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Clinical and therapeutic significance of genetic variation in the *GRIN* gene family encoding NMDARs

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Abstract

Considerable genetic variation of N-methyl-D-aspartate receptors (NMDARs) has recently become apparent, with many hundreds of *de novo* variants identified through widely available clinical genetic testing. Individuals with *GRIN* variants present with neurological conditions such as epilepsy, autism, intellectual disability (ID), movement disorders, schizophrenia and behavioral disorders. Determination of the functional consequence of genetic variation for NMDARs should lead to precision therapeutics. Furthermore, genetic animal models harboring human variants have the potential to reveal mechanisms that are shared among different neurological conditions, providing strategies that may allow treatment of individuals who are refractory to therapy. Preclinical studies in animal models and small open label trials in humans support this idea.

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e. None reported.

However, additional functional data for variants and animal models corresponding to multiple individuals with the same genotype are needed to validate this approach and to lead to thoughtfully designed, randomized, placebo-controlled clinical trials, which could provide data in order to determine safety and efficacy of potential precision therapeutics.

Keywords

GRIN; NMDARs; intellectual disability; epilepsy

Diagnosis and phenotyping

Developmental encephalopathies[1] are disorders associated with delayed childhood development, including impairments in cognition, communication, as well as fine motor and gross motor skills. Impairments in sensory systems such as vision and hearing are also often apparent. When these developmental delays involve multiple modalities, they are often referred to as global delay. When developmental delays persist for more than 5 years, they are almost certain to alter the individual for a prolonged period, thereby compromising their ability to carry out functions needed to live an independent life. When this is the case, the term Intellectual Disability (ID) is used, which is usually categorized as mild, moderate, severe or profound [2]. The alterations in developmental trajectories, including regression or loss of previously acquired skills, can be distinct for each disorder and thus an essential component of phenotyping. Further classification of epilepsy in terms of seizure characteristics and EEG properties is a necessary feature of phenotyping [3, 4]. Individuals harboring GRIN variants also show altered brain structure revealed through imaging, aberrant muscle tone, movement disorders such as ataxia, behavioral disturbances, as well as symptoms that can be understood through autonomic dysfunction. Efforts to harmonize the description of these characteristics has been developed (e.g. Human Phenotype Ontology, HPO, https://hpo.jax.org/app/) and could help synchronize phenotypic descriptions in the peer-reviewed literature[5].

Genotyping

Whole exome sequencing (WES) is widely considered to be the first stage of diagnostic testing when neurodevelopmental disorders are suspected[6], which includes individuals who show concerns for developmental delay, intellectual disability, and/or seizures. While gene panel testing typically utilizes the same technology as next generation sequencing, it still has limitations in terms of analysis compared to WES, such as a reduced capability to detect genomic copy number variants (CNVs, including deletion or duplication). Variation in genomic copy number will usually affect multiple genes, and is detected using chromosomal or SNP microarrays. Genetic diagnosis[7-10] for rare diseases has become an important means to end the cycle of unproductive diagnostic testing, and offers the prospect of catalyzing the development of precision therapies[11-13], even for open label “N=1” trials for the most severely affected individuals [14]. Genetic diagnosis via WES has changed therapeutic treatments, can produce improved outcomes[15, 16], and when applied early, is cost-effective [17]. Although care guidelines for certain well

known disorders are continually being refined each year[18-20, 21], diagnosis remains a critical component for rare diseases as it can facilitate natural history studies, provide more accurate prognosis, and help clinicians determine guidelines for the care of individuals with newly discovered genetic disorders. Despite these positive attributes, WES still has several important limitations. These include: causative genes may not be identified for very rare disorders, especially when recessive, some genetic alterations (e.g. repeat expansions, complex rearrangements or very low-grade mosaics) may not be identified due to technical difficulties, and data describing intronic elements capable of controlling translation and RNA splicing are not accessible. For these reasons, many individuals with potential genetic disorders will remain undiagnosed, and this is a driving force arguing for the utility of whole genome sequencing, including for those already subjected to WES.

Classification of Variants

Genetic variation in humans differs for each gene, which necessitates statistical analysis to validate gene constraints as well as the susceptibility of each gene for neurological (or other) diseases. One of the more established scores relevant for this is the *Z score* for missense variants. In addition, a score for the *probability of being loss-of-function intolerant* (pLI) and for the *observed / expected metric* (OE) (https://gnomad.broadinstitute.org/help/constraint) are commonly used. A variant Z score is interpreted as positive if it is greater than 3.09, which suggests that a given transcript is intolerant of variation, and thus constrained. In addition, a pLI score that is greater than 0.9 implies intolerance to null variants, including frameshift, nonsense, and splice variants. Using the upper end of the OE confidence interval (LOEUF, < 0.35) is another way to establish a firm threshold for intolerance to null variants.

Criteria for the classification of sequence variants[22] into five categories has been established by The American College of Medical Genetics and Genomics. These standard terms include 1) pathogenic, 2) likely pathogenic, 3) variants of uncertain significance (VUS), 4) likely benign, and 5) benign. This variant classification considers multiple criteria such as the type of variant, its origin, location, the functional consequences, conservation, allele frequency, predictive computational metrics, and other factors. It is also important to evaluate whether copy number variants (CNV) are present. Indeed, large CNVs affect multiple genes that may contribute to the clinical phenotype in a complex manner, whereas small CNVs could alter the transcription of just a single gene.

Clinical reports describing genetic variation often refer to variants as pathogenic or likely pathogenic, but can also classify a variant as VUS, indicating that it is unclear whether it does or does not participate in clinical aspects of an individual's disorder. The final determination of whether a variant can alter the function of the encoded protein necessarily requires the evaluation of all avaliable data by a specialist who is familiar with both the clinical disorder as well as the gene. In a subset of cases, determination of the functional properties for the protein encoded by the variant is advisable, even in some cases for null variants that may have dominant negative properties upon the expression of the other allele. The interpretation of genetic variants in a manner that can unequivocally account for an individual's phenotype remains a goal that awaits a description of the complete spectrum of variation in humans, including across ethnic backgrounds and in health and disease.

GRIN variants

The GRIN gene family contains *GRIN1*, *GRIN2A-D*, *GRIN3A-B*. Of these, variants in *GRIN1*, *GRIN2A*, *GRIN2B*, and *GRIN2D* genes have been identified in individuals with various neurodevelopmental disorders (Figure 1); the association of *GRIN2C*[23] and *GRIN3*[24] with disease remains unclear (see Table 1, Figure 1B). As expected, there are both similarities as well as differences among these genes in terms of the spectrum of genetic variants that appear to contribute to human pathological conditions.

GRIN genes code for different NMDAR GluN subunits. Functional NMDARs (Figure 1A) require 2 subunits coded by *GRIN1* and 2 subunits coded by any *GRIN2* or *GRIN3*. This specificity is regulated in anatomical, developmental, and neuronal subtype specific manners. NMDARs are assembled from subunits within the endoplasmic reticulum; the spectrum of features that regulate assembly, such as the relative availability of any given subunit, are not fully understood. NMDARs are essentail to multiple key roles including neuronal migration, synaptic connectivity, neuronal pruning and survival, and synaptic plasticity (reviewed in this Special Edition and see [25]). Thus, it is not surprising that variation of NMDARs plays a role in human disease.

GRIN1 is a phylogenetically conserved gene. Through statistical representations in the neurotypical population, *GRIN1* appears intolerant of both missense (Z = 6.22) and null variation (pLI = 0.98; LOEUF = 0.31), assessed via gnomAD (v2.1.1, https://gnomad.broadinstitute.org/). Pathogenic heterozygous missense variants have arisen *de novo*, and are clustered near one another in a limited number of domains with important functions, such as the agonist binding domain (made up of two different portions of the polypeptide chain), pore-forming transmembrane domains, and the linker regions that connect the agonist binding domain to the channel pore and control the opening of the channel following agonist binding[26, 27] (Figure 1). The amino-terminal domain (also know as N-terminal domain, or NTD) that resides distal to the membrane and the C-terminal domain (CTD), which resides intracellularly, are more tolerant to variation, and thus appear less likely to contribute to *GRIN1*-related developmental disorder. However, this notion may change with further study of the actions and binding partners for these two regions as we still have an incomplete understanding of their roles.

Despite the high constraint metrics, null *GRIN1* variants have not yet been associated with neurological disease. Several individuals who possess heterozygous *GRIN1* null variants are reported to not show any distinct clinically relevant phenotypes[26]. One family with a homozygous *GRIN1* null variant that was associated with fatal epileptic encephalopathy has been reported[26], suggesting that null variants can contribute to *GRIN1*-related disorders but only when affecting both alleles. This is currently the only report of an autosomal recessive form of any GRIN-related disorder. Despite the description of two families with rare homozygous missense variants [26, 28], autosomal recessive *GRIN1*-related developmental disorders due to missense variation have not yet been unequivocally confirmed.

In contrast to *GRIN1*, through similar statistical representations in the neurotypical population (gnomAD v2.1.1, https://gnomad.broadinstitute.org/), *GRIN2A* is intolerant of null variation (pLI = 1.00; LOEUF = 0.19), but not necessarily intolerant for missense variants (Z = 2.83). Presumed pathogenic heterozygous missense variants are typically *de novo* and are localized in a similar fashion as for *GRIN1* and other GRIN genes to the regions encoding the agonist binding domain, the pore-forming transmembrane domain, and the short linkers that connect the agonist binding domain and the channel pore (Figure 1). Similar to *GRIN1*, the NTD as well as the intracellular CTD show more tolerance to missense variation [29].

In addition, *GRIN2A* null variants are associated with a disease spectrum that can be clinically mild, with affected individuals in some cases able to reproduce (Table 1). Thus, *GRIN2A* null variants are unique among pathogenic variants in the GRIN gene family, as they are occassionally found to be inherited.

Similar to *GRIN1*, the *GRIN2B* gene through similar statistical representations in the neurotypical population (gnomAD v2.1.1, https://gnomad.broadinstitute.org/), is intolerant for missense (Z = 5.42) and null variation (pLI = 1.00; LOEUF = 0.06). Pathogenic heterozygous missense variants are typically *de novo* and show a similar pattern of localization to critical domains in the subunit encoded by *GRIN2B* (Figure 1). Variants are highly concentrated in the agonist binding sites, in addition to the transmembrane and linker regions, with minimal pathogenic variants in the NTD or the CTD [30-32]. *GRIN2B* null variants are associated with *GRIN2B*-related neurodevelopmental disorder, and are usually found to arise *de novo* [30, 31].

Like *GRIN1* and *GRIN2B*, the *GRIN2D* gene through similar statistical representations in the neurotypical population (gnomAD v2.1.1, https://gnomad.broadinstitute.org/), is intolerant for missense (Z = 4.85) and null variation (pLI = 1.00; LOEUF = 0.17). Pathogenic heterozygous missense variants are typically *de novo*[33, 34]. *GRIN2D*-related disorders are the least frequently observed among GRIN disorders, and thus it is premature to draw conclusions about potential clustering of pathogenic missense variants in any region of the protein encoded by *GRIN2D* (Figure 1B and Table 1). Null variants in *GRIN2D* gene (other than large scale deletions) have not yet been reported.

Clinical characteristics

Patients with *GRIN1*-related neurodevelopmental disorder show multiple deficits, including ID, epilepsy, hypotonia, and for some individuals, movement disorders. All affected individuals evaluated to date show variable levels of ID:, including 5% mild, 7% moderate, 71% severe, or 17% profound [31].

Sixty-five percent of individuals presented with epilepsy (Figure-1C, Table 2). The onset ranges from birth to 11 years of age, and two thirds demonstrated resistance to conventional antiseizure treatment. Seizure types include generalized seizures (68 %; with multiple semiologies), focal seizures (20 %), and epileptic spasms (13 %). Additional clinical characteristics (Figure-1C, Table 2) include hypotonia (66 %), movement disorders

(48 %), cortical visual impairment (CVI, 34 %), as well as oculogyric crises (11 %). Some individuals show features of autism spectrum disorders, or exhibit other behavior problems such as stereotypic movement disorder (32 %), sleep problem (15 %), and self-harm behavior (7 %) [31]. A subset of individuals showed an unusual type of cortical malformation that consisted of extensive bilateral polymicrogyria together with lateral ventriculomegaly, enlarged extra-axial spaces, reduced thickness of the corpus callosum, basal ganglia dysplasia, and decreased white matter volume [35].

Neurodevelopmental disorders in *GRIN2A* individuals are associated predominantly with epilepsy and ID. However, as many as 37 % of the individuals demonstrate normal intelligence and 63 % have ID (46% mild, and 22%with moderate, 11% severe, 21% profound) [29]. Brain imaging is usually normal and only a minority (14 %) reveal nonspecific changes[29]. Epilepsy is present in almost all *GRIN2A* individuals (Figure-1C, Table 2) with onset from birth to 8 years of age. Interestingly, seizures may resolve between 8 and 20 years of age. Fifty-seven percent present with focal seizures; 40 % showed a centrotemporal focus similar to Rolandic epilepsy. EEG demonstrated in 34 % continuous spikes and waves during slow wave sleep (CSWS). Additional challenges including hypotonia (29 %), movement disorders (27 %), autism spectrum disorders (9 %), and/or psychiatric disorders, such as schizophrenia (3 %) [29] (Table 2). A unique feature associated within the GRIN-associated disorders is the breadth of language/speech problems observed in *GRIN2A*-related developmental disorders, which include dysarthria, dysphasia, speech dyspraxia, speech regression with residual impairments in more than a third (Table 2) and 19 % had aphasia [29, 36].

Individuals affected with *GRIN2B*-associated disorders exhibit ID, hypotonia, epilepsy, and movement disorders. All affected (so far) have DD preceding certain degrees of ID (Figure-1C, Table 2). A wide range of ID is observed, which includes 6% mild, 21% moderate, and 73% severe-to-profound (http://grin-portal.broadinstitute.org/#tab-1201-3).

Some form of epilepsy is present in half of the affected individuals and shows an onset between birth to 9 years of age. Seizures are medically refractory for half. The spectrum of seizure characteristics is similar to that observed for *GRIN1* variants, which include 35% epileptic spasms, 48% focal seizures, and 58% generalized seizures [31].

Additional clinical characteristics are perhaps less frequent and/or are somewhat milder then *GRIN1*-associated neurodevelopmental disorders. However, the spectrum of clinical characteristics is similar to *GRIN1* with hypotonia (56%) and spasticity (23%), autism spectrum disorder (26%), movement disorders (10%), and cortical visual impairment (8%) [31] (Table 2). Cortical malformations with polymicrogyria and basal ganglia dysplasia in *GRIN1* is mirrored in a subset of individuals with *GRIN2B* disorders [30].

As described above, variation in *GRIN2D* appears far less frequent than that in *GRIN1*, *GRIN2A*, or *GRIN2B* [37, 38]. One population-based study reported no truncated *GRIN2D* variants, suggesting a crucial role in early development and survival [39]. However, a different conclusion was reached by other investigators[24], who raised the idea that intronic variations (i.e. missense) might be related to the risk for schizophrenia[23].

GRIN2D missense variants have been observed in individuals with severe, drug-resistant epileptic encephalopathy with an early onset [33, 34, 38, 40, 41]. Functional analysis of variants introduced into *GRIN2D* cDNA have shown gain of function characteristics[33, 38], possibly with a compensatory reduced expression[34]. Only 28 *GRIN2D* variants are currently documented in the literature[25].

GRIN3A, which encodes a glycine binding subunit that can coassemble with GluN1 to form a glycine sensitive receptor in neurons[42], is expressed throughout the CNS[25]. Various studies suggest a role in multiple behaviors[43]. Genetic variations in *GRIN3A* genes have been associated with bipolar disorder [44], however, no further evidence has suggested *GRIN3A* is involved in neurological disorders. *GRIN3B*-encoding the GluN3B subunit is expressed in brainstem and spinal cord [45]. One study of the function of *GRIN3B* gene in motor neuron diseases reported a SNP that was caused a null allele was present in about 10% of the general population [46]. Additional research evaluating truncating variants in individuals with neurodevelopmental disorders identified *GRIN3A* and *GRIN3B* truncation variants in the control cohorts[24, 39]. These observations cannot determine whether or not *GRIN3* plays a role in human disease.

Functional assessment of GRIN variants

The determination of how a GRIN variant might alter protein function usually requries electrophysiological and biochemical assays. The goal of these functional assays is to determine whether a variant results in a loss-of-function (LoF), a gain-of-function (GoF), does not influence receptor function, or produces some complex mixture of effects on protein function. NMDARs have many different functional properties, so testing typically needs to be a comprehensive evaluation of agonist potency, receptor function, endogenous modulation, and protein expression and trafficking. Further, these assessments are limited to what we currently know about NMDAR function. It is important to take this comprehensive approach because, a priori, it is impossible to know which function(s) a given variant will impact. A case in point is the recently decribed *GRIN1*_p.P532H variant, which resides in the glycine binding domain but exerts its effects on glutamate binding to the GluN2 subunit[47]. Functional testing often occurs on several levels. The first tier of experimentation typically assesses how a variant alters function of the NMDAR as expressed in a a non-neuronal heterologous expression system, for example, *Xenopus* oocytes or cultured fibroblasts (e.g. HEK293 cells) that can be manipulated to express the recombinant subunits of interest, thereby allowing the study of a purified population of receptor by various assays. The second tier of experimentation can include experiments to assess how a variant might alter NMDAR function in a cultured neuron. This can be followed by study of the variant introduced into a whole animal either by knockin methods or viral-mediated gene transfer. The secondary tier of study involving native neurons are meant to extrapolate the phenotype and neuronal characteristics that are expected to be observed in affected individuals. Whether or not a given variant is ultimately proven to contribute to disease characteristics may involve studies that go beyond the expertise of a neurologist or clinical geneticist tasked with making a determination of likely pathogenticity.

Functional analysis is a necessary step that provides a wealth of opportunities to advance understanding of the condition. It allows stratification of individuals for future approaches to precision therapies, as individuals with variants that produce similar changes in receptor function are more likely to yield similar results in clinical trials and clinical practice than variants that simply happen to be close to each other on the polypeptide chain. Precision therapeutics requires objective evidence of benefit through well-designed clinical trials (safety and efficacy), and stratification of similar individuals increases the likelihood that clinical trials will be able to reach meaningful conclusions.

Testing in animals often does recapitulate key features observed in affected individuals. However, in other situations, the variant might perturb neurological systems that are altered in a way that does not fully reproduce the observed clinical features. For example, a missense variant from one individual with epilepsy might, when knocked into mice, elevate or reduce seizure threshold. Failure of complete reproduction of the clinical features does not invalidate *in vivo* models showing a clear and measureable deficit[48]. Moreover, such deficits can be studied for altered neurodevelopment, rescue pharmacology, or for the refinement of genetic strategies. That is, these models can be useful tools to gain insight into various paths to mitigate deficits in affected individuals.

The number of known genetic variants in NMDARs vastly outnumbers those for which we have some functional information. However, an appreciation of the utility of comprehensive functional characterization in terms of diagnoses, stratification, and future potential treatments is driving increased effort toward functional characterization. Among the more than 700 *GRIN* variants, published functional evaluation exists for for less than half [25] (see http://functionalvariants.emory.edu/ and http://grin-portal.broadinstitute.org/). Much of this work has progressed at the level of tier 1 in heterologous expression systems, and several parameters can be measured *in vitro* in recombinant receptors. However, clearly this initial approach has the limitations that heterologous expression does not enable detection of functional changes that require the unique developmental and anatomical context and features of the neuronal environment explored in tier 2 studies. Variants in the intracellular CTD[49, 50] do not typically alter functional parameters in heterologous systems. However, the CTD is known to interact with a large number of scaffolding proteins and intracellular signaling systems in neurons, and thus neuronal assays are needed to potentially see the ramifications of variation in the CTD. In this regard, cells or tissues from transgenic animals are needed to evaluate how CTD variants alter NMDAR function. Accessible functional parameters that can be assessed include glutamate and co-agonists glycine potency, voltage-dependence and potency for channel block by extracellular Mg^{2+}, sensitivity to endogenous extracellular modulators such as Zn^{2+}, single channel open probability, receptor deactivation time course in order to predict the synaptic response time course, desensitization, synaptic plasticity, and receptor trafficking, including both receptor subcellular localization and cell surface expression [29, 34, 37, 51-59].

Missense variants can in principle alter any properties of the NMDAR. For this reason, it is important to study as many functional attributes of the receptor as possible, since one variant can (and often does) alter multiple parameters. For example, a variant can produce a change in one parameter that increases current flow, and a unrelated change in another parameter

that reduces current flow. While human variation is heterozygous for missense variants of NMDAR subunits, in tier 1 testing missense variants are typically tested in a homozygous state and co-expressed with a wild-type co-subunit. For example, wild-type GluN1 will be co-expressed with a GluN2B variant. For clarity, this demonstrates the potential for the variant to alter NMDAR function.

To recap the prior descriptions, human NMDAR subunit variations include missense (which could be GoF, LoF or unknown), nonsense (resulting in a truncated subunit) or deletion of the entire subunit. These are heterozygous alterations, meaning there is another unaffected allele coding for a normal subunit. It remains unclear if heterozygous deletion of an entire NMDAR subunit will behave simply as a heterozygous missense LoF variants, the latter of which may have a dominant negative effect by assembling with other GluN subunits in the endoplasmic reticulum. In support of this dominant negative role of LoF missense variants, as noted previously and in Table 1, heterozygous loss of *GRIN1* does not show any distinct clinically relevant phenotypes[26]. Further, nonsense variation that results in a truncated NMDAR subunit may have variable effects, including as dominant negative, that may depend on the length of truncation. This could occur if variants that reduced function or truncated the receptor depleted the pool of partner GluN subunits, and making instead receptor complexes that do not reach the cell surface. The determination of functional consequences in this situation may require knock-in animals that contain the variant in one allele, and thus mimic more closely the human variation, allowing assessment of trafficking as well as circuit development and normal behaviors. This construct validity is the necessary first step towards face validity of effective precision therapy applied to an animal model, to ensure that ultimately precision therapy (see “Preclinical pharmacological studies“) can be translated into safe and efficacious clinical trials and ultimately applications in affected individuals.

In vivo models of GRIN variation

In an effort to move a step closer to human physiology, the generation of mouse models containing human-specific variants allows for the detailed exploration of GRIN variants. More specifically, genetically-modified mice are essential for the elucidation of how these variants impact the brain on a developmental, circuit, cellular, and molecular level, as well as provide investigators with a mammalian platform for testing therapeutic approaches. Although homozygous null (knockout) mutations for each of the seven GRIN genes have been generated, information gleaned from the study of these mice should be viewed cautiously, as the majority with GRIN variants only have one impacted allele As discussed above, gene deletions can affect individuals differently than LoF missense and nonsense variants, which suggests that LoF missense and nonsense variants have the capacity to act as dominant negatives. Thus, heterozygous null (knockout) or variant knock-in mouse models-whether nonsense, missense, or deletion – are highly relevant to the human condition and should thus be given precedent when making comparisons to affected individuals.

The first targeted mutation in the GRIN gene family was the homozygous null mutation in the *Grin1* gene, which resulted in perinatal lethality[60, 61]. *Grin1* null mice die due to respiratory failure as well as failure to suckle, highlighting the omnipresent roles

of NMDARs in all facets of early brain function, including those in the brainstem. Like homozygous *Grin1* null mice, homozygous *Grin2b* null mice also suffer perinatal lethality[62]. Although *Grin2b* knockout mice do breathe, they do not suckle and thus do not survive the neonatal period. The homozygous null mutations of *Grin2a*[63, 64], *Grin2c* [65], and *Grin2d* [66] appear less severe than those homozygous null *Grin1* and *Grin2b* mice, with each being fertile and viable, albiet with abberations in a variety of behavoiral and cognitive tasks[38, 67-73]. However, these characterizations largely preceded an appreciation of the scope of GRIN disorders, and additional study of null mutations, especially in heterozygous animals, could provide meaningful insights into the impact of LoF GRIN variants. In this vein, subsequent studies of homozygous and heterozygous *Grin2a* null mice revealed that these mice displayed epileptiform discharges, although these changes in circuit excitability appear to be developmentally transient [74, 75].

To date, multiple mouse models carry missense mutations or human variants in *Grin1*, *Grin2a*, and *Grin2b genes*, with additional models of human *GRIN* variants actively being developed. The first missense variants of *Grin1* (p.N616Q and p.N616R, which reside within the ion channel) revealed two important concepts for GRIN disorders [76]. First, heterozygous mice with a *Grin1* loss-of-function missense variant are capable of generating a more severe phenotype than heterozygous null mice (however see [77]). While heterozygous *Grin1* null mice have no clear phenotype, heterozygous p.N616Q mice have diminished maternal behaviours and heterozygotes for p.N616R are unable to suckle, with both mouse models dying in the early neonatal periods. The only similar mapped *GRIN1* human variant in the pore-forming region, p.N616K, is likely GoF (http://grin-portal.broadinstitute.org/), but further details are unpublished. It should be noted, however, that data needed to make strong conclusions regarding LoF variants and heterozygous null mice are still lacking, as the location of this variant along the different domains of the receptor likely plays a decisive role in determining its severity (i.e. variants in the pore-forming region are predicted to be more severe than those in the NTD)[29]. Second, missense variants for *Grin1* can demonstrate a broad range of phenotype characteristics that depends on the the amino acid exchange encoded by the missense variant. Additional studies of homozygous missense *Grin1* mutations (that do not currently map to human variants) were either lethal[78, 79] or showed altered behaviors [78, 80, 81]. In studies of *Grin1* haploinsufficiency(Figure-2A) [77], behavioral abnormalities were rescued in adult mice by Cre recombinase gene editing [82].

Recently, patient-specific knock-in mouse models have been generated for variants in *GRIN2A* (p.S644G, GoF[58]; p.N615K, GoF[83]) and *GRIN2B* (p.C456Y, mixed[59]). In both *Grin2a* GoF variants, homozygosity was either preadolescent lethal, dying around P15, (Figure-2B) [58] or associated with a worsening phenotype [83]. Notably, heterozygous GoF variants displayed a range of phenotypes, with either reduced seizure thresholds (Figure-2C) and hippocampal thinning [58] to changes in circuit output, such as a reduction in EEG power in the γ-range [83]. The study of *Grin2b* p.C456Y highlighted a limitation of our current classification system, as this variant presented with mixed (gain and loss) functional changes to receptor function when studied in vitro[30, 51, 55]. However, the totality of these changes resulted in a decrease in *Grin2b* expression, coupled with reduced hippocampal long-term depression (Figure-2D,E). With the mixed functional effects observed in vitro,

there were no *a priori* ways to predict how this variant might impact neurodevelopment and total brain function in a mouse, calling to attention the importance of patient-derived animal model testing. Moreover, this study suggested that treatment with cycloserine early, (but not late), in development could improve behavioral abnormalities. These data seem to be in contrast to those described in the knockdown *Grin1* mouse model where behavorial deficits could largely be restored by gene correcting therapy in adulthood (Figure-3A) [77]. These differences could be due to the different roles that GluN1 and GluN2B play in brain function or differences in temporal expression patterns. These results also reveal a disparity in chemical versus genetic therapeutic strategy. Regardless, the sum of our data on patient-derived mouse models suggest that each human variant might require its own unique model to identify the optimal therapeutic approches. In order to expedite this process, future *in vivo* model development and characterization should build a map of examplar human *GRIN* variants with gain, loss, and mixed function variants spanning each domain of a particular subunit. This approach might allow future prediction of phenotypic consequences, best clinical trial stratification, and best therapeutic options for each affected individual based on where their variant lies along the different modular domains of the receptor.

Preclinical pharmacological studies

Given that a *GRIN* variant can often be defined as gain or loss of function, how best can pharmacological modulation be identified that can alleviate symptoms? Pharmacological modulation of clinical symptoms secondary to modulation of NMDAR properties could be beneficial if, and only if, current symptoms are actively being mediated by the GoF or LoF for the NMDAR. However, this is likely an overly simplistic viewpoint, and it seems almost certain that compensatory changes and/or maladaptive plasticity due to the variant will have altered some aspect of overall brain development (e.g., findings noted with altered brain imaging). Such compensation may produce unintended consequences of pharmacological treatments when they alter NMDAR function. In this situation, precision medicine based on correcting the NMDAR variants function *in vitro* may not be helpful to affected individuals (although classifying individuals based on variant function and location along the affected subunit would likely bring more empirical evidence to improve existing treatments). This is the main reason why increased emphasis on rodent models of *GRIN* variants is needed.

Using *in vitro* heterologous systems, NMDAR channel blockers and negative allosteric modulators have shown to retain high potency and efficacy at some GoF variants, and this *in vitro* data is a powerful tool to inform pharmacological agent testing in mouse models [25, 30, 33, 51, 53, 58, 84-87]. Differential sensitivity of each GoF *GRIN* variant [53, 84, 85, 87]. emphasizes the need to determine the sensitivity of each GRIN variant to potential pharmacological treatments. However, compounds with the highest potency or those that are the most selective *in vitro* seldom prove to be the best for use in animals. For example, as mentioned previously, mice with a homozygous variant in *Grin2a* (p.S644G, GoF) die before the third week of life. Chronic treatment with either dextromethorphan (channel blocker), Nuedexta (dextromethorphan + quinidine), or memantine (channel blocker) significantly delayed the onset of lethal seizures (Figure-3C) [58]. Surprisingly, the most efficacious treatment was Nuedexta, even though the IC_{50} for dextromethorphan on homozygous *GluN2A*-p.S644G recombinant receptors is 22 μM, which is very similar to that of

memantine at 30 μM[58]. Although, the difference in efficacy is likely due to quinidine's effect at prolonging dextromethorphan's half-life, this study demonstrates a pressing need for drug validation in rodent models of *GRIN* variants in order to better understand the functional specificity of NMDAR channel blockers and negative allosteric modulators.

The treatment and pharmacological outlook for the use of NMDAR positive allosteric modulators may be more straightforward. Relying on endogenous chemical backbones, such as neurosteroids (i.e. endogenous neurosteroid 24(S)-hydroxycholesterol), FDA-approved aminoglycosides or co-agonists at the glycine-binding site (i.e. D-serine, L-serine, and D-cycloserine) can help to mitigate common reasons why pharmacological agents fail *in vivo* (i.e. brain penetration and bioavailability), while still being highly efficacious on a set of loss-of-function *GRIN1*, *GRIN2A* and *GRIN2B* variants[47, 51, 52, 56, 88, 89]. Early treatment (before adulthood) with D-cycloserine on young transgenic mice harboring a patient-derived loss-of-function *GRIN2B-p.*C456Y variant mitigated NMDAR-dependent synaptic long-term depression and corrected aberrant anxiety behavior (Figure-3D,E) [59]. These preclinical studies provide foundational evidence for clinically useful pharmacological agents for the treatment of *GRIN* variants as stand-alone options or synergist options in addition to genetic manipulation.

Human studies

Multiple pathogenic variants in *GRIN* genes have been described as GoF or LoF for the NMDAR, which provides clinicians with opportunities to potentially mitigate dysfunction through the use of either NMDAR-specific blockers/inhibitors or enhancers/potentiators. An initial proof of principle was established by treating a male individual with early-onset epileptic encephalopathy and drug-resistant seizures due to a pathogenic *de novo* GOF missense variant in *GRIN2A* (p.L812M) with the FDA-approved NMDAR channel blocker memantine. This treatment resulted in a significant reduction in seizure frequency[84, 87]. Additional individuals with *GRIN* variants have been treated with memantine, but only several of them have been reported in the peer-reviewed literature.

There are a limited numbers of publications if solely considering cases with a demonstrably pathogenic *GRIN* variant with a confirmed GoF consequence for the NMDAR. For *GRIN1*, recently a GOF missense variant that showed enhanced potency for a set of FDA-approved NMDAR channel blockers was described (*GRIN1*-p.M641I), which creates a situation where clinical treatments would preferentially block variant but not wild type receptors [87]. The affected individual responded favorably to memantine with a significant reduction in seizure frequency and severity of spasms, with evidence of efficacy when seizures/spasms worsened during accidental discontinuation of memantine [87]. For *GRIN2A*, only one other individual (p.S644G) has been reported in the literature to have a significant reduction in seizure frequency after adding memantine and later dextromethorphan, similar to the GRIN2A variant p.L812M [58]. For *GRIN2B*, four affected individuals have been reported [30]. All of these individuals showed subtle and subjective improvements in awareness, behavior or sleep. However, none had quantifiable improvements in these modalities by objective assessment. For *GRIN2D*, two affected individuals have been reported that showed mild to moderate improvement in seizure frequency following the addition of

memantine to their treatment regimen. The older one subsequently developed refractory status epilepticus, which showed dramatic electroclinical improvement during treatment with ketamine and magnesium [33]. Only one affected person with LOF *GRIN* variant has been reported to experience subjective behavioral improvements as well as improved sleep and motor development during administration of L-serine [89]. Across these studies, a safety assessment cannot be provided due to the small number of individuals studied; it may be dangerous to generalize further. These early but limited studies suggest that precision medicine for *GRIN*-related disorders may be possible, but there remains a pressing need to assess these treatments in well-designed, double-blinded, placebo-controlled clinical trials that systematically and quantitatively assess multiple parameters of safety and efficacy.

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Highlights

- We review genetic variation of NMDA receptors associated with neurological disease
- Genetic variation of NMDA receptors can alter their function
- Initial studies suggest links between functional alterations and treatment strategies
- Additional studies, including animal models, are needed to validate this approach

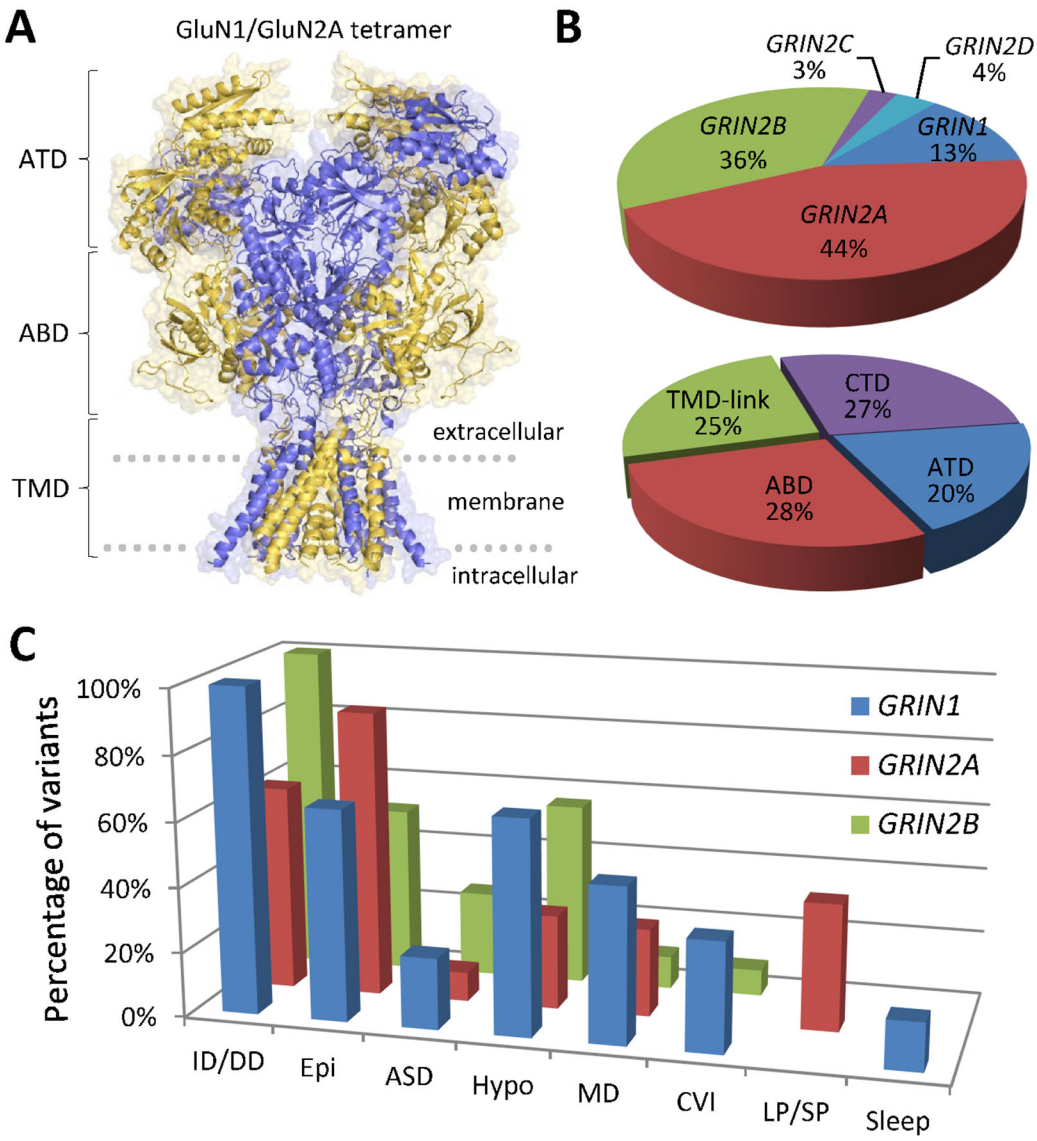

Figure 1. *GRIN* variants are associated with various neurologic disorders.

A), Architecture and domain organization for the NMDAR. *B*), Summary of NMDAR variants in different GRIN subunits and domains. *C*), Summary of *GRIN* variant-associated phenotypes. ATD: amino terminal domain, ABD: agonist binding domain, TMD-link: transmembrane domains (M1-4) and linker regions, CTD: intracellular carboxy-terminal domain. ASD: Autism Spectrum Disorder, CVI: cortical visual impairment, Epi: epilepsy/seizures, Hypo: hypotonia, ID/DD: intellectual disability/developmental delay; LP/SP: language/speech problems; MD: movement disorders; Sleep: sleep problems. Many individuals showed multiple phenotypes, which are only a snapshot of the current literature, which is disproportionally weighted by different diagnostic approaches and ascertainment.

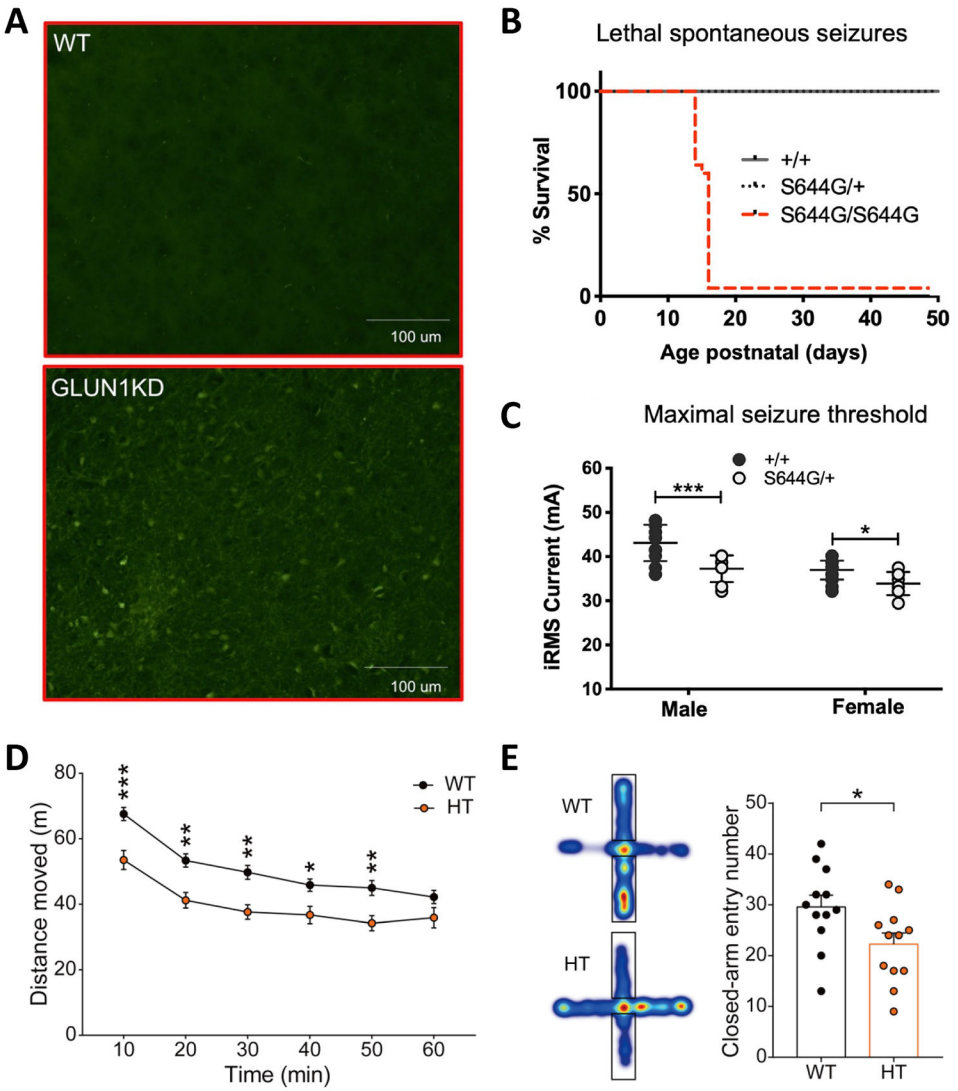

Fig. 2. Mouse models of human *GRIN* variants.

A), Fluoro-Jade C staining showed that neurons of *Grin1*-KD mice in the striatum surrounding the anterior commissure are degenerating in the adult brain. *B*), Survival curve showing the rate and onset between postnatal days 15 and 17 of *Grin2a*p. S644G genotype-dependent in F2 hybrid male and female mice. *C*), Heterozygous (het) *Grin2a*-p.S644G adult mice have lower seizure threshold in minimal seizure end points. *D*), Hypoactivity in *Grin2b*$^{+/C456Y}$ mice (P68–78) in the open-field test. *E*), Anxiolytic-like behavior in *Grin2b*$^{+/C456Y}$ mice (P70–124) in the elevated plus-maze, as shown by entries into in closed arms. Modified with permission from (Intson et al., 2019) (*A*), (Amador et al., 2020) (*B,C*), and (Shin et al., 2020) (*D,E*).

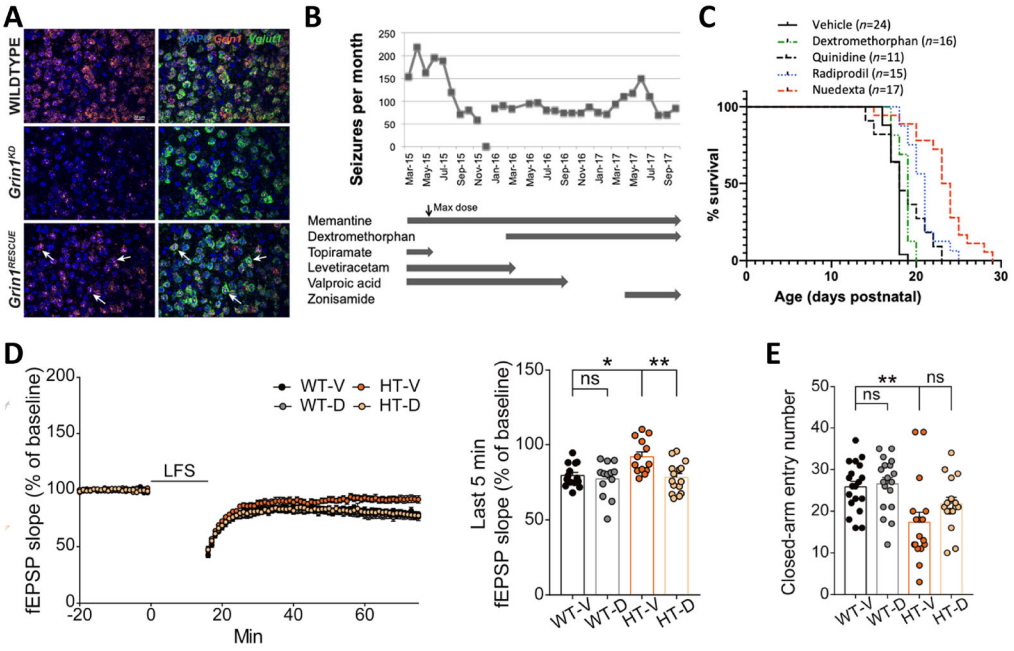

Figure 3. Therapeutic strategies for the treatment of GRIN variants.

A), *Grin1* mRNA expression in Vglut+ cells in the adult mouse somatosensory cortex (1.53 mm lateral from midline). *Grin1* (orange) and *Vglut1* (green) mRNA was visualized in mouse sagittal sections (20 μm) with fluorescent in situ hybridization in WT, *Grin1KD*, and *Grin1*RESCUE mice. *B*), Impact of drug therapy on clinical seizures for the individual with *GRIN2A*-p.S644G variant. *C*), Pharmacological rescue of lethal seizures of p.S644G/p.S644G homozygotes, showing the respective survival after daily injections of dextromethorphan, quinidine, radiprodil, and Nuedexta®. *D*), Early chronic oral D-cycloserine (DCS) treatment (40 mg/kg) normalizes LFS-LTD at SC-CA1 synapses in juvenile *Grin2b*$^{+/C456Y}$ mice (P17–20). *E*), Early chronic oral DCS treatment (40 mg/kg) improves anxiolytic-like behavior in adult *Grin2b*$^{+/C456Y}$ mice (P63–73). Modified with permission from[77] (*A*), [58](*B*,*C*), and [59](*D*,*E*).

Table 1.

NMDAR subunit null mouse models and prevalence of null mutations in affected individuals.

Protein Subunit Name	Heterozygous Null Phenotype	Homozygous Null Phenotype	Individuals with Large-Scale Disruptions (LSD) or Premature Stops
Severe Phenotypes in Mice			
GluN1	Viable and fertile[61, 90]; accelerated cortical neuron development[90]	Lethal – No suckling response and poor lung development[61]; altered brainstem nuclei development[60]	4 total (4/91; 4.4%): 4 preSTOP
GluN2B	Viable and fertile[62]	Lethal – No suckling response with altered brainsteam nuclei development[62]	36 total (36/258; 14%): 14 LSD and 22 preSTOP
Viable, but with deficits			
GluN2A	Viable and fertile[63]; minute and transient changes in brain microstructure assessed via diffusion tensor imaging[74]	Viable and fertile [63]; diminished spatial learning[63] with largely transient epileptiform activity[74, 75]; alterations in pup vocalizations[74]	53 total (53/311; 17%): 29 LSD and 24 preSTOP
GluN2C	Viable and fertile[65]; alterations in sensorimotor gating [91]	Viable and fertile[65]; some alterations in working memory[70] and sensorimotor gating [91]	1 total (1/19; 5.3%): 1 preSTOP
GluN2D	Viable and fertile[92]	Viable and fertile; hypolocomotion and increased anxiety-like behaviors[72, 73, 92]	Yet to be reported
GluN3A	Viable and fertile[93]	Viable and fertile[93]; impaired locomotor activity[94]; changes to spatial and aversion learning[94, 95]	2 total (2/13; 15%): 2 preSTOP
GluN3B	Viable and fertile[96]	Viable and fertile[96]; impaired motor learning and coordination[96]	Yet to be reported

Heterozygous and homozygous null (knockout) mouse models have been generated for each of the seven *GRIN* genes. Mice containing homozygous null mutations for GluN1 and GluN2B are postnatal lethal, while heterozygous null offspring survive normally. In general, homozygous null mice, regardless of which gene has been disrupted, display some sort of aberrant phenotype that could be extrapolated to patients. It should be noted, however, only one large-scale chromosomal disruption has been reported to be homozygous in human, an inherited *GRIN2A* deletion affecting both alleles, with all others reported being heterozygous. Given the overall lack of characterization of heterozygous null *GRIN* mouse models, it is difficult to determine whether they can truly mimic features of the human condition. Large-scale disruptions (LSD) refers to chromosomal deletions, duplications, inversions, insertions, or translocations; premature stops (preSTOP) refers to a nonsense mutation resulting in a premature stop codon in the mRNA.

Table-2.

Summary of phenotypes associated with *GRIN* variants

	GRIN1	GRIN2A	GRIN2B
Intellectual disability	100%	63%	100%
Epilepsy/seizures	65%	88%	57%
Muscular hypotonia	66%	29%	56%
Movement disorders	48%	27%	56%
Autism spectrum disorder	22%	9%	26%
Cortical visual impairment	34%	–	8%
Language/speech problems	–	39%	–
Schizophrenia	–	3%	–
Sleep problems	15%	–	–

Many patients showed multiple phenotypes, which are only snapshot of the current literature, which is disproportionally weighted by different diagnostic and ascertainment procedures.