

## REVIEW

# Disease-Associated Variants in *GRIN1*, *GRIN2A* and *GRIN2B* genes: Insights into NMDA Receptor Structure, Function, and Pathophysiology

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## Summary

N-methyl-D-aspartate receptors (NMDARs) are a subtype of ionotropic glutamate receptors critical for synaptic transmission and plasticity, and for the development of neural circuits. Rare or *de-novo* variants in *GRIN* genes encoding NMDAR subunits have been associated with neurodevelopmental disorders characterized by intellectual disability, developmental delay, autism, schizophrenia, or epilepsy. In recent years, some disease-associated variants in *GRIN* genes have been characterized using recombinant receptors expressed in non-neuronal cells, and a few variants have also been studied in neuronal preparations or animal models. Here we review the current literature on the functional evaluation of human disease-associated variants in *GRIN1*, *GRIN2A* and *GRIN2B* genes at all levels of analysis. Focusing on the impact of different patient variants at the level of receptor function, we discuss effects on receptor agonist and co-agonist affinity, channel open probability, and receptor cell surface expression. We consider how such receptor-level functional information may be used to classify variants as gain-of-function or loss-of-function, and discuss the limitations of this classification at the synaptic, cellular, or system level. Together this work by many laboratories worldwide yields valuable insights into NMDAR structure and function, and represents significant progress in the effort to understand and treat *GRIN* disorders.

## Keywords

NMDA receptor • *GRIN* genes • Genetic variants • Electrophysiology • Synapse • Animal models

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## Introduction

N-methyl-D-aspartate receptors (NMDARs), encoded by *GRIN* genes, are ionotropic glutamate receptors present at virtually all excitatory synapses in the central nervous system. Classical NMDARs have a characteristic biophysical signature, with a requirement for the binding of two agonists (glutamate and glycine/D-serine), strong block by Mg<sup>2+</sup> at resting membrane potentials, high Ca<sup>2+</sup> permeability, and relatively slow activation and deactivation kinetics [1]. These properties enable NMDARs to serve as coincidence detectors of presynaptic glutamate release and postsynaptic depolarization that removes the Mg<sup>2+</sup> block. The resulting NMDAR-mediated Ca<sup>2+</sup> influx is a key signal regulating activity-dependent changes in synaptic strength [2] that underlie the development of neural circuits and their

ability to process and store information [1]. Given this physiological role, dysfunction of NMDAR signaling is considered to be a significant factor in the etiology of diverse neurological disorders. In recent years, rare or *de-novo* variants of *GRIN* genes have been identified in patients with neurodevelopmental syndromes characterized by intellectual disability, developmental delay, with features of autism, schizophrenia, or epilepsy [3], sometimes collectively referred to as *GRIN* disorders.

There are seven NMDAR genes, *GRIN1*, *GRIN2A-D* and *GRIN3A-B*, encoding GluN1, GluN2A-D and GluN3A-B subunits, respectively. NMDARs are assembled as heterotetramers of two obligatory GluN1 subunits and two GluN2 and/or GluN3 subunits [1]. Aside from the GluN1 subunit, GluN2A and GluN2B are the most abundant subunits in principal neurons in the forebrain [4], with the GluN2B expression beginning early during embryonic development, followed by the expression of GluN2A postnatally; the expression of both GluN2A and GluN2B persists until and throughout adulthood [1]. NMDAR complexes can be diheteromeric (i.e., containing two GluN1 and two identical GluN2 subunits) or triheteromeric (containing two GluN1 and two different GluN2 subunits). Receptors containing different combinations of GluN2 subunits have different functional and pharmacological properties [5] and likely play different physiological roles [6]. It has been suggested that the majority of NMDARs in the adult forebrain are GluN1/GluN2A/GluN2B triheteromers [7,8] (but see [9]), whose structure and function has been explored in recent years, thanks to new methods for selectively expressing or isolating triheteromeric receptor complexes [5,10,11].

NMDAR subunits have a modular design: each subunit contains an extracellular amino-terminal domain (ATD), an agonist-binding domain (ABD), a transmembrane domain (TMD), and a cytoplasmic carboxy-terminal domain (CTD), interconnected by polypeptide linkers [1]. Recent structural studies of NMDAR complexes in different conformational states [12] or exploring receptor conformational dynamics [13,14,15] have greatly improved our understanding of NMDARs at the molecular level. NMDAR gating is a coordinated process involving agonist-induced structural rearrangements of the core gating machinery formed by the ABDs and the TMDs of all four subunits. The gating mechanism is regulated by voltage-dependent channel block by  $Mg^{2+}$ , and by endogenous allosteric modulation by  $H^+$ ,  $Zn^{2+}$ ,  $Ca^{2+}$ , and several classes of naturally present small molecules [16]. All domains participate in

endogenous allosteric modulation. The role of the ATDs in mediating the effects of  $H^+$  and  $Zn^{2+}$  is particularly well described [17]. Our own recent work has contributed to the characterization of NMDAR modulation by endogenous neurosteroids [18,19,20,21,22] and membrane cholesterol [23,24], that interact primarily with the TMDs.  $Ca^{2+}$ -dependent NMDAR modulation involves membrane-proximal regions of the CTDs [25,26,21]. While the intrinsically disordered CTDs resist structural characterization, they are known to contain residues that can undergo post-translational modification, and binding sites for intracellular signaling and scaffolding proteins. The CTDs thus play an important role in regulating NMDAR trafficking, signaling, and even gating [27]. Disease-associated *GRIN* gene variants are found in all receptor domains, and the variant pathogenic effects may be related to any aspect of receptor function, including the gating process, as well as changes in receptor allosteric modulation, post-translational modification, protein-protein interactions or trafficking.

Either excessive or insufficient NMDAR function is associated with neurological or neuropsychiatric disorders, suggesting a narrow physiological window of NMDAR activity [4]. Consistent with this, *GRIN* genes have low frequencies of variation in the healthy population [28,29,3,1]. In recent years, many *GRIN* gene variants have been identified through high-throughput sequencing in patients with neurological and neuropsychiatric symptoms [28,30,3]. To date, over 700 disease-associated *GRIN* gene variants absent in the healthy population (<https://gnomad.broadinstitute.org>) have been described, distributed among the different NMDAR subunits, but predominantly found in GluN2A (311 variants, 43 %), GluN2B (258 variants, 35 %) and GluN1 (91 variants, 13 %) [1]. Several different databases collect information about disease-associated *GRIN* gene variants (<https://www.ncbi.nlm.nih.gov/clinvar>; <https://webapp2.pharm.emory.edu/cferv>; [https://www.grin-database.de/gen\\_table](https://www.grin-database.de/gen_table); <https://alf06.uab.es/grindb/home>). Most frequent are missense variants in exon regions that result in a single amino acid substitution in the protein sequence. Some patients carry nonsense or frameshift variants that introduce a premature stop codon. Variants in 5' or 3' untranslated regions, introns, or splice sites, or large-scale chromosomal rearrangements have also been reported [31]. In an overwhelming majority of cases the variants arise *de novo* and the variant is present in only one allele of the affected gene [3].

To understand how different variants may lead to

disease, many groups have embarked on functional characterization of individual patient-derived *GRIN* gene variants. Here we review the published reports of the functional evaluation of disease-associated variants in *GRIN1*, *GRIN2A*, and *GRIN2B* genes. Most of the reports so far have examined recombinant receptors composed of GluN subunits heterologously expressed in non-neuronal cell types, such as *Xenopus* oocytes or HEK293 cells. Typically, these studies use well-established patch-clamp electrophysiology and immunofluorescence microscopy methods, allowing some comparison across studies and research groups. We have focused on four parameters most commonly used to assess NMDAR function: receptor affinity for glutamate, receptor affinity for glycine, channel open probability ( $P_o$ ), and receptor cell surface expression. Based on such parameters, pathogenic variants can be classified as loss-of-function (LoF) or gain-of-function (GoF), and therefore as candidates for therapeutic positive or negative pharmacological modulation, respectively. We discuss whether and how the variant position within the subunit amino acid sequence may predict its receptor-level functional consequence(s) [32] and its LoF or GoF classification.

Individual variant pathogenicity at the receptor level, however, may not accurately predict its functional effects at the synaptic, cellular, or system level. Relatively fewer reports include functional data from *in-vitro* neuronal preparations and only a handful of *in-vivo* studies in animal models exist to date. We briefly summarize this work and argue for the need to include these more physiologically relevant experimental models in future studies of the etiology of *GRIN* disorders.

## Functional characterization of *GRIN* gene variants in non-neuronal systems

Our literature search found 74 studies [32a] of recombinant receptors heterologously expressed in non-neuronal systems (Tables 1, 2, 3) together reporting functional data for 45 disease-associated GluN1 variants, 72 GluN2A variants, and 70 GluN2B variants (49 %, 23 %, and 27 % of disease-associated GluN1, GluN2A, and GluN2B variants reported in [1], respectively). This represents a substantial body of work that is beginning to yield insights not only into the mechanisms of pathogenicity of different *GRIN* gene variants, but also into NMDAR structure and function more generally.

Importantly, patients are typically heterozygous for their *GRIN* gene variant, so any individual patient's

NMDARs may be functionally heterogeneous: some may be composed entirely of unaffected subunits and some may include one or two subunits containing the potentially pathogenic variant combined with unaffected subunits of various types. It is therefore necessary to study not only diheteromeric receptors containing two variant subunits, but also triheteromeric receptors containing only one potentially pathogenic variant subunit. Typically, the function of triheteromeric receptors containing one variant subunit was found to be intermediate between the function of wild-type receptors and diheteromeric receptors containing two variant subunits [33,34,35,36,37,38]. For some variants, triheteromeric receptors show a functional deficit comparable to diheteromeric receptors (e.g., GluN2A-D731N [39]; GluN2A-N616K [40]). While studies of triheteromeric receptors are essential, here we focus on results obtained for diheteromeric receptors (Tables 1, 2, 3, Figs 1, 2, 3, 4), because these data represent the majority of the available information.

It is useful when studies directly compare effects of different variants, ideally with multiple functional parameters assessed in the same system [34,41,42,40,43,44,45]. These data often illustrate that individual variants can affect multiple functional parameters, sometimes in contradictory directions, complicating simple variant classification as LoF or GoF [43,44,45]. Some studies have proposed ways to integrate information about multiple functional parameters into one readout, for example by estimating how a variant would affect synaptic charge transfer or  $Ca^{2+}$  influx, to evaluate the overall impact of the variant on physiologically relevant NMDAR signaling [34,44,46].

Though imperfect, variant classification as LoF or GoF has prompted pharmacological investigations with the goal of identifying suitable NMDAR modulators that could correct the functional change associated with different disease-associated variants. Most generally, negative allosteric modulators show promise in treating certain GoF variants [47,48], while positive modulators may compensate for the effects of LoF variants [49,50]. Ultimately, pharmacological intervention should be more sophisticated and specifically target the functional parameters altered by the variant. Importantly, individual variants can show reduced or enhanced sensitivity to different modulators [51,42,52,53,50,48], further emphasizing the need to tailor therapy to the specific variant present. While we do not discuss rescue pharmacology of disease-associated *GRIN* gene variants any further in this review, the relevant references are

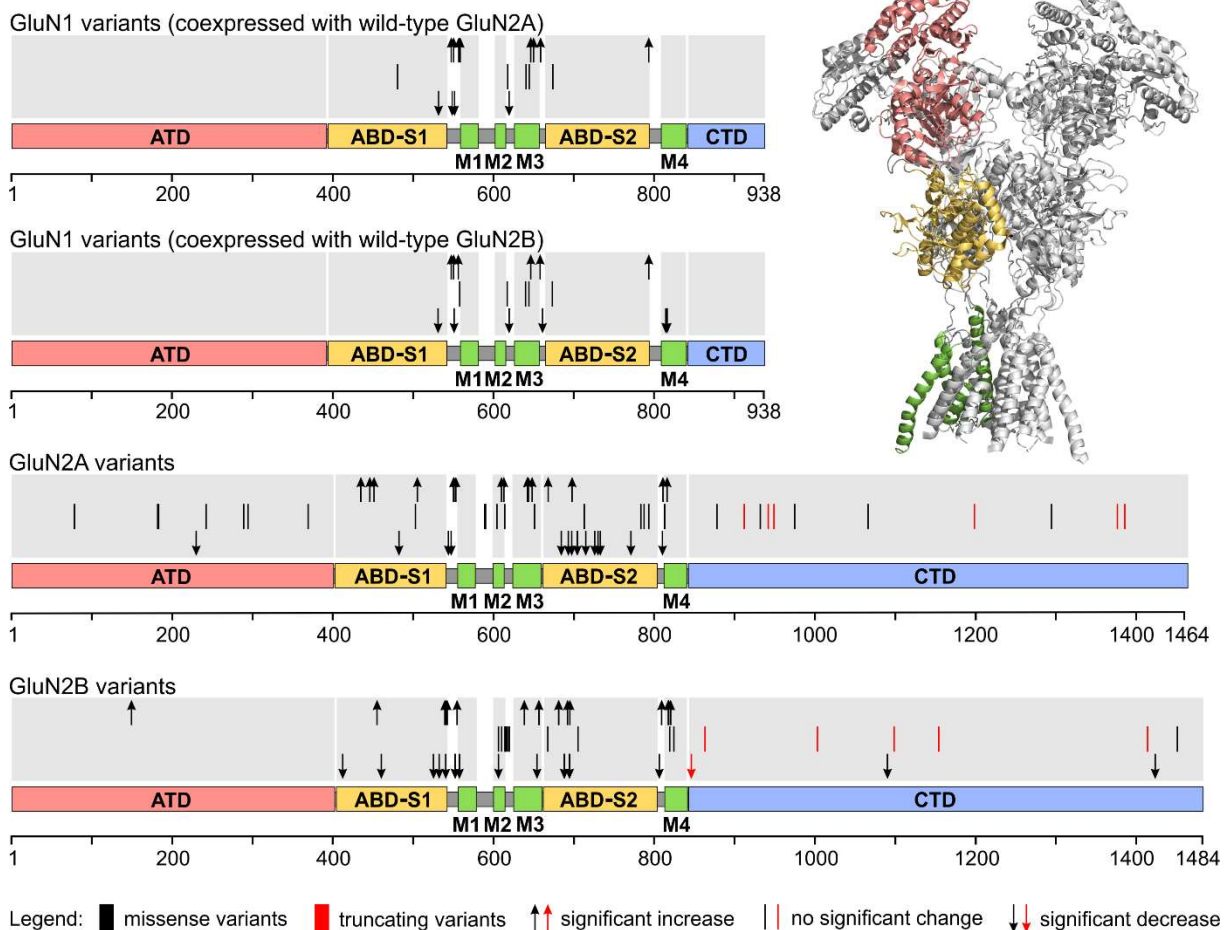
included. Several clinical case studies illustrate the benefit of pharmacological treatment for some carriers of pathogenic *GRIN* gene variants [54,28,55,56,57,58], and it is results like these that motivate the continued effort to characterize the functional impact of different *GRIN* gene variants in order to design appropriate therapy.

## Glutamate and glycine affinity

The ABD of the GluN1 subunit binds the NMDAR co-agonist glycine or D-serine, while the ABDs of GluN2 subunits bind the agonist glutamate. NMDAR affinity for glutamate depends on the types of GluN2 subunits present in the receptor complex, with GluN2A associated with a lower glutamate affinity than GluN2B [1]. Receptor affinity for glutamate influences the rate of

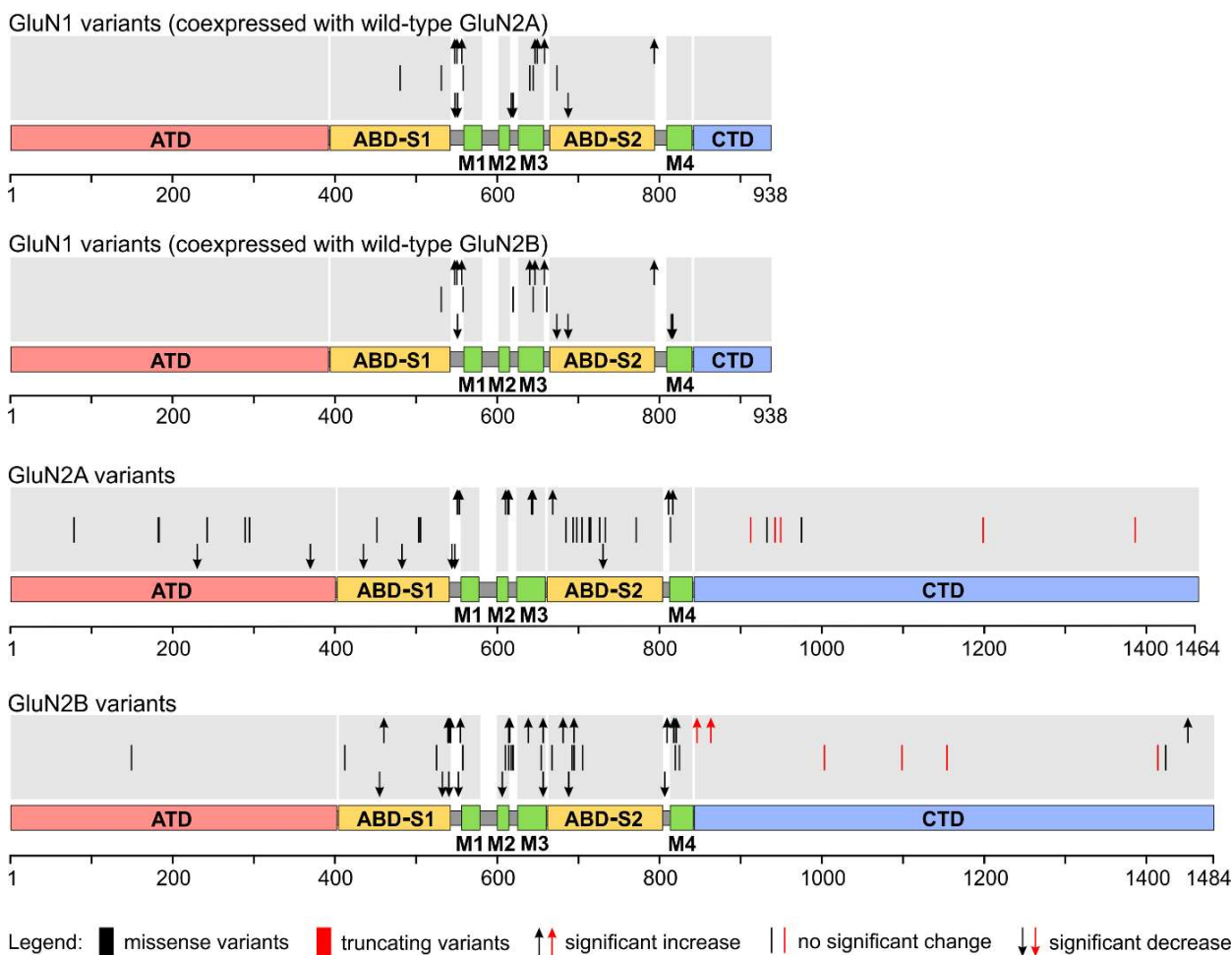
receptor deactivation following a brief synaptic-like glutamate transient [59], such that a lower-affinity receptor–glutamate interaction (typical for GluN2A-containing receptors) results in faster kinetics of receptor deactivation, while a higher-affinity interaction (characteristic for GluN2B-containing receptors) leads to slower deactivation. Receptor deactivation kinetics in turn influence the charge transfer and the  $\text{Ca}^{2+}$  influx through synaptically activated NMDARs, with consequences for downstream signalling [1]. In addition, the quality control of the nascent receptor complexes in the endoplasmic reticulum (ER) is based, in part, on glutamate and glycine affinity, and receptors with very low agonist and/or co-agonist affinity fail to reach the cell surface [60].

### Glutamate affinity



**Fig. 1.** Effects of disease-associated *GRIN* gene variants on glutamate affinity. Relationship between the amino acid positions of individual variants (scale shown below the schematic of the domain structure of each subunit) and the observed effect on glutamate affinity (increase, decrease, no change), as determined for diheteromeric recombinant receptors in non-neuronal cells. ATD, amino-terminal domain; ABD-S1 and ABD-S2, agonist-binding domain segments S1 and S2; M1–M4, transmembrane domain (TMD) helices; CTD, carboxy-terminal domain. Inset shows human GluN1/GluN2A diheteromeric complex (PDB: 7EU7) with GluN1 ATD, ABD, and TMD highlighted in colors corresponding to the schematic of the domain structure; the CTD is not included in this receptor structure.

## Glycine affinity

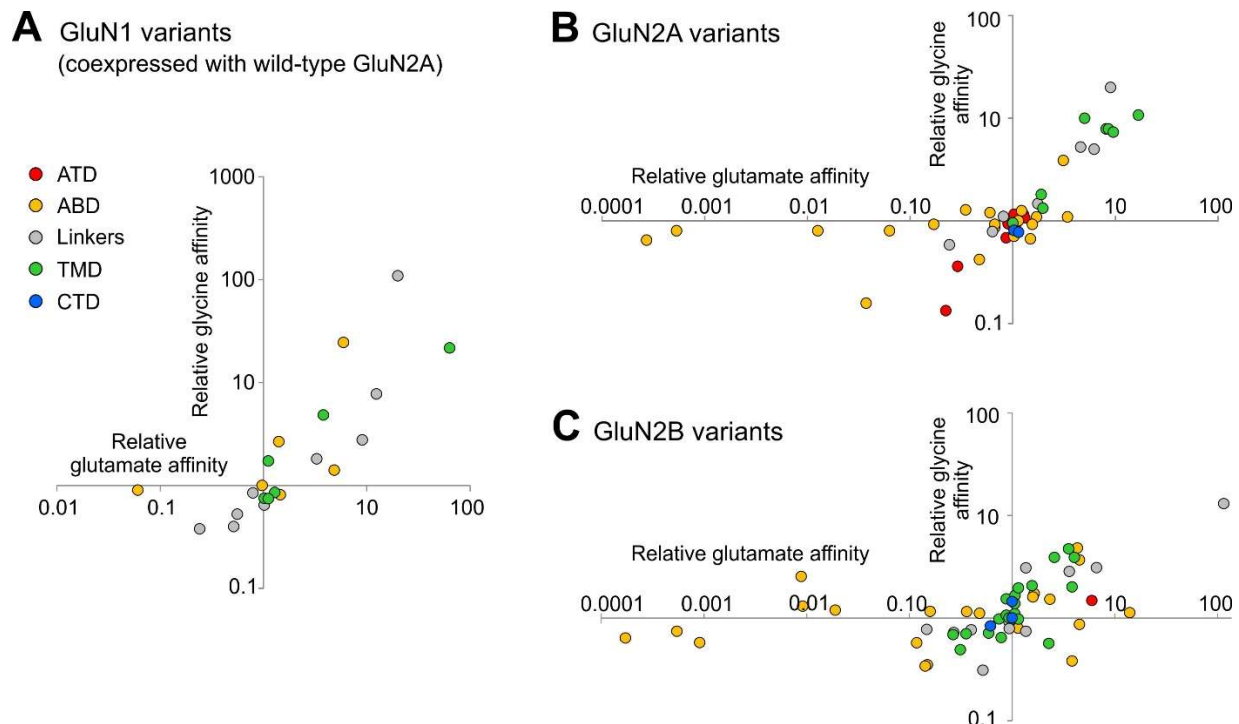


**Fig. 2.** Effects of disease-associated *GRIN* gene variants on glycine affinity. Relationship between the amino acid positions of individual variants (scale shown below the schematic of the domain structure of each subunit) and the observed effect on glycine affinity (increase, decrease, no change), as determined for dimeric recombinant receptors in non-neuronal cells. Abbreviations as in Figure 1.

Glutamate and glycine affinity thus represent key functional parameters influencing NMDAR signalling. In whole-cell patch-clamp measurements of agonist-evoked currents, a change in the receptor affinity for glutamate or glycine is quantified as a change in the concentration of glutamate or glycine that evokes a half-maximal current response ( $EC_{50}$ ), with a higher affinity associated with a lower  $EC_{50}$  value and *vice versa*.

For disease-associated variants for which agonist/co-agonist affinity was reported we summarize the consensus regarding the observed changes (increase, decrease, or no change), or indicate if different studies reached different conclusions (Tables 1,2,3). We plot the qualitative change in glutamate or glycine affinity as a function of the amino acid position of the variant (Figs. 1,2). Missense variants causing significant changes in glutamate or glycine affinity tend to be localized in the

ABDs, ABD-TMD linkers, and in the TMDs [34,41,42,61,32]. Variants in the ATDs and the CTDs are considerably understudied, but those that have been evaluated tend to leave the agonist/co-agonist affinity intact, with some exceptions [62,63]. It is difficult to predict the direction of the change based on the location of the variant in the subunit amino acid sequence. This could be related to the nature of the amino acid substitution in the variant, as illustrated by two different disease-associated variants affecting serine S541 in GluN2B: a substitution of this serine by glycine (S541G) results in a significant increase of both glutamate and glycine affinities but a substitution of the same serine by arginine (S541R) results in a significant decrease of glutamate and glycine affinities [45]. Cases where the same residue is altered by more than one disease-associated variant (Tables 1, 2, 3) need further study.



**Fig. 3.** Relationship between disease-associated missense *GRIN* gene variant effects on glutamate and glycine affinity. Glutamate vs. glycine affinity for individual missense *GRIN* gene variants relative to wild-type as determined for diheteromeric recombinant receptors in non-neuronal cells. The position of each variant within the domain structure of the subunit is indicated by color.

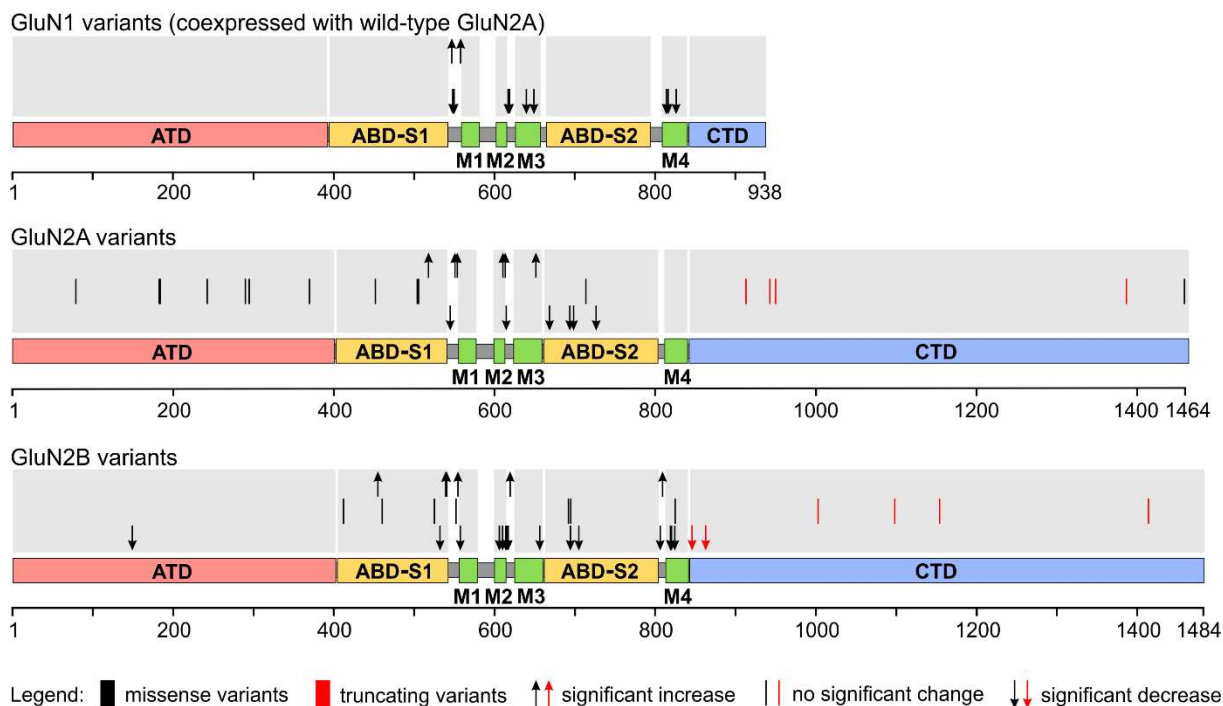
Interestingly, it is very common that variants in GluN1 change not only the affinity for glycine that binds to the GluN1 ABD, but also the affinity for glutamate that binds to the ABDs of the unaffected GluN2 subunits. Similarly, GluN2 variants are often associated with a change in the affinity not only for glutamate but also for glycine. This observation may be related to the fact that glutamate and glycine binding sites in the NMDAR complex are allosterically coupled [64,1]. Indeed, Figure 3 shows that the changes in glutamate and glycine affinity observed for individual variants are correlated. Several variants show a very large (>10-fold) change in glutamate affinity, with variants associated with the strongest decrease in glutamate affinity located in the GluN2A or GluN2B ABDs. For glycine, >10-fold increase in affinity can be seen for several variants but none show >10-fold decrease in affinity. The transient concentration of glutamate released into the synaptic cleft is supersaturating for the glutamate binding sites at the GluN2 subunits [65,1], thus even a substantial decrease in glutamate affinity may be relatively well tolerated. On the other hand, the binding sites for glycine/D-serine at the GluN1 subunits are likely not saturated by ambient co-agonist concentrations [66,67], so a substantial decrease in glycine affinity may be too damaging.

### Channel open probability

Upon agonist/co-agonist binding, the channel may undergo the transition from the closed to the open state. Based on kinetic models of NMDAR activation [59,68], channel open probability ( $P_o$ ) can be expressed in terms of the rate constants of transitions to and from the open state. The  $P_o$  is influenced by the combination of GluN2 subunits in the NMDAR complex, with GluN2A associated with a higher  $P_o$  than GluN2B [1]. In single-channel experiments the  $P_o$  typically refers to the proportion of the total recording time that the channel spends in the open state under conditions of steady-state activation by saturating agonist/co-agonist concentrations [38]. In whole-cell patch-clamp experiments, the  $P_o$  can be estimated from the time course of receptor inhibition by an open-channel blocker MK-801 [42], or from the degree of potentiation induced in receptors containing GluN1-A652C (or GluN2A-A650C) by a cysteine-modifying reagent MTSEA that locks receptors in the open state [34,40].

NMDAR  $P_o$  has been evaluated for only a small proportion of disease-associated variants (Fig. 4). Given that channel gating fundamentally involves the TMDs, the  $P_o$  is particularly influenced by missense variants in the ABD-TMD linkers [34,45] and the TMD helices

## Channel open probability



**Fig. 4.** Effects of disease-associated *GRIN* gene variants on NMDAR  $P_o$ . Relationship between the amino acid positions of individual variants and the observed qualitative effect on NMDAR  $P_o$  (increase, decrease, no change), as determined for diheteromeric recombinant receptors in non-neuronal cells. Abbreviations as in Figure 1.

[42,38,40]. Membrane regions determine additional aspects of NMDAR channel function, such as  $Ca^{2+}$  permeability and  $Mg^{2+}$  block, so disease-associated variants located in the TMDs often have a complex receptor-level functional phenotype [38,40,69,48]. Similar to the analysis of agonist/co-agonist affinities, missense variants in the ATDs or the CTDs generally do not change the  $P_o$ , but additional variants in these domains should be tested.

## Receptor cell surface expression

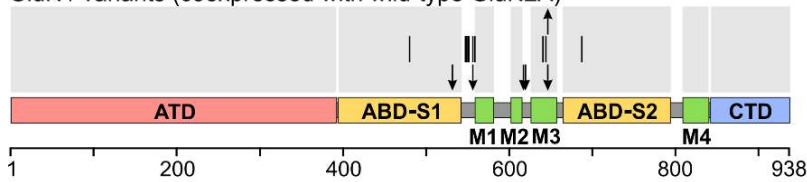
The assembly of NMDAR complexes and their delivery to the cell surface is a key prerequisite for proper receptor signalling, therefore receptor surface expression is an essential parameter influencing the functional outcome associated with individual disease-associated variants. Heterotetrameric NMDAR complex is assembled in the ER [60], with the ATDs, ABDs, and TMDs all participating in proper protein folding and subunit assembly. In addition, several different polypeptide motifs promoting ER retention or ER export have been described, most located in the CTDs of GluN1 or GluN2A/GluN2B subunits [70]. In neurons, the abundance of NMDAR

complexes in the postsynaptic membrane is regulated by the interplay between receptor internalization driven by endocytic signal sequences in the CTDs and receptor anchoring in the synapse via interactions between the CTDs and synaptic scaffolding proteins [27]. To analyze receptor surface expression, most studies have used the endogenous trafficking system of stable cell lines (HEK293T or COS-7). A commonly used approach is to express variant or wild-type GluN subunit with a tag fused to its extracellular end. Surface and intracellular expression of the studied subunit can then be assessed by sequential immunofluorescent labeling performed under non-permeabilizing and permeabilizing conditions, respectively [41,42,43,53,71]. Some studies have used surface protein biotinylation assays [37,72], or expressed  $\beta$ -lactamase fused to the ATD of the studied GluN subunit and performed a colorimetric measurement of  $\beta$ -lactamase activity on the surface of transfected cells [34,40,45].

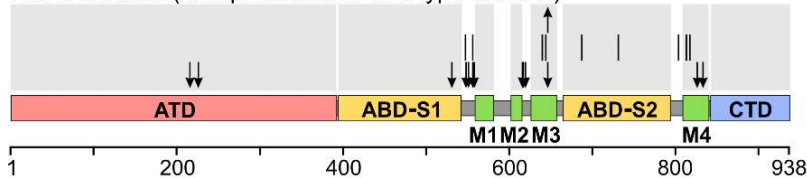
Disease-associated missense variants in the ABDs and the TMDs in all subunits can influence receptor surface expression (Fig. 5). Interestingly, while many ABD or TMD variants decrease receptor surface expression [34,37,41,42,53], only a few have been found to have the opposite effect [42,73,71,45]. This suggests

## Surface expression

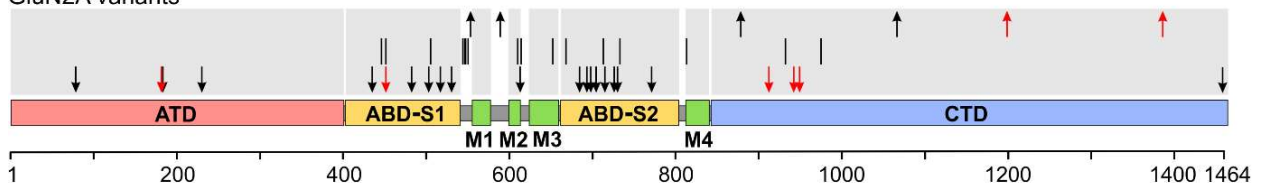
GluN1 variants (coexpressed with wild-type GluN2A)



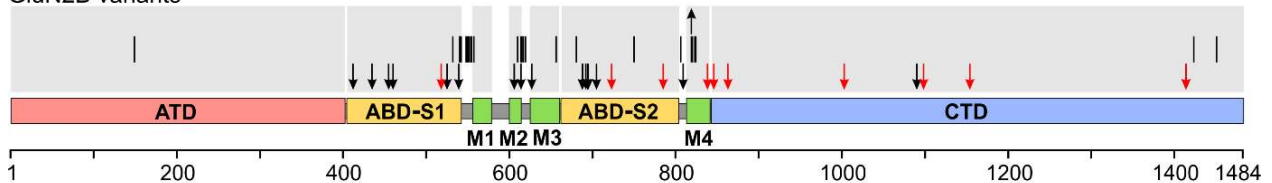
GluN1 variants (coexpressed with wild-type GluN2B)



GluN2A variants



GluN2B variants



Legend: ■ missense variants ■ truncating variants ↑↑ significant increase || no significant change ↓↓ significant decrease

**Fig. 5.** Effects of disease-associated *GRIN* gene variants on receptor surface expression. Relationship between the amino acid positions of individual variants and the observed effect (increase, decrease, no change) on the variant-containing subunit surface expression, as determined for recombinant receptors in non-neuronal cells. Abbreviations as in Figure 1.

that the nascent receptor quality control is very strict, and any conformational changes that may affect tetramer stability or impair agonist binding or receptor gating prevent receptor trafficking to the cell surface. In contrast to the agonist/co-agonist affinity and the  $P_o$  that tend to be relatively unaffected by missense variants in the ATDs and CTDs, receptor surface expression is sensitive to variants in these domains, possibly because disease-associated variants may impair receptor assembly regulated by the ATDs, or disrupt the trafficking and sorting signal sequences present in the CTDs [43,73,74]. Non-neuronal cell lines used for surface expression analyses express only some scaffolding proteins, but biochemical assays can be used to show that certain disease-associated variants in the GluN2A or GluN2B CTDs disrupt the binding of the affected subunits to synaptic scaffolding proteins such as PSD-95 [62,75,63], predicting a deficit in synaptic

targeting of these variant-containing subunits.

### Disease-associated *GRIN* gene variants resulting in protein truncation

Some disease-associated *GRIN* gene variants introduce a premature stop codon predicted to create a truncated protein. Protein-truncating variants (PTVs) account for 20-25 % of disease-associated *GRIN* gene variants and they tend to be associated with a less severe clinical presentation [29,76,1]. Interestingly, GluN1 PTVs in the heterozygous condition are non-pathogenic [76]. Some authors consider PTVs to be null variants [29], assuming that the variant-containing mRNA is degraded by nonsense-mediated decay, producing simple haploinsufficiency. However, it is likely that mRNA surveillance mechanisms may activate compensatory



changes in the expression of the unaffected allele or other functionally related genes [77]. Further, nonsense-mediated decay is less efficient in neurons, particularly early in development, leaving open the possibility that truncated proteins may be expressed to some extent [78].

Nevertheless, GluN1, GluN2A, or GluN2B subunits truncated in any domain other than the CTD are virtually absent from the cell surface [79,76] (Fig. 5); such PTVs can thus be considered functionally null. In contrast, for PTVs affecting the CTD, the truncated protein is expressed and can be analyzed functionally [22] (Figs. 1, 2, 4). All CTD truncating variants studied so far lead to altered subunit surface expression, but for the most part they do not affect the receptor functionally [22]. Only the variants causing the most extensive CTD truncation (e.g., GluN2B-R847X and GluN2B-I864SfsX20) show some effects on agonist/co-agonist affinity, receptor desensitization, and the P<sub>o</sub> [22]. While more work is needed, data so far suggest that, compared to missense variants, *GRIN* gene PTVs in any domain other than the CTD form a more homogeneous group leading to haploinsufficiency, with relatively milder symptoms in carriers. These findings may open new possibilities for non-pharmacological (genetic) therapy for patients with more damaging *GRIN* variants that could possibly be silenced by a newly introduced premature stop codon in the aberrant *GRIN* allele.

### Functional characterization of *GRIN* gene variants in neurons *in vitro*

Much useful functional information about disease-associated *GRIN* gene variants has been gained from studies in non-neuronal heterologous expression systems, but there are aspects of NMDAR function that are influenced by conditions specific to neurons. NMDARs operate in a highly specialized subcellular compartment – the synapse – where they interact with a specific microenvironment characterized by distinct membrane composition and a dense network of scaffolding and signaling proteins. Neuronal NMDARs assemble as various combinations of different subunits, and the effects of disease-associated variants should be characterized in this context. To study a disease-associated variant in neurons it is necessary to work out a strategy for expressing the variant-containing subunit in the presence or absence of native subunits expressed endogenously [80]. This is a challenge and an opportunity: with appropriate experimental design studies in neurons can

elucidate effects of disease-associated variants under more physiological and clinically relevant conditions.

To study NMDARs with a defined subunit composition in neurons, one group focused on the obligatory GluN1 subunit, using shRNA knockdown of native GluN1 combined with the expression of variant-containing GluN1 subunits [61,53,71]. This strategy, assuming efficient knockdown, results in receptors containing two copies of the variant-containing GluN1 subunit, similar to non-neuronal heterologous expression of diheteromeric receptors. Indeed, in these studies, experiments in neurons largely confirmed effects of GluN1 variants observed in non-neuronal models.

It is more complicated to characterize effects of disease-associated GluN2 subunit variants, because the different GluN2 subunit types may partially substitute for each other. Since NMDARs in principal neurons in the cortex and hippocampus predominantly contain GluN2A and/or GluN2B subunits [4], Cre-Lox recombination in excitatory neurons prepared from *Grin2a<sup>fl/fl</sup>/Grin2b<sup>fl/fl</sup>* mice effectively eliminates the majority of native GluN2 subunit expression [6]. In a series of elegant molecular replacement experiments, GluN2A or GluN2B subunits containing specific disease-associated variants were introduced together with Cre-recombinase in neurons from *Grin2a<sup>fl/fl</sup>/Grin2b<sup>fl/fl</sup>* mice [62,81,82]. In the simplest case, native GluN2A and GluN2B are both eliminated and replaced only with wild-type or variant GluN2A [81] or only with wild-type or variant GluN2B [62,82]. The results of such experiments tend to confirm the LoF or GoF phenotypes of the variants, similar to results observed in non-neuronal systems. Most revealing, however, are experiments in *Grin2a<sup>fl/fl</sup>* mice where native GluN2A is replaced with variant-containing GluN2A, while the native GluN2B expression is left intact [81], or experiments in *Grin2b<sup>fl/fl</sup>* mice where native GluN2B is replaced with variant-containing GluN2B, while the native GluN2A expression is intact [82]. In the first case, surprisingly, the outcome of expressing either LoF or GoF GluN2A variants is similar, with excitatory postsynaptic currents (EPSCs) characterized by slower deactivation kinetics. This reflects either the GoF of the GluN2A variant or, in case of the LoF GluN2A variants, the increased relative contribution of native GluN2B subunits to synaptic NMDAR currents [81]. In the second case, the expression of either LoF or GoF GluN2B variants in the presence of native GluN2A results in EPSCs with faster deactivation kinetics, in part because the GoF phenotype of certain GluN2B variants is lost in triheteromeric receptors containing the GluN2B

variant together with wild-type GluN2A [82]. These results suggest that for some variants the functional phenotype at the receptor level may not accurately predict the functional phenotype at the synaptic level, underscoring the need for more work in neuronal preparations.

Many studies have expressed variant subunits in neurons on the background of endogenous expression of native subunits. This approach is technically relatively simple and can address cell surface or synaptic expression of subunits harboring disease-associated variants in the presence of neuronal trafficking and scaffolding machinery. Several studies have expressed tagged variant-containing subunits in neurons and used immunofluorescence microscopy to evaluate the levels of dendritic surface expression of the different variants [34,62,79,75,76,61,53,71,22]. Synaptic localization of variant subunits can be examined more specifically, for example by quantifying colocalization between the tagged subunit and endogenous PSD-95 [22]. A few reports include surface expression data for the same variants in a non-neuronal system and in neurons, in some cases finding comparable results [79,61,53,71], and in other cases finding qualitative or quantitative differences between variant subunit trafficking in neurons and in non-neuronal systems [34,76,22].

Functionally, expressing a disease-associated variant subunit in neurons on the background of endogenous expression of native subunits may approximate the situation in patients heterozygous for the variant, if the method used does not result in significant overexpression of the introduced subunit [51,79]. Such experiments tend to show that in the presence of native wild-type subunits, the functional consequences of the variant are more subtle than in non-neuronal heterologous expression systems, with wild-type subunits partially mitigating the effects of the pathogenic variant [51]. Studies of this type can also begin to address possible indirect effects of changes in NMDAR signaling due to disease-associated variants on synapse structure and function and the development of neural networks [79,75,63].

### Functional characterization of *GRIN* gene variants in animal models

Methods used to introduce subunits containing disease-associated variants to neurons in culture or in acute slices only achieve transient expression of the target

subunit in a subset of cells in the preparation. As a result, these experiments can only describe relatively acute and cell-autonomous effects of disease-associated variants [80]. However, in patients, the *GRIN* gene variant is permanently present in all cells, and patient symptoms may be related to circuit-level effects of the variant, possibly influenced by compensatory changes of gene expression, synapse structure or function, or circuit connectivity. Transgenic animal models can be used to reveal how changes in NMDAR signaling due to the widespread and chronic presence of the pathogenic *GRIN* gene variant may affect the nervous system function and development.

Mouse strains with targeted disruption of *Grin1* [83], *Grin2a* [84], or *Grin2b* [85] may serve as models of null variants in the corresponding human *GRIN* genes, with heterozygous animals representing the condition of patients. Mouse strains with truncated *Grin2a* or *Grin2b* gene leading to the expression of GluN2A or GluN2B subunits lacking the CTDs have also been created [86]. While homozygous animals of these strains have been characterized, data about heterozygous animals are limited. Heterozygous *Grin1*<sup>-/+</sup> mice are healthy and their neurons have normal NMDAR responses [83], consistent with the finding that truncating human *GRIN1* variants in the heterozygous condition are not pathogenic [76]. Recently, some studies have examined selected parameters in heterozygous *Grin2a*<sup>+/-</sup> mice [87,88,89] and *Grin2b*<sup>+/-</sup> mice [90], generally observing a milder phenotype in heterozygous compared to homozygous animals. Still, much more work is needed to understand the effects of *GRIN2A* or *GRIN2B* haploinsufficiency at the cellular, circuit, and system level. Additional useful data may emerge from using a zebrafish (*Danio rerio*) model to study the developmental roles of different NMDAR subunits [91,92].

Very few transgenic mouse models of specific patient-derived *GRIN* gene variants have been created to date. In 2010, a large-scale mutagenesis project at RIKEN generated a mouse strain expressing GluN1-R844C [93], a variant subsequently identified in two patients with severe intellectual disability, motor disorder, and seizures [94]. At the receptor level this variant has no apparent functional phenotype and in heterozygous mice it is associated with hyperactivity and increased novelty-seeking behavior [93].

Three mouse strains expressing patient-derived GluN2A variants, all associated with intellectual disability and epilepsy, have been described. The variant GluN2A-S644G [95] increases glutamate and glycine affinity and

prolongs NMDAR EPSC deactivation, and in heterozygous animals it is associated with abnormal network activity, hyperactive behaviors, and mixed effects on seizure susceptibility [95]. The variant GluN2A-K879R increases subunit surface expression [74], and in heterozygous animals it is associated with increased NMDAR EPSC amplitude but faster deactivation, altered levels of surface expression of NMDAR and AMPAR subunits, synaptic plasticity deficits, and impaired cognitive function [74]. The variant GluN2A-V685G reduces NMDAR glutamate affinity and surface expression [34], and reduces NMDAR EPSC amplitude, yet this LoF variant increases seizure susceptibility in heterozygous mice, possibly due to circuit-level effects on excitation/inhibition balance [96].

To our knowledge, two mouse models of disease-associated GluN2B variants, both found in patients with intellectual disability and autism, have been created to date. The variant GluN2B-C456Y [90] strongly reduces receptor surface expression [34] and in heterozygous animals it is associated with smaller and faster NMDAR EPSCs, mild deficits in long-term synaptic depression, and hypoactivity and reduced anxiety [90]. The variant GluN2B-L825V reduces receptor P<sub>o</sub> [42] (but see [38]), and in heterozygous animals it leads to NMDAR EPSCs with faster deactivation, hypoactivity in the open field, impaired sensorimotor gating, and cognitive inflexibility [97].

Together, these examples illustrate how difficult it is to extrapolate the receptor-level functional phenotype of a given variant to its circuit-level or system-level effects that ultimately underlie symptoms in carriers. Many more disease-associated *GRIN* gene variants need to be studied in animal models and their impact should be examined thoroughly, not only in the canonical circuits (hippocampus, cortex), but in multiple brain regions, over the course of development, and in both sexes. A better understanding of how different *GRIN* gene variants lead to neurological and neuropsychiatric disease is a prerequisite for finding effective therapies for *GRIN* disorders.

## Conclusions

Here we present a comprehensive review of the literature on the functional evaluation of human disease-associated variants in *GRIN1*, *GRIN2A* and *GRIN2B* genes. Most information so far comes from studies of receptor-level effects of *GRIN* gene variants in non-neuronal systems. These studies establish that variants in the core gating region comprised of the ABDs, the TMDs, and the ABD-TMD linkers frequently lead to profound changes of receptor function manifested as changes in agonist/co-agonist affinity, channel open probability, and/or receptor surface expression. Variants in the ATDs and the CTDs can significantly affect receptor surface expression, but have been relatively overlooked so far. An individual variant often influences multiple functional parameters, which may complicate variant classification as LoF or GoF, and the receptor-level functional impact may not accurately predict the consequences observed at the synaptic, cellular, or circuit level in neuronal preparations or animal models. Taking advantage of the more physiological experimental models in the future should lead to further advances in the understanding of the role of NMDAR signaling in healthy nervous system function, and in the etiology of *GRIN* disorders.

## Conflict of Interest

There is no conflict of interest.

## Acknowledgements

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## Abbreviations

ATD, amino-terminal domain; ABD-S1 and ABD-S2, agonist-binding domain segments S1 and S2; CTD, carboxy-terminal domain; del, deletion; dup, duplication; ins, insertion; fs, frame shift; M1–M4, transmembrane domain (TMD) helices; Ter, termination codon.

**Table 1.** Functional characterization of disease-associated variants in *GRIN1*/GluN1.

DNA	Protein	Coexpressed with GluN2A				Coexpressed with GluN2B			Functional characterization references			
		P <sub>o</sub>	Glu <sup>1</sup>	Gly <sup>2</sup>	Surface Expression	Glu <sup>1</sup>	Gly <sup>2</sup>	Surface Expression	Non-neuronal cells	Neurons		
c.649C>T c.679G>C c.803G>A c.977del	R217W D227H W247Ter P305RfsTer21					↓		↓	[94], [43] [43] [76] [76]			
	c.1378A>G c.1567C>T c.1595C>A	I460V* R523C P532H		↔	↔	↔				[98] [99] [100]	[98]	
		c.1643G>A c.1705C>T c.1645A>C c.1652T>C c.1656C>G c.1666C>T c.1670C>T c.1670C>G	R548Q R548W S549R L551P D552E Q556Ter P557L P557R	↑	↑	↑	↔	↑	↑	↔	[45] [43] [94], [45] [45] [94], [37], [46] [94], [76] [73] [94], [37]	
			c.1676A>G c.1679_1681dup	Q559R S560insS	↑	↑	↔	↔	↔	↔	↓	[45] [94], [45], [101]
c.1851C>A c.1852G>C c.1854_1859dup c.1858G>C	S617C G618R I619_G620dup G620R			↓	↔	↓	↓	↔		↓	[43] [94] [43] [94], [102], [40], [43]	
	c.1923G>A c.1921A>G c.1933G>T c.1939T>C c.1940A>C c.1940A>G c.1950C>G		M641I M641V A645S* Y647H* Y647S Y647C N650K	↓	↔	↔	↓	↔	↑	↔	[94], [56], [46], [53], [57] [43] [94], [53], [98] [98] [94], [53] [103] [94], [71]	[53] [53], [98] [98] [53] [71]
			c.1975C>T c.1984G>A	R659W E662K		↑	↑		↑	↑		[103] [94], [101]
c.2021A>T c.2063C>A c.2196T>G c.2365G>A c.2381G>A				N674I S688Y D732E D789N R794Q		↔	↔	↔	↔	↓	↓	[103] [61] [43] [103] [103]
			c.2414C>T c.2413C>T	P805L P805S						↔	↔	[43] [43]
		c.2441C>A c.2443G>A c.2444G>T c.2449T>C c.2515A>G c.2479G>A c.2500G>C		A814D G815R G815V F817L M818V G827R E834Q	↓				↓	↓	↔	[43] [94], [38], [46] [94], [38] [94], [38] [43] [94], [38], [43] [43]
			c.2530C>T	R844C							[94]	[93]

<sup>1</sup>Affinity for glutamate <sup>2</sup>Affinity for glycine ↑ = increase compared to WT ↓ = decrease compared to WT ↔ = no change compared to WT

Color denotes the domain: red = ATD; yellow = ABD; grey = linkers; green = TMD; blue = CTD

Abbreviations: del, deletion; dup, duplication; ins, insertion; fs, frame shift; Ter, termination codon. All variants refer to the sequence of GluN1-1a. \*Variants GluN1-I460V, GluN1-A645S, and GluN1-Y647H were studied in GluN1-3b [96] where they correspond to I481V, A666S, and Y668H, respectively. In this and all other Tables, when the literature provides multiple measurements of the same parameter for the same variant, we consider this parameter to be significantly increased (decreased) if most studies report a significantly increased (decreased) value. Where no clear consensus exists, different reported outcomes are indicated.

**Table 2.** Functional characterization of disease-associated variants in *GRIN2A*/GluN2A.

DNA	Protein	P <sub>o</sub>	Glu <sup>1</sup>	Gly <sup>2</sup>	Surface Expression	Functional characterization references	
						Non-neuronal cells	Neurons
c.172G>T c.236C>G c.544del c.547T>A c.551T>G c.692G>A c.728C>T c.869C>T c.883G>A c.1108C>T	E58Ter					[104]	
	P79R	↔	↔	↔	↓	[41], [35]	[35]
	E182NfsTer22				↓	[76]	
	F183I	↔	↔	↔	↔	[35]	
	I184S	↔	↔	↔	↓	[35], [105]	
	C231Y	↔	↓	↓	↓	[41], [35]	
	A243V	↔	↔	↔	↔	[35], [46]	
	A290V	↔	↔	↔	↔	[35]	
G295S	↔	↔	↔	↔	[35]		
R370W	↔	↔	↓	↔	[35]	[35]	
c.1306T>C c.1341T>A c.1354G>A c.1354insT c.1447G>A c.1510C>T c.1517T>C c.1553G>A c.1592C>T	C436R		↑	↓	↓	[41], [35], [34], [49]	[81], [82]
	N447K		↑		↔	[107]	
	V452M	↔	↑	↔	↔	[34]	
	V452CfsTer11				↓	[76]	
	G483R		↔	↓	↓	[41], [34], [49], [108]	
	R504W	↔	↓	↔	↓	[34]	
	V506A	↔	↑	↔	↔	[34]	
	R518H	↑			↓	[105], [34], [106]	[81]
T531M				↓	[34], [109]	[81]	
c.1634C>T c.1639_1641del c.1642G>A c.1651G>A c.1655C>G c.1661G>C c.1757G>A c.1770A>C c.1771G>A	S545L	↓	↓	↓	↔	[45]	
	S547del				↔	[45]	
	A548T		↓	↓	↔	[37], [49]	
	E551K	↑	↑	↑	↔	[47], [45]	
	P552R		↑	↑	↔	[47], [37], [110]	[37]
	S554T	↑	↑	↑	↑	[45]	
	R586K					[111]	[111]
	K590N				↑	[73]	
G591R		↔			[104]		
c.1815A>G c.1832T>A c.1841A>G	I605M		↔			[104]	
	L611Q	↑	↑	↑	↔	[40], [48]	
	N614S	↑	↑	↑	↓	[40], [48], [46]	
c.1845C>A c.1844A>G	N615K	↓	↔	↑	↔	[112], [54], [40], [69], [113], [48], [114]	[69]
	N615S*						[115]
c.1923G>A c.1928C>A c.1930A>G c.1945C>G c.1954T>G c.1959G>A	M641I					[56]	
	A643D		↑	↑		[116], [49]	
	S644G		↑	↑		[47], [95]	[95]
	L649V		↑			[47]	
	F652V	↑	↔			[110], [106]	
	M653I					[104]	
c.2007G>T c.2054T>C c.2081T>C c.2093A>G c.2095C>T c.2100C>G c.2113A>G c.2140G>A c.2146G>A c.2179G>A c.2191G>A c.2200G>C c.2314A>G c.2351G>C c.2364G>A c.2380C>A	K669N	↓	↑	↑	↔	[34]	[81]
	V685G		↓	↔	↓	[34], [49]	
	I694T	↓	↓	↔	↓	[34]	
	Y698C		↓			[104]	
	P699S	↔/↓	↑	↔	↓	[34], [49], [108]	
	Y700Ter					[104]	
	M705V		↓	↔	↓	[41], [34], [49], [46]	
	E714K	↔	↔	↔	↔	[41], [34]	
	A716T		↓	↔	↓	[105], [34], [49], [108]	
	A727T	↓	↓	↔	↓	[34], [49], [108], [104]	
	D731N		↓	↓	↓	[41], [39], [34], [49]	
	V734L		↓	↔	↔	[34], [49], [108]	
	K772E		↓	↔	↓	[34], [49]	
	G784A		↔			[104]	
	M788I		↔			[104]	
	L794M		↔			[104]	
	c.2427C>A c.2432A>C	S809R					[104]
Q811P			↓			[104]	
c.2434C>A c.2441T>C c.2449A>G	L812M		↑	↑		[47], [54], [33], [104]	
	I814T		↔	↔	↔	[41], [38], [46]	
	M817V		↑	↑		[38], [47], [36]	
c.2636A>G c.2738C>A c.2797G>A c.2829C>G c.2848C>T c.2927A>G c.3199C>T c.3596del c.3884T>C c.4128del c.4161C>G c.4375A>G	K879R		↔		↑	[74], [73]	[74]
	S913Ter	↔	↔	↔	↓	[22]	[22]
	D933N		↔	↔	↔	[41]	
	Y943Ter	↔	↔	↔	↓	[22]	[22]
	Q950Ter	↔	↔	↔	↓	[22]	[22]
	N976S		↔	↔	↔	[41]	
	R1067W		↔	↔	↑	[73]	
	P1199RfsTer32		↔			[117]	[117]
	I1295T		↔			[104]	
	L1377fs		↔			[104]	
	Y1387Ter	↔	↔	↔	↑	[22]	[22]
	S1459G	↔	↔	↔	↓	[75], [118]	[75], [118]

<sup>1</sup>Affinity for glutamate <sup>2</sup>Affinity for glycine ↑ = increase compared to WT ↓ = decrease compared to WT ↔ = no change compared to WT

Color denotes the domain: red = ATD; yellow = ABD; grey = linkers; green = TMD; blue = CTD

Abbreviations as in Table 1. \*The variant GluN2A-N615S has not been found in humans, but it is closely related to the patient variant GluN2A-N615K and has been characterized in a transgenic mouse model [112].

**Table 3.** Functional characterization of disease-associated variants in *GRIN2B*/GluN2B.

DNA	Protein	P <sub>o</sub>	Glu <sup>1</sup>	Gly <sup>2</sup>	Surface Expression	Functional characterization references	
						Non-neuronal cells	Neurons
c.448A>G	I150V	↓	↑	↔	↔	[44]	
c.1238A>G	E413G	↔	↓	↔	↓	[119], [50], [49], [108], [120], [34]	[34], [121]
c.1306T>C	C436R				↓	[49], [34]	[82]
c.1367G>A	C456Y	↑	↑	↓	↓	[49], [108], [34]	[82], [90]
c.1382G>T	C461F		↓	↑	↓	[49], [51], [34]	[51], [34], [82]
c.1555C>T	R519Ter				↓	[76]	[76]
c.1576T>C	S526P	↔	↓	↔	↓	[44]	
c.1598G>A	G533D	↑	↑	↑	↓	[44]	
c.1619G>A	R540H	↓	↓	↓	↔	[50], [30], [122], [34], [51], [82]	[82]
c.1623C>G	S541R		↓	↓	↔	[28], [49], [45]	
c.1621A>G	S541G	↑	↑	↑	↔	[45]	
c.1627G>C	G543R		↑	↑	↔	[47], [45]	
c.1646C>T	A549V				↔	[45]	
c.1649T>C	F550S				↔	[45]	
c.1652T>C	L551S				↔	[45]	
c.1658C>T	P553L		↓			[37], [42], [51]	[51]
c.1657C>A	P553T	↔	↓	↓	↔	[45], [55]	[55]
c.1664G>A	S555N	↑	↑	↑	↔	[45]	
c.1664G>T	S555I				↔	[45]	
c.1672G>A	V558I	↓	↓	↔	↔	[50], [28], [42]	
c.1821G>T	W607C	↓	↓	↓	↓	[50], [40], [48], [42]	
c.1820G>C	W607S	↓	↔	↓	↓	[44]	
c.1832G>T	G611V	↓	↔	↔	↔	[40], [28], [48]	
c.1844A>T	N615I	↓	↔	↔	↔	[50], [30], [40], [122], [48], [42], [51], [114]	[51]
c.1845C>G	N615K	↓	↔	↑	↓	[40], [48]	
c.1848C>G	N616K	↓	↔	↑	↔	[40], [48]	
c.1853T>G	V618G	↓	↔	↔	↔	[50], [94], [40], [122], [48], [42], [51], [114]	[51]
c.1858G>A	V620M	↑	↔	↔	↔	[40], [48]	
c.1883C>T	S628F				↓	[50], [42]	
c.1906G>C	A636P					[50]	
c.1916C>T	A639V		↑	↑		[47]	
c.1963A>T	I655F		↓	↔		[28]	
c.1970A>G	E657G		↑	↓	↔	[50], [42]	
c.1971G>C	E657D	↓	↑	↑	↔	[44]	
c.2002G>A	D668N		↔	↔		[50]	
c.2044C>T	R682C		↑	↑	↔	[50], [34], [112]	
c.2045G>A	R682H		↑	↑	↔	[44]	
c.2065G>T	G689C		↓	↓	↓	[52]	[52]
c.2065G>A	G689S		↓	↓	↓	[44], [52]	[52]
c.2079A>T	R693S	↔	↑	↔	↓	[44]	
c.2084T>G	I695S	↓	↓	↑	↓	[44]	
c.2084T>C	I695T	↓	↓	↔	↓	[44]	
c.2087G>A	R696H	↔	↑	↔	↓	[34], [82]	[82]
c.2116A>G	M706V	↓	↔	↔	↓	[44], [28], [108]	
c.2172-2A>G	G724Ter				↓	[79]	[79], [123]
c.2252T>C	I751T				↔	[58]	
c.2355del	D786MfsTer23				↓	[76]	[76]
c.2419G>A	E807K	↓	↓	↓	↔	[50], [44]	
c.2430C>A	S810R		↑	↑		[28]	
c.2429G>A	S810N	↑	↑	↑	↓	[44]	
c.2443G>A	G815R					[38]	
c.2453T>C	M818T		↑	↑		[47], [28]	
c.2455G>A	A819T		↑	↑		[47], [28]	
c.2459G>C	G820A	↓	↔	↔	↔	[38], [50], [28], [42]	
c.2459G>A	G820E	↓			↑	[38], [58], [42]	
c.2461G>T	V821F		↑	↑	↔	[44]	
c.2471T>G	M824R				↔	[42]	
c.2473T>G	L825V	↔/↓	↔	↔	↔	[38], [50], [28], [42], [51]	
c.2477G>A	G826E	↔				[38]	
c.2515G>T	E839Ter				↓	[76]	[76]
c.2539C>T, c.2539-2540CG>TA	R847Ter	↓	↓	↑	↓	[76], [22]	[76], [22]
c.2589del	I864SfsTer20	↓	↔	↑	↓	[22]	[22]
c.3012C>G	Y1004Ter	↔	↔	↔	↓	[22]	[22]
c.3272A>C	K1091T		↓		↓	[63]	[63]
c.3295del	R1099AfsTer51	↔	↔	↔	↓	[22]	[22]
c.3465C>ACATCTA	Y1155Ter	↔	↔	↔	↓	[22]	[22]
c.4244C>T	S1415L		↔	↔	↓	[62]	[62]
c.4244del	S1415Ter	↔	↔	↔	↓	[22]	[22]
c.4270C>T	L1424F		↓	↔	↔	[62]	[62]
c.4355C>T	S1452F		↔	↑	↔	[62]	[62]

<sup>1</sup>Affinity for glutamate <sup>2</sup>Affinity for glycine ↑ = increase compared to WT ↓ = decrease compared to WT ↔ = no change compared to WT

Color denotes the domain: red = ATD; yellow = ABD; grey = linkers; green = TMD; blue = CTD

Abbreviations as in Table 1.

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