they cause are largely heterogeneous. A surprising finding was that two variants, V667I (n = 3) and L670F (n = 2), were found in more than one patient, with a total of 13 individuals containing a DEE-associated *GRIN2D* variant (Table 2).^[39, 89]

The mean age of onset is 11 ± 3.6 months, with a median age of onset of 6.5 months (Figure 2C). *GRIN2D*-related DEE does not appear to be gender-related as the male to female ratio is 5:7, with gender unknown for the A675T patient.^[89]

There appears to be little commonality in the type of seizures elicited by *GRIN2D* mutations, even amongst individuals carrying the same *GRIN2D* variant. All three V667I patients display different seizure patterns, with one having atypical absence seizures, one having partial complex seizures, and another having generalized seizures.^[39] The same is true of the two patients harboring the L670F variant: one has epileptic spasms while the other has generalized tonic-clonic (GTC) seizures.^[89] The heterogeneity of seizure types amongst patients with the same *GRIN2D* variant indicates a highly complex nature of epileptogenesis. Unfortunately, this complexity and disease heterogeneity amongst individuals containing the same mutation appears to be common, as individuals with Dravet's syndrome – a genetic epilepsy caused by a haploinsufficiency in the *SCN1A* gene – also exhibit multiple different seizure phenotypes.^[93] While a full list of the different seizure types caused by *GRIN2D* variants can be found in Table 2, two of the most common are focal motor to GTC (n = 4) and epileptic spasms (n = 4).

DEE-associated *GRIN2D* patients also exhibit diverse types of electrical activity recorded via EEG, although EEG patterns are more uniform than seizure type (Table 2). The majority (75%, 9/12; EEG pattern for Patient #8 is unavailable) of patients exhibited multifocal epileptiform activity, indicating that more than one brain region may be affected. This finding is not surprising given the expression patterns of GluN2D throughout the brain and their ability to regulate inhibitory tone via GABAergic interneurons. The two most common EEG patterns were multifocal spikes (n = 5) and hypsarrhythmia (n = 4; Figure 2D). The other three patients – Patient #5-V667I, Patient #6-L670F, and Patient #13-R1313W – all had focal epileptiform activity that presented as spike-and-wave discharges or paroxysmal fast activity.^[12, 39] Again, patients containing the same *GRIN2D* variant did not exhibit similar EEG recordings, as one of the three V667I patients and one of the two L670F patients presented focal interictal EEG recordings.^[39, 89]

Furthermore, patients with *GRIN2D* mutations express a host of neurological and developmental abnormalities. As mentioned previously, the GluN2D subunit is heavily involved in motor control likely via robust expression in the spinal cord and basal ganglia. Thus, it is not surprising that 69% (9/13) of DEE-associated *GRIN2D* variants are associated with hypotonia and poor motor control (Figure 2E). However, the type and location of hypotonia experienced spans the gamut between minor hypotonia with dyskinetic and choreiform movements (Patient #3) to severe hypotonia with tetraplegia (Patient #13). Approximately 45% (5/11; data not available for Patient #4 and Patient #8) experienced some form of cortical atrophy when overall brain morphology was assessed via magnetic resonance imaging (Figure 2E). Only 38% (5/13) *GRIN2D* DEE-associated patients have cerebral visual impairment, indicating an improper relay between the information sensed by the retina and that received by the visual cortex. These results are somewhat surprising given the strong GluN2D expression in rod bipolar cells and in interneurons in the cerebral cortex, as described earlier.^[23, 59]

Autism and the presence of autistic-like features and behaviors are also fairly common, as 31% (4/13) patients with DEE-associated *GRIN2D* variants are on the autism spectrum. Most present with poor eye contact and social withdrawal. 23% (3/13) of patients have plantar deformities, with two having *pes planus* and one having 'abnormal plantars'. Other behavioral difficulties manifest as problems with sleeping (n = 2), feeding (n = 2), breathing (n = 1), and speaking (n = 1). As with seizure type and EEG pattern, neurological and developmental symptoms are highly varied among individuals with the same *GRIN2D* variant.

3.4 Treating patients with *GRIN2D* variants

Unfortunately, another unifying characteristic of these 13 DEE *GRIN2D* patients is a resistance to typical AED therapy. Specifically, 75% (9/12; Patient #2 has not reported any formal drug therapy) of these patients were either refractory or only saw partial recovery from seizure burden on AEDs (Figure 2E).^[39, 88, 89] Patient #7 showed a modest improvement of EEG recordings on steroids, whereas Patient #9 and Patient #11 were seizure free using typical AEDs. Additionally, these data only reflect information on seizure burden and EEG readouts in response to drug therapy as data regarding recovery from secondary behavioral, neurological, and developmental symptoms are not available. However, given the severity of DEE, it seems unlikely that any conventional drug therapy would be able to ameliorate all the *GRIN2D* variant-inflicted symptoms.

The three patients containing the V667I mutation are unique in that all three were exposed to targeted drug therapies that were specific for NMDARs. After exome sequencing, all three patients were placed on a combination therapy of memantine and magnesium.^[39, 89] Memantine, as mentioned previously, is an NMDAR pore blocker that is efficacious at all NMDAR subunits, and shows an increase in potency at GluN2D receptors.^[35] Additionally, magnesium was chosen as this divalent cation can block the NMDAR pore at polarized membrane potentials. Of the three patients receiving the memantine/magnesium cocktail, only one (Patient #3) responded positively. One patient did not have a response, whereas the other patient worsened and saw an increased seizure burden. When examining age of onset, Patient #3 was diagnosed and placed on this targeted treatment earlier than the other two patients.^[39, 89] This suggests that not only is precision medicine needed but perhaps there exists a critical time window in which therapeutics may be most beneficial. While the exact mechanism behind the exacerbated seizures for the V667I patient on memantine/magnesium is unclear, this study provides critical information needed to combat the severe group of neurological diseases generated via *GRIN2D* variants.

4. Conclusions

The confluence of the advances in genetics, structural biology, and functional analysis will provide an opportunity to gain insight into the relationships between human disease and *GRIN2D*/GluN2D NMDA receptors and provide new insight into new strategy of personalized medicine to improve diagnosis and effective treatment. In turn, the evaluation of the disease associate *GRIN2D* variants will provide new insight into GluN2D NMDAR function.

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Figure-1. Distinct functional features of GluN2D-containing NMDARs.

A, Space-filled ribbon structure of a homology model of human GluN1/GluN2D receptor generated from GluN1/GluN2B crystallographic data (PDB-4PE5; *Karakas and Furukawa, 2014; Lee et al., 2014*). ATD: amino terminal domain, ABD: agonist-binding domain, TMD: transmembrane domain, comprised by M1, M2, M3, and M4. **B**, Concentration-response curves for glutamate (in the presence of 30 μM glycine) for GluN1/GluN2A (Blue) and GluN1/GluN2D (Red) generated from two-electrode voltage-clamp current recordings at $V_{HOLD} -40$ mV. **C**, Representative traces of whole cell voltage clamp current recordings in response to 1 mM glutamate from human GluN1/GluN2A- and GluN1/GluN2D-transfected HEK293 cells at 60 mV. **D**, Representative unitary currents (activated by 1 mM glutamate and 50 μM glycine) from outside-out single channel patch clamp recordings from human GluN1/GluN2A- and GluN1/GluN2D-transfected HEK293 cells at $V_{HOLD} -80$ mV. C, close; O, open. H. Yuan, unpublished data.

Figure-2. Developmental and epileptic encephalopathy (DEE)-associated human *GRIN2D*/*GluN2D* missense variants.

A,B, Locations of DEE-associated GRIN variants (indicated by BOLD/PURPLE). S1,S2 comprise the ABD; TMD-link: transmembrane domains (comprised by M1, M2, M3, and M4) and linker regions (S1-M1 linker, M1-M2 linker, M2-M3 linker, M3-S2 linker, and S2-M4 linker); see Fig-1 for domain organization; References are in the text. **C**, Box-and-whisker plot for the age of onset of 12 patients harboring 9 unique DEE-associated *GRIN2D* variants. Mean age of onset is 11 ± 3.6 months, with a median age of onset of 6.5 months. Onset data for Patient #8 was not available. **D**, Different types of electroencephalographic (EEG) patterns were observed from 12 patients harboring 9 unique DEE-associated *GRIN2D* variants. EEG type was not available for Patient #8. The most common EEG recording was multifocal discharges (5/12), followed by hypsarrhythmia (4/12), focal spike-and-wave discharges (2/12), and paroxysmal fast activity (1/12). **E**, Various clinical features observed in DEE-associated *GRIN2D* variant-containing patients. Besides the unifying phenotypes of DEE (epilepsy and developmental delay/intellectual disability), 69% of patients are hypotonic, 31% of patients display autistic-like features and behaviors, 45% of patients experienced cortical atrophy in brain MRI, and 75% are refractory to typical AED therapy. Magnetic resonance imaging (MRI) data was not available for Patient #4 and Patient #8. Patient #2 has not started any formal drug therapy and was not included in the calculation.

Table 1.Summary of missense mutations and rare variants in *GRIN2D*/GluN2D NMDARs

	<i>Gene</i>	Genotype	Protein	Location	Phenotype	References
1	<i>GRIN2D</i>	c.1345G>A	p.D449N	ABD (S1)	Epi, ID	Tsuchida et al., Clinical Genetics 2018
2	<i>GRIN2D</i>	c.1396C>G	p.L466V	ABD (S1)	SCZ	Tarabeux et al., Transl Psychiatry 2011
3	<i>GRIN2D</i>	c.1718C>T	p.S573F	pre-M1	Epi; ID	XiangWei et al., Brain 2019
4	<i>GRIN2D</i>	c.1774A>C	p.M592L	M1	ASD	Tarabeux et al., Transl Psychiatry 2011
5	<i>GRIN2D</i>	c.1999G>A	p.V667I	M3	Epi; ID; 3 pts	Li et al., Am J Hum Genet. 2016; XiangWei et al., Brain 2019
6	<i>GRIN2D</i>	c.2008C>T	p.L670F	M3	Epi, ID; 2 pts	XiangWei et al., Brain 2019
7	<i>GRIN2D</i>	c.2023G>A	p.A675T	M3	Epi; ID	XiangWei et al., Brain 2019
8	<i>GRIN2D</i>	c.2033C>A	p.A678D	M3	Epi; ID	XiangWei et al., Brain 2019
9	<i>GRIN2D</i>	c.2043G>C	p.M681I	M3	Epi, ID	Tsuchida et al., Clinical Genetics 2018
10	<i>GRIN2D</i>	c.2080A>C	p.S694R	ABD (S2)	Epi, ID	Tsuchida et al., Clinical Genetics 2018
11	<i>GRIN2D</i>	c.2197A>G	p.M733V	ABD (S2)	SCZ	Tarabeux et al., Transl Psychiatry 2011
12	<i>GRIN2D</i>	c.2701G>A + c.2702C>T	p.A901I	CTD	SCZ	Tarabeux et al., Transl Psychiatry 2011
13	<i>GRIN2D</i>	c.2776G>A	p.A926T	CTD	ASD	Tarabeux et al., Transl Psychiatry 2011
14	<i>GRIN2D</i>	c.2944G>C	p.A982P	CTD	SCZ	Tarabeux et al., Transl Psychiatry 2011
15	<i>GRIN2D</i>	c.3812C>T	p.S1271L	CTD	Epi; ID	XiangWei et al., Brain 2019
16	<i>GRIN2D</i>	c.3937C>T	p.R1313W	CTD	Epi, ID	XiangWei et al., Brain 2019

ASD: Autism Spectrum Disorder, Epi: epilepsy/seizures, ID: Intellectual Disability, SCZ: Schizophrenia

ATD: amino-terminal domain, ABD: agonist-binding domain, TMD: transmembrane domains (M1, M2, M3, M4), CTD: carboxy-terminal domain

Table-2.

Summary of DEE-related *GRIN2D* variants and patient’s phenotypes

	Patient-1	Patient-2	Patient-3	Patient-4	Patient-5	Patient-6	Patient-7	Patient-8	Patient-9	Patient-10	Patient-11	Patient-12	Patient-13
<i>GRIN2D</i> variants (NM_000836)	c.1345G>A;p. (Asp449Asn)	c.1718C>T; p. (Ser573Phe)	c.1999G>A; p. (Val667Ile)	c.1999G>A;p. (Val667Ile)	c.1999G>A;p. (Val667Ile)	c.2008C>T; p. (Leu670Phe)	c.2008C>T; p. (Leu670Phe)	c.2023C>A; p. (Ala678Asp)	c.2033C>A; p. (Ala678Asp)	c.2043G>C;p. (Met681Ile)	c.2080A>C;p. (Ser694Arg)	c.3812C>T; p. (Ser1271Leu)	c.3937C>T; p. (Arg1313Trp)
Variant location	ABD	pre-M1	M3	M3	M3	M3	M3	M3	M3	M3	ABD	CTD	CTD
Gender	male	female	male	female	female	male	Female	N/A	Female	male	female	male	female
Age at onset	3 days	2mo	9mo	4mo	2mo	< 1yo	4mo	N/A	3y, 5mo	2yo	2yo	2mo	<1yo
Seizure types and EEG	startling seizure; EEG: intermittent multifocal discharges at 15 mon old	focal motor to GTCS; EEG: slow background, multifocal spikes	atypical absence; EEG: bilateral central spikes; hypersarhythmia	complex partial seizure; EEG: frequent spikes and waves during awake and hypersarhythmia during sleep	generalized seizure; EEG: sharp and spike wave discharges	epileptic spasms; EEG: diffuse paroxysmal abnormalities	focal motor to GTCS; EEG: continuous hypersarhythmia with bilateral synchrony	epileptic spasms; EEG: N/A	focal motor → GTCS; EEG: frequent multifocal spikes; runs of faster alpha activity, multifocal spikes	febrile convulsion, atlebrile generalized or unilateral convulsions; myoclonic seizure; EEG: Frequent multifocal spikes and diffuse spwc and sharp waves	tonic seizures, atonic seizures, atypical absence; EEG: Diffuse spwc. Focal spikes. Continuous diffuse spwc during sleep	epileptic spasms, myoclonic jerks; EEG: modified hypersarhythmia; sporadic focal epileptic activity, runs of faster beta activity	GTCS, focal clonic, myoclonic, epileptic spasms; EEG: frequent/ almost continuous sharp-waves with high amplitude
Developmental delay and other neurological features	no head control, no rolling over, no speech; hypotonia, hyperreflexia; Disturbed sleep pattern	walked at 2yo; poor eye contact, autistic behaviors	severe DD; mild hypotonia, dyskinetic and choreiform movements; cerebral visual impairment, oculomotor apraxia, changing tone, periodic breathing pattern	sitting alone at 3.5yo, walking with support at 3.5yo, speaking single words at 6.5yo; axial hypotonia, mild appendicular hypertonia; facial dysmorphism, bilateral mild fifth finger clinodactyly, mild pes planus; poor sleep	rolling over at 9 mon old, unable to sit alone; truncal hypotonia, and orofacial appendicular hypertonia, atetoid limb movements, autistic features; microcephaly, severe pes planus	severe DD; severe hypotonia; cerebral visual impairment, feeding difficulties	severe DD; hypotonia, dyskinetic and choreiform movements; visual impairment with inconstant fixation	DD	walked at 2yo; single words at 2.5yo; hypotonia; poor eye contact, autistic behaviors	sitting alone at 18 mon old, DQ < 10 at 7 yo; myoclonus, ballismus, autism	Walking alone at 14 mon old, speaking two-word sentences at 36 mon old, IQ 64 at 7yo, full-scale IQ 58 at 11yo; ADHD	Severe DD; severe axial hypotonia, continuous movements; cerebral visual impairment, pyramidal signs with abnormal plantars (2yo), failure to thrive	severe: severe hypotonia, tetraplegia, wheelchair user, scoliosis, cerebral visual impairment, visual impairment, amaurosis, feeding difficulties
Brain MRI	loss of white matter, thin corpus callosum at 7.5 mon old	Normal	mild cerebral atrophy	N/A	normal	cortical atrophy	mild cerebral atrophy	N/A	Normal	normal at 6yo	normal at 3yr	normal	mild cerebral atrophy
Response to AEDs	PB, CZP, VPA: partially effective, CLB, Vlt, B6; not effective	no formal therapy	seizure free on memantine, IVIG, oral steroids and Mg	Intractable, ACTH, LEV, Memantine, Mg, ketamine, pentobarbital, MDZ	intractable; seizure free on memantine, sulfamie, lamotrigine, and valproic acid	no response	mild amelioration of EEG on steroids – no clinical overt seizures	no response	seizure free on VPA, LEV, and clonazepam	Intractable: VPA, CLB, LTG, LEV, LCM	ESM: effective for atonic seizures and atypical absence, CBZ, VPA, CZP; not effective	relatively controlled by VPA, TPM and ELF (in combination with VNS)	no response
Functional consequences	N/A	↑glu/D-serine potency, ↑Mg inhibition, ↓pH inhibition, ↓Popen, ↓current amplitude, ↓receptor cell surface expression	↑glu/gly potency, ↓Mg/pH/zinc inhibition, ↑Popen, ↑deactivation tau, causes neuronal cell death, dendritic swelling	↑glu/gly potency, ↓Mg/pH/zinc inhibition, ↑Popen, ↑deactivation tau, causes neuronal cell death, dendritic swelling	↑glu/gly potency, ↓Mg/pH/zinc inhibition, ↑Popen, ↑deactivation tau, causes neuronal cell death, dendritic swelling	↑glu/gly/D-serine potency, ↓pH inhibition, ↑Popen, ↑deactivation tau, ↓charge transfer, ↓receptor cell surface expression	↑glu/gly/D-serine potency, ↓pH inhibition, ↑Popen, ↑deactivation tau, ↓charge transfer, ↓receptor cell surface expression	↑glu/gly/D-serine potency, ↓pH inhibition, ↓current amplitude, ↓receptor cell surface expression	↑glu/gly/D-serine potency, ↓current amplitude, ↓receptor cell surface expression; a significant ↓ in neuronal viability	N/A	N/A	↓gly potency, No effects on Mg/pH inhibition, no effects on Popen and current amplitude, ↓receptor cell surface expression	↓gly potency, No effects on Mg/pH inhibition, no effects on Popen and current amplitude, ↓receptor cell surface expression

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	Patient-1	Patient-2	Patient-3	Patient-4	Patient-5	Patient-6	Patient-7	Patient-8	Patient-9	Patient-10	Patient-11	Patient-12	Patient-13
Source	Tsuchida et al., 2018	Xiang Wei et al., 2019	XiangWei et al., 2019; Li et al., 2016	Li et al., 2016	Li et al., 2016; Xiang Wei et al., 2019	XiangWei et al., 2019	Xiang Wei et al., 2019	XiangWei et al., 2019	Xiang Wei et al., 2019	Tsuchida et a., 2018	Tsuchida et a., 2018	XiangWei et al., 2019	XiangWei et al., 2019

AED, anti-epileptic drug; DD, developmental delay; GTCS, generalized tonic clonic seizures; LEV, levetiracetam; NA, not available; TPM, topiramate; VGB, vigabatrin; VNS, vagal nerve stimulator; ELF, ethylloflazepate; VPA, valproate.