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# *De Novo* Mutations and Rare Variants Occurring in NMDA Receptors

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## Abstract

A significant number of variants/mutations in the *N*-methyl-$_D$-aspartate glutamatergic receptor (NMDAR) gene family (*GRIN*) have been identified along with stunning advances in the technologies of next generation of whole-exome sequencing. Mutations in human *GRIN* genes are distributed throughout the entire gene, from amino terminal domain to C-terminal domain, in patients with various neuropsychiatric disorders, including autism spectrum disorders, epilepsy, intellectual disability, attention deficit hyperactivity disorder, and schizophrenia. Analyzing the currently available human genetic variations illustrates the genetic variation intolerance to missense mutations differs significantly among domains within the *GRIN* genes. Functional analyses of these mutations and their pharmacological profiles provide the first opportunity to understand the molecular mechanism and targeted therapeutic strategies for these neurological and psychiatric disorders, as well as unfold novel clues to channel function.

### Keywords

glutamate receptor; NMDA receptor; *GRIN*; GluN; channelopathy; functional genomics

## Introduction

*N*-methyl-$_D$-aspartate receptors (NMDARs), ligand-gated inotropic glutamatergic channels, mediate a $Ca^{2+}$-permeable, slow component of synaptic current that plays key roles in the

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formation and maturation of excitatory synapses and brain circuits [1]. NMDARs, hetero-tetramers, are comprised of two GluN1 subunits and two GluN2 subunits. The glycine-binding GluN1 subunit, product of a single gene *GRIN1* with eight splicing variants, has an ubiquitous expression throughout the brain. The four glutamate-binding GluN2 subunits (2A, 2B, 2C, and 2D, encoded by genes of *GRIN2A, 2B, 2C,* and *2D*) present different temporal and spatial expression profiles, and distinct pharmacological and biophysical properties. The expression of GluN2A and GluN2C subunits are predominant in postnatal period and peak in adult, whereas high levels of GluN2B and GluN2D subunits are expressed at embryonic/prenatal stages. The expression levels of GluN2B and GluN2D subunits in most brain regions decreases with age leading to localization after birth in certain brain regions [2]. Each subunit in the NMDARs shares a same structure and contains four semi-autonomous domains: an amino-terminal domain (ATD), a clamshell-shaped agonist binding domain (ABD), four channel-forming transmembrane domains (TMDs: M1, M2, M3, and M4; with M2 re-entrant loop to form the channel pore), and an intracellular carboxy-terminal domain (CTD). The ABDs fold into clamshell-shaped bi-lobed structures, hosting a binding pocket for agonists [3]. Activation of NMDAR channels requires binding of both glutamate and glycine simultaneously. The agonist binding promotes the ABD clamshell closure, which induces movement of linker regions connected the ABD to the transmembrane domains that causes conformational changes and the cation-selective pore to open. Opening of NMDAR channels results in an elevation in the $Ca^{2+}$ levels intracellularly and membrane depolarization [1]. It has been established that NMDARs play critical roles in normal brain function, such as synaptic plasticity, learning, memory, motor and sensory function, and brain development, as well as in various pathological conditions, including stroke, epilepsy, Parkinson's disease, Alzheimer's disease, Huntington's disease, pain and schizophrenia [1,4].

Recent advances in the technologies of next-generation whole exome sequencing have yielded a significant number of rare variants and *de novo* mutations in the *GRIN* genes which are associated with a number of neuropsychiatric disorders [5–7]. In this review, we will summarize the recent studies of NMDAR mutations and rare variants, discuss the current understanding of the molecular mechanism of the impact on clinical phenotype and disease progression underlying these mutations and rare variants, and highlight exploration of mechanism-based novel therapeutic strategies.

## NMDAR mutations and rare variants are associated with various neurological and psychiatric disorders

Analysis of genetic variation to missense mutations in the healthy population (e.g. documented in ExAC Browser) by using residual variation intolerance score shows that the *GRIN* genes are intolerant to variation (have fewer SNPs than expected) [8•,9], suggesting genetic variation may be more likely to influence disease. Following the first report of potential disease-causing NMDAR mutations [10•], a large number of genetic variants (>200) in NMDARs subunits have been reported. Here, we focus on the genetic variants on NMDAR subunits in literatures which are absent in gnomAD database (http://gnomad.broadinstitute.org/; assessed on August 2nd, 2017) since the variants in gnomAD

were presumably identified in healthy population without neurologic and neuropsychiatric disorders. Among 258 reported mutations and rare variants, there are 65% (167/258) of missense, 9% (22/258) of nonsense, 9% (23/258) of frameshift, 5% (14/258) of splice site, and 12% (32/258) others (i.e. chromosomal translocation, inversion, deletion) (Figure 1A). The mutations and rare variants are scattered across all NMDAR subunits with 10% (26/258) in GluN1 [11–28], 39% (100/258) in GluN2A [10•,12,16,22,24,29–50], 45% (116/258) in GluN2B [10•,12,13,16,18,19,21,22,24,29,39,42,44••,49–73••], 3% (9/258) in GluN2C [11,12,36], and 3% (7/258) in GluN2D [12,74••] (Figure 1B).

These NMDAR mutations and rare variants are present in patients with various neurodevelopmental and neuropsychiatric disorders, such as epilepsy/seizures (EPI), intellectual disability (ID), attention deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), as well as schizophrenia (SCZ) (Figure 1C,D; Suppl. Table S1). Many of these conditions are comorbid. Generally, 77% (198/258) of the patients harboring NMDAR mutations have intellectual disability (including developmental delay and mental retardation), 49% (127/258) with epilepsy, 19% (49/258) with autism spectrum disorder, 5.0% (12/258) with schizophrenia, and 2.0% (5/258) with attention deficit hyperactivity disorder (Figure 1C,D). Epilepsy was the largest group observed in 78% (78/100) of the patients with *GRIN2A*/GluN2A variants, followed by intellectual disability (70%, 70/100) and autism spectrum disorder (10%, 10/100) (Figure 1C,D). The *GRIN2A*-related epilepsies often include some aspects of the epilepsy-aphasia syndrome and comprise Landau-Kleffner syndrome (LKS), as well as idiopathic focal epilepsy (IFE), atypical benign partial epilepsy of childhood (ABPE), benign partial epilepsy of childhood with centrotemporal spikes (BECTS), continuous spike-and-wave during slow wave sleep (CSWSS), benign childhood epilepsy (BCE), atypical childhood epilepsy with centrotemporal spikes (ACECTS), early-onset epileptic encephalopathy (EOEE), and unclassified childhood-onset epilepsy. Intellectual disability was the largest group occurred in 87% (101/116) of the patients with *GRIN2B*/GluN2B variants, followed by epilepsy (29%, 34/116) and autism spectrum disorder (27%, 31/116) (Figure 1C,D). Most mutations in GluN2B were in patients with intellectual disability, whereas GluN2A mutations were associated with epilepsy most frequently in the cohort of patients studied.

Moreover, mutations have been found in all domains (ATD, ABD, TM-linker, and CTD) throughout the mature protein (Figure 2), with the large numbers in ABD and TM-link regions. Interestingly, analyzing the genetic intolerance to missense mutations in protein domains within GluN subunits (e.g. GluN1, GluN2A and GluN2B subunits) in healthy population shows the certain regions in ABD and TM-link are among the sub-regions of the relevant gene-encoded proteins with the most heavily missense depletion [44••,75], indicating genetic variation in these regions may be more likely to cause disease.

## Estimation of overall impact of the mutations on NMDAR function

It is impossible to predict how the rapidly expanding number of disease-related rare variants and mutations in *GRIN* gene family impact brain function and neuronal health without functional assessment of the NMDAR protein. This lack of functional data precludes the accurate diagnosis and proper treatment. In past several years, of 258 published mutations

on NMDAR subunits, functional analyses have been performed on 66 mutations (Suppl. Table S1), including 13 mutants from GluN1 [11,23,26,75], 25 from GluN2A [10•,31–34,44••,48,75–79••], 27 from GluN2B [10•,44••,60,61,72•,73••,75], and one from GluN2D [74••]. These functional assessments range from evaluation of a single parameter (i.e. current amplitudes) to more comprehensive analyses of multi-parameters, including pharmacological properties (i.e. agonist potency, sensitivity to negative modulators, and current amplitude), biophysical properties (i.e. channel activation time course, deactivation, desensitization, charge transfer, single channel open probability), receptor biogenesis and forward trafficking, and/or neuronal excitotoxicity assay. We summarized the functional studies with more than one parameter evaluated on 52 missense mutations, 3 located in ATD, 25 in ABD, 23 in TM-link, and 1 in CTD (Figure 3). Interestingly, 87% mutations in ABD and 96% in TM-link showed functional changes and the functional consequences of these mutations differ among domains. 70% of ABD mutations presented loss-of-function, whereas 56% of TM-link mutations showed gain-of-function (Figure 3B). No trend is evident for the mutations located in ATD and CTD, since there are only a limited number of the mutations with functional data. Two TM-link mutations, GluN2A-P552R and GluN2D-V667I, have been shown to induce excitotoxicity in transfected cultured neurons [74••,75], indicating these gain-of-function mutations may underlie the patients' phenotypes of intractable seizures and epileptic encephalopathy. In addition, a functional analysis was performed on several variants reported in gnomAD database, some of which showed functional changes [44••,77•,79••–81].

Similar neurologic phenotypes (i.e. seizures) can result from both gain- (enhanced NMDAR function; i.e. GluN2A-P552R [75] and GluN2A-K669N [44] and loss-of-function mutations (reduced NMDAR function; i.e. GluN2A-A548T [75] and GluN2A-V685G [44] in the same gene. This observation suggests compensatory mechanism may contribute to patient's phenotype. Moreover, mutations influenced NMDAR function by multiple aspects and may have apparent conflicting consequences on the receptor function. For instance, mutant GluN2B-C456Y showed enhanced glutamate potency, but reduced glycine potency and decreased receptor expression on cell surface [44••]. Thus evaluation of only one aspect of NMDAR function may result in an incomplete or even misleading conclusion. Therefore, comprehensive evaluation of mutant NMDAR function is necessary to estimate overall impact of the mutations on NMDAR function. A recent study [44••] reported an approach that integrates measured multiple parameters on NMDAR mutations to estimate overall impact of these mutations on receptor function (synaptic and non-synaptic responses). The changes in synaptic NMDAR charge transfer for mutations relative to wild type receptors were evaluated by the product of the weighted deactivation time rate and the relative current amplitude (Figure 4A). Non-synaptic NMDAR charge transfer was assessed in steady-state non-synaptic agonist concentrations (Figure 4B). This approach combines quantitative assessments of the various effects that mutations have on receptor's pharmacological properties, intrinsic biophysical properties, and surface expression to discern the mechanisms underlying the mutant-associated phenotypes.

The in-depth and comprehensive functional evaluation is required to fill the expanding gulf between the volume of genetic information describing disease-related NMDAR mutations

and our understanding mechanisms of how the mutant influence the receptor function, especially study on *in vivo* models (i.e. transgenic mouse).

## Pharmacologic modulation of mutant NMDARs

The diagnosis of human NMDAR mutations that increase or decrease NMDAR activation makes it possible that each mutated receptor can be positively and negatively modulated. A set of FDA-approved NMDAR channel blockers with low affinity (e.g. memantine, dextromethorphan and its metabolite dextrorphan, amantadine, and ketamine) that can inhibit NMDAR function have been evaluated in a subset of gain-of-function NMDAR variants [44••,72•,74••–76•,78], some of which have been shown to be safe in a pediatric population. The different gain-of-function NMDAR mutants showed differential responses on the sensitivity to FDA-approved blockers that inhibit mutated NMDARs, suggesting a necessity for specific electrophysiological assessment of the response of each mutation to NMDAR blockers considered for treatment. In addition, the sensitivity of GluN2A- selective non-competitive antagonist TCN-201 and GluN2B selective negative allosteric modulator radiprodil were also tested on GluN2A and GluN2B gain-of function mutations [75,78,82]. These compounds may attenuate NMDAR hyperactivity, slow neuro-excitotoxicity, and also partially restore the circuit imbalances that result from altered NMDAR function to the wild type receptors. The “n of 1” trial, an useful approach, has been employed on a subset of epileptic patients with GoF *GRIN* mutations. Add-on memantine treatment has been used in children with intractable seizures and epileptic encephalopathy harboring potential gain-of-function mutations in *GRIN2A, GRIN2B,* and *GRIN2D* genes with a mixed response. In these cases, the patient with GluN2A-L812M [76•] and the patient with GluN2D-V667I (the second proband in Li et al., 2016 [74••]) had a favorable response to memantine treatment with significant reduction of the patients’ seizures burden. However, add-on memantine therapy was showed no effectiveness or unclear results in reducing intractable seizures in four patients harboring GluN2B mutations (GluN2B-G611V, -N615I, -V618G, and -M818T; [72•]) and another patient with GluN2D-V667I (the first proband in Li et al., 2016 [74]), whose seizures were controlled by a combination therapy with ketamine and magnesium sulfate [74••]. The difference in memantine response in these cases reflects the complexity of precision medicine. Well-designed double-blinded prospective clinical trials are necessary to determine the outcomes of memantine treatment.

As for rectifying loss-of-function mutations, the strategies enhancing NMDAR activity were also evaluated. Several positive allosteric modulators, pregnenolone sulfate, spermine, and FDA-approved tobramycin, potentiated several GluN2A and GluN2B loss-of-function mutations located in ABD through enhancement of current amplitude, and/or prolongation of deactivation time course [44••]. A GluN2A-selective positive allosteric modulator (a thiazolopyrimidinone compound) significantly increased the calcium influx of a subset of GluN2A loss-of-function mutations located in agonist binding domain, and partially or fully rectified the altered functions (changes in agonist potency) that caused by these mutations to wild type receptor levels [79]. Co-agonist D-serine was able to attenuate hypofunction of GluN2B-P553T-containing NMDARs, associated with Rett-like patient with severe encephalopathy, and the patient has shown motor, cognitive, and communication improvements after 17 months of D-serine dietary supplementation [73••]. These data

suggest that further work evaluating positive allosteric modulators as a strategy for enhancing mutant NMDAR function could be beneficial. A significant number of disease-associated NMDAR mutants appear to cause protein folding or assembly defects. An alternative strategy for variants that reduce receptor protein levels at the cell surface is to develop pharmacological chaperones, which could facilitate NMDAR biogenesis, correcting protein folding and NMDAR assembly, thereby enhancing protein stability and forwarding trafficking. Taken together, the NMDARs with rare variants can be positively or negatively modulated, which may highlight the continued development of clinically-available NMDAR modulators.

## Conclusion and future direction

Although sequencing provides unprecedented opportunities for target identification, a large and expanding gulf has developed between the volume of genetic information describing disease-associated rare variants and de novo mutations in patients and our understanding of how these genetic variants affect the function of the proteins they encode. The lack of functional understanding blunts the promise that genetic analysis holds for improving treatment. Furthermore, it prevents translation of genetic information into a better understanding of the basis for disease. Efforts to fill a gap in this rapidly emerging field will provide a functional understanding of how all mutations impact NMDAR properties. We expect that these studies will be instrumental in allowing clinical diagnostic criteria to be developed, which will also facilitate better identification of patients with NMDAR mutations. In addition, we expect the evaluation of these mutations will unfold new clues to channel function.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **ABD** | agonist-binding domain |
| **ABPE** | atypical benign partial epilepsy |
| **ACECTS** | atypical childhood epilepsy with centrotemporal spikes |
| **ADHD** | attention deficit hyperactivity disorder |

| | |
|---|---|
| **ASD** | autism spectrum disorders |
| **ATD** | amino terminal domain |
| **BCE** | benign childhood epilepsy |
| **BECTS** | benign epilepsy with centrotemporal spikes |
| **CSWSS** | continuous spike-and-wave during sleep syndrome |
| **CTD** | cytosolic carboxyl terminal domain |
| **EOEE** | early-onset epileptic encephalopathy |
| **EPI** | epilepsy/seizures |
| **GoF** | gain-of-function |
| **ID** | intellectual disability |
| **IFE** | idiopathic focal epilepsy |
| **LoF** | loss-of-function |
| **LKS** | Landau-Kleffner syndrome |
| **NMDAR** | N-methyl-D-aspartate receptor |
| **SCZ** | schizophrenia |
| **TM-link** | transmembrane domains (M1-4) and linker regions |

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• of special interest

•• of outstanding interest

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## Highlights

1. NMDAR rare variants are associated with various neuropsychiatric disorders
2. Functional consequences of variants differ among domains
3. Evaluation of multiple parameters is necessary to define gain- or loss-of-function
4. NMDAR variants can be positively or negatively modulated pharmacologically•

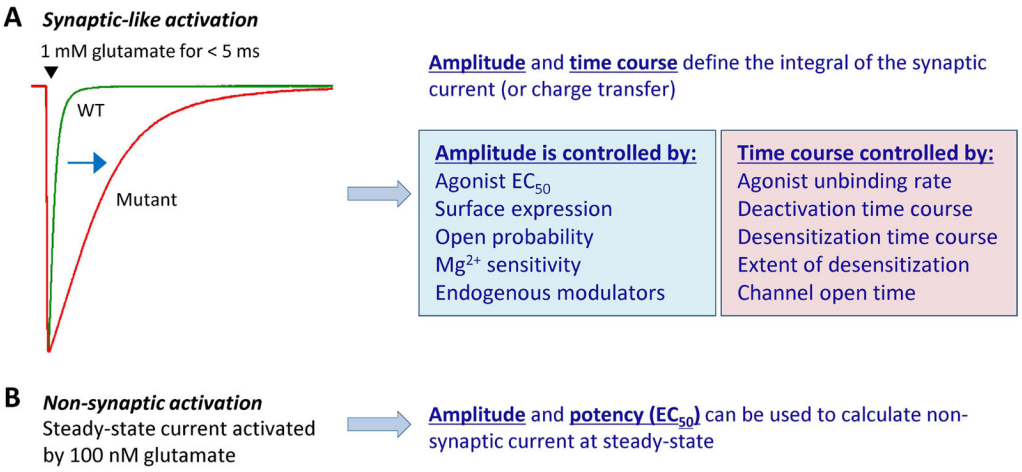



**Figure 1. NMDAR mutations are identified in patients with various neurodevelopmental and neuropsychiatric disorders**

(*A,B*) Pie charts depict the proportion of mutation types (*A*) and mutations found in different GluN subunits (*B*). (*C,D*) Summary of the types of mutations and variants that occurred in NMDARs and associated phenotypes. All published mutations absent from gnomAD database (http://gnomad.broadinstitute.org/; assessed on August 2nd, 2017) are included. Many mutations have multiple phenotypes, which are only snapshot of the current literature, which is disproportionally weighted by different diagnostic procedures. “Others” includes large-scale chromosomal deletion, translocation, inversion, and duplication, ADHD: attention deficit hyperactivity disorder, ASD: autism spectrum disorder, EPI: epilepsy/seizures, ID: intellectual disability (including developmental delay and mental retardation), SCZ: schizophrenia.

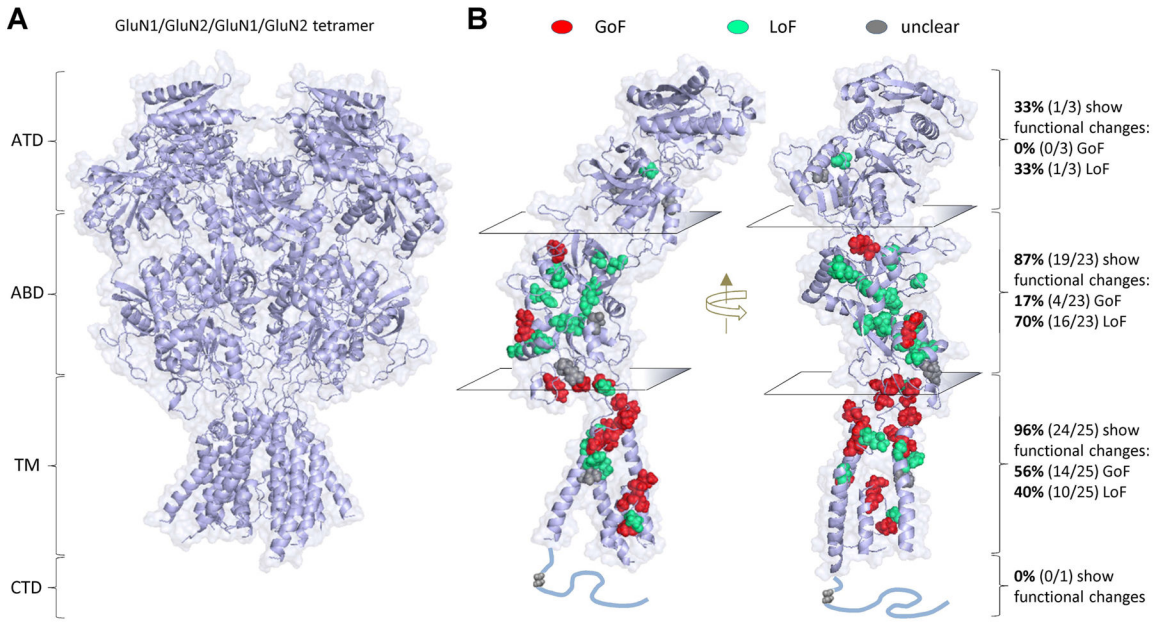
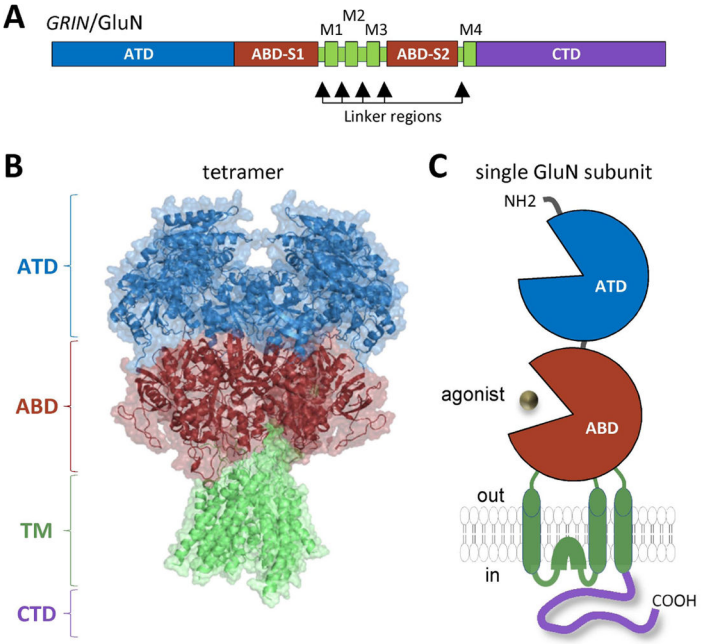
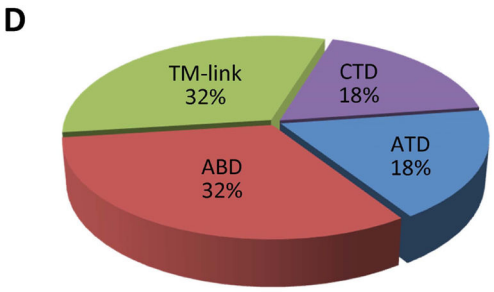



**Figure 2. Location of NMDAR mutations**

(*A–C*) Architecture and domain organization for the NMDAR. (*A)* linear representation for a GluN subunit; (*B)* an NMDA receptor tetramer (side view) (83); (*C)* a GluN subunit topology (side view). (*D,E*) Summary of NMDAR mutation locations (missense, nonsense, and frame-shift mutations are included, not the splice site mutations and large-scale chromosomal mutations). ATD: extracellular amino terminal domain, ABD: agonist binding domain, TM-link: transmembrane domains (M1-4) and linker regions, including S1-M1 linker, M1-M2 linker, M2-M3 linker, M3-S2 linker, and S2-M4 linker, CTD: intracellular carboxy-terminal domain.



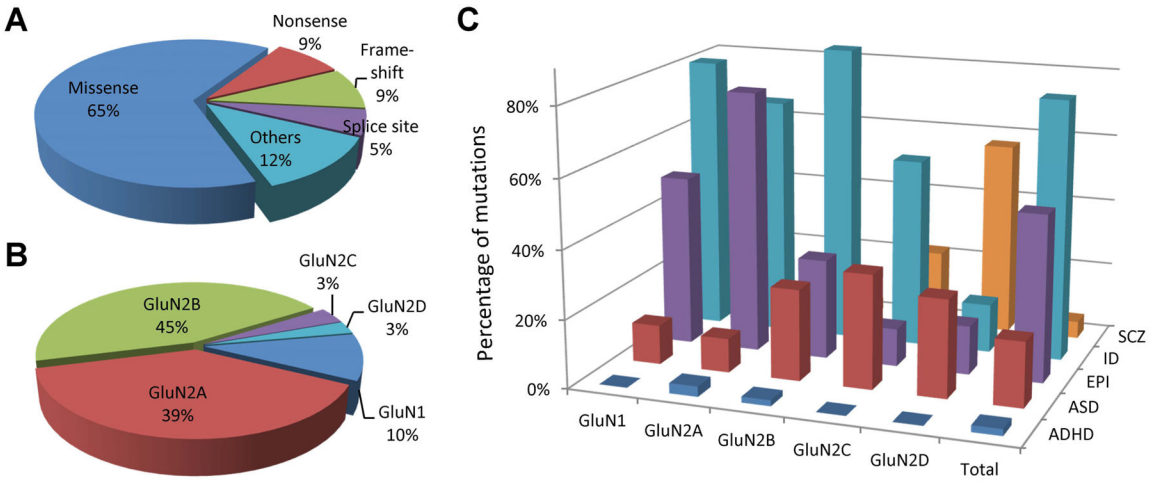




E Summary of NMDAR mutation locations

| | ATD | ABD | TM-link | CTD | Total |
|---|---|---|---|---|---|
| GluN1 | 4 | 4 | 17 | 1 | 26 |
| GluN2A | 17 | 29 | 15 | 14 | 75 |
| GluN2B | 15 | 33 | 33 | 14 | 95 |
| GluN2C | 2 | 0 | 1 | 6 | 9 |
| GluN2D | 0 | 2 | 2 | 3 | 7 |
| Total | 38 | 68 | 68 | 38 | 212 |

**Figure 3. Functional consequences of mutations differ among domains**

(*A*) Ribbon structure of a tetramer NMDA receptor (GluN1/GluN2/GluN1/GluN2). The CTD, carboxyl terminal domain, is not present in the crystal structure (83) and therefore not shown. (*B*) Residues harboring missense mutations with different functional consequences are highlighted by different colors: RED, gain-of-function (GoF); GREEN, loss-of-function (LoF); GRAY: functional status unclear (unclear). The mutations with functional study on single parameter are excluded from this summary.




**D** Summary of NMDAR mutations identified in patients with neurological diseases

| | Missense | Nonsense | Frame-shift | Splice site | Others | Total | ADHD | ASD | EPI | ID | SCZ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **GluN1** | 24 | 1 | 1 | 0 | 0 | **26** | 0 | 3 | 13 | 21 | 1 |
| **GluN2A** | 56 | 10 | 9 | 7 | 18 | **100** | 3 | 10 | 78 | 70 | 2 |
| **GluN2B** | 75 | 11 | 9 | 7 | 14 | **116** | 2 | 31 | 34 | 101 | 4 |
| **GluN2C** | 5 | 0 | 4 | 0 | 0 | **9** | 0 | 3 | 1 | 5 | 2 |
| **GluN2D** | 7 | 0 | 0 | 0 | 0 | **7** | 0 | 2 | 1 | 1 | 4 |
| **Total** | **167** | **22** | **23** | **14** | **32** | **258** | **5** | **49** | **127** | **198** | **13** |

**Figure 4. Estimation of overall impact of the mutations on NMDAR function**

Synaptic (*A*) and non-synaptic activity (*B*) can be evaluated by integration of multiple parameters. (*A*) Representative current traces by whole-cell voltage clamp recording on HEK293 cells transfected with an example mutant with prolonged response time course.