Common synaptic phenotypes arising from diverse mutations in the human NMDA receptor subunit GluN2A

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Dominant mutations in the human gene GRIN2A, encoding NMDA receptor (NMDAR) subunit GluN2A, make a significant and growing contribution to the catalogue of published single-gene epilepsies. Understanding the disease mechanism in these epilepsy patients is complicated by the surprising diversity of effects that the mutations have on NMDARs. Here we have examined the cell-autonomous effect of five GluN2A mutations, 3 loss-of-function and 2 gain-of-function, on evoked NMDAR-mediated synaptic currents (NMDA-EPSCs) in CA1 pyramidal neurons in cultured hippocampal slices. Despite the mutants differing in their functional incorporation at synapses, prolonged NMDA-EPSC current decays (with only marginal changes in charge transfer) were a common effect for both gain- and loss-of-function mutants. Modelling NMDA-EPSCs with mutant properties in a CA1 neuron revealed that the effect of GRIN2A mutations can lead to abnormal temporal integration and spine calcium dynamics during trains of concerted synaptic activity. Investigations beyond establishing the molecular defects of GluN2A mutants are much needed to understand their impact on synaptic transmission.
N-methyl-D-aspartate receptors (NMDARs) are conserved synaptic proteins underlying the late component of postsynaptic potentials at excitatory synapses. The receptor plays key roles in sculpting nervous system development and spine morphology, shaping temporal integration of synaptic potentials, regulating synaptic efficacy, and the formation of new declarative memories. Core to NMDARs achieving these roles is their high-calcium permeability, depolarisation-dependent block of extracellular magnesium ions from the channel pore, and the slow time course of NMDAR channel closure (deactivation) following brief exposure to neurotransmitter glutamate.

In adulthood, conventional NMDARs are most frequently heterotetramers composed of obligatory GluN1 subunits and regulatory GluN2 subunits, while unconventional glycine-gated GluN1/3 heterotetramers are transiently expressed during early postnatal development, persisting into adulthood only in the Medial Habenula. The GluN2 subunits contemlate the ligand-binding site indispensable for the glutamate-gated operation of NMDARs at excitatory synapses. Remarkably, four GluN2 subunits, subsumed by A–D, account for much of the diversity observed for a wide range of functional characteristics of NMDARs.

NMDAR subunit expression varies between neuronal types and during development thus providing of means of adapting the NMDAR-mediated component of excitatory postsynaptic potentials (EPSPs) to the physiological context. Principal neurons account for the majority of neurons in the adult forebrain, where they predominantly express GluN2A and 2B, and to a much lesser extent GluN2C or D. While GluN2B predominates at birth and continues to make a major contribution to synaptic NMDARs into adulthood, GluN2A is upregulated during early postnatal development, by neuronal activity and sensory experience, and accounts for much of the observed developmental speeding of NMDAR-mediated excitatory postsynaptic currents (NMDA-EPSPs) and the ligand-binding domain (LBD) or the ion channel. Missense mutations, either mutating the ligand-binding site indispensable for the glutamate-gated opening of the fundamental operating units for glutamate-gated opening of the NMDAR or transmitted with dominant inheritance, cause substitutions of amino acids and can vary considerably in their consequence on protein function and expression.

Most research on GRIN2A mutations has investigated the effects of missense mutations on GluN2A protein function in heterologous expression systems. A common outcome of these studies is that epilepsy-associated GRIN2A mutations have strikingly variable functional consequences on NMDARs. Until recently, the impact of GluN2A mutations on the NMDAR-mediated component of synaptic transmission has received relatively little attention. While modelling of synaptic charge transfer has been made for some GRIN2A mutations, these models do not consider how expressing the mutant GluN2A receptor could feedback on synaptic transmission, or to what extent and how other native NMDAR subunits continue to support NMDAR-mediated synaptic transmission. In this study, we have evaluated and compared the phenotypes and mechanisms of synaptic dysfunction in CA1 neurons expressing epilepsy-associated gain- and loss-of-function GRIN2A mutants.

**Results**

**Contrasting gain- and loss-of-function epilepsy-associated missense mutations in GRIN2A.** More than 40% of mutations discovered in GRIN2A are missense mutations (Fig. 1a), with the majority of them (approx. 60%) being in the sequence encoding the ligand-binding domain (LBD) or the ion channel. Missense mutations cause substitutions of amino acids and can vary considerably in their consequence on protein function and expression. Considerable experimental data have been published characterising how disease-linked mutations in GRIN2A affect the surface expression and biophysical properties of GluN2A-containing NMDA receptors in heterologous expression systems, namely in HEK cells and Xenopus oocytes. We compiled data for 20 different mutations from various publications and databases (Fig. 1b: blue spheres). The most comprehensive sets of measurements have been made for mutations in the LBD and it’s linkers to the ion channel, which are the fundamental operating units for glutamate-gated opening of the channel (Fig. 1b). The measured variables graphed in Fig. 1c reflect receptor activation (agonist potencies), channel activity (open probability and current deactivation), surface expression, or a combination of the above (current density). The effects of the mutations were up to 4 orders magnitude different from WT receptors with the effects being generally larger for glutamate potency and current density (Fig. 1c). Loss-of-function effects were most frequently observed for current density (79%), surface expression (77%) and open probability (63%), while receptor deactivation time was the only receptor property to be dominated by gain-of-function effects (59%) (Fig. 1c).

Although mutation effects can be easily identified for specific receptor properties (Fig. 1c), overall classification of mutations can become difficult as we illustrate with some examples. The R518H mutation was identified in a patient with CSWSS and was initially described as a gain-of-function mutation based on the longer open time of the channel in single-channel recordings. Since then, others have characterised the mutation further and reported that the mutant is poorly expressed on the cell surface and gives negligible whole-cell currents, which raises uncertainty on the overall effect of this mutation. Another complicated example is the case of K669N. This mutant expresses in a patient with CSWSS and was initially described as a gain-of-function mutation based on the longer open time of the channel in single-channel recordings. Since then, others have characterised the mutation further and reported that the mutant is poorly expressed on the cell surface and gives negligible whole-cell currents, which raises uncertainty on the overall effect of this mutation. Another complicated example is the case of K669N. This mutant expresses normally and exhibits a 3–4-fold higher agonist potency and deactivation time constant compared to WT receptors. However, the open probability of the channel is estimated to be 4–5 times lower making the overall impact of this mutation also unclear.

The examples described above motivated us to adopt factor analysis to assist in interpreting the dataset of GluN2A mutation.
effects (Fig. 1d, e). Principle component analysis provides a means to reduce multivariate data down to fewer, more manageable variables (components). The first two components calculated from the 6 variables collectively accounted for the majority of the variance (71 + 16 = 87%). After extraction and oblique rotation of these components, the first and second components (i.e., factors) had the highest loadings for glutamate potency (1/EC50) and current density respectively (Fig. 1d).
Consistent with previous observations that GluN2 mutants with low glutamate potency also tend to exhibit poor surface expression\textsuperscript{23}, we found a moderate, but significant, positive correlation between the factors 1 and 2 (Fig. 1e).

The fragmented bivariate distribution of factor scores for the mutations suggests possible segregation of the mutants into different classes (Fig. 1e). Mutations with similar effects were classified by k-means clustering. The four clusters identified were: (a) gain-of-function mutants for factor 1 (4/20), referred to as GOF type 1; (b) loss-of-function mutants mostly for the factor 1 (3/20), referred to as LOF type 1; (c) severe loss-of-function mutations mostly for factor 2 (3/20), referred to as LOF type 2; and (d) WT-like mutations with overall little effect in one or both factors (11/20); (Fig. 1e). While the clustering of mutations is rather arbitrary and the wider population of mutations may exist rather as a continuum, the analysis serves to summarise and broadly classify this set of mutations. Given some of the assumptions and ambiguity in estimating glutamate potency for R518H and T531M (see methods), their cluster membership may not be accurate but it is clear that these represent mutations with a severe LOF phenotype. We cloned representative mutations from the different clusters that had most extensive functional characterisation that was published at the time (labelled in Fig. 1e), and we went on to examine their functional incorporation at synapses.

**Functional incorporation of gain-of-function, but not loss-of-function GluN2A mutants at synapses in CA1 neurons.** To assess functional incorporation of mutant GluN2A at excitatory synapses we have adopted a molecular replacement strategy in mouse organotypic hippocampal slices.\textsuperscript{63} CA1 neurons in slices from adult mice are reported to express GluN2A and GluN2B subunits and their combined deletion effectively depletes excitatory synapses of NMDA receptors.\textsuperscript{23} Conditional deletion of the native mouse grina and b alleles was achieved using grin2a\textsuperscript{fl/fl} grin2b\textsuperscript{fl/fl} mouse lines. In these mice, loxP sites are orientated as direct repeats and inserted into introns flanking exons that encode critical structural components of receptor\textsuperscript{23,64}. This genetic manipulation predisposes the intervening region to deletion by Cre-recombinase.\textsuperscript{65} We prepared slices of hippocampus from double homozygote neonatal mice (grin2a\textsuperscript{fl/fl} grin2b\textsuperscript{fl/fl}) and cultured them in vitro for a week before co-transfecting individual CA1 neurons with cDNA for Cre-recombinase (fused in-frame with Green Fluorescent Protein, Cre-GFP) together with wildtype or mutant human GRIN2A cDNA. Slices were cultured for a further 1.5 weeks before performing recordings of evoked NMDA-mediated synaptic currents simultaneously from transfected and untransfected neurons at 15–20 DIV (Fig. 2a).

Using the experimental paradigm described above, we were able to compare the amplitudes of NMDA-EPSCs both within pairs of transfected and untransfected neurons, and the statistical interaction for the NMDA-EPSC properties between the GluN2A mutants (see Experimental procedures). Cre-GFP alone reduced the peak NMDA-EPSCs to 12% of the peak amplitude in neighbouring untransfected neurons (95% CI [9, 17]) (Fig. 2b: DKO) indicating that the NMDA-EPSCs were largely mediated by GluN2A and/or GluN2B two subunits. With the further addition of DQP-1105 (10 µM), a selective antagonist of GluN2C/D, NMDA-EPSCs were barely resolvable above baseline noise with the peak amplitudes, being 6% that of neighbouring untransfected neurons (95% CI [4, 9]) (Fig. 2b: DKO-DQP).

Since most of the current was mediated by GluN2A/B, we continued with experiments to rescue NMDA-EPSCs from the GluN2A/B double knockout (DKO) neurons using mutant human GluN2A variants. We found strong evidence in favour of an effect of expressing different GRIN2A mutants on NMDA-EPSC amplitudes (Fig. S1.1, F (5,87) = 11.4, p < 0.001, BF\textsubscript{10} = 4.14 × 10\textsuperscript{8}). Overall, the mutants differed significantly from WT in their ability to rescue NMDA-EPSCs (Fig. 2c and Fig. S1.1, F (1,87) = 31.2, p < 0.001) and we could resolve a significant difference between putative LOF (C436R, T531M, R518H) and GOF (K669N and L812M) mutations (Figs. 2c and S1.1, F (1,87) = 25.9, p < 0.001). The NMDA-EPSC peak amplitudes for LOF mutants relative to WT (100%) were 35% for C436R, 38% for T531M and 37% for R518H, 95% CIs [25,49], [25,57] and [26,53], respectively (Fig. 2c). In contrast, the K669N and L812M exhibited more effective functional rescue of the peak NMDA-EPSC, them being 64% for both K669N and L812M, 95% CIs [46,89] and [46,91], respectively (Fig. 2c). Together, these data show that the GOF GluN2A mutants were more effective (than LOF GluN2A mutants) at rescuing NMDA function at synapses in neurons largely devoid of native GluN2 expression.

The GOF mutants tested were previously reported to have much slower deactivation time constants when expressed in heterologous expression systems.\textsuperscript{25,58} We next examined the decay time constants of the NMDA-EPSCs from the GOF mutations and compared these to WT and GluN2A and the more slowly deactivating GluN2B subunit (Figs. 2d and S1.2). Compared to WT (100%), the decay of NMDA-EPSCs in neurons rescued with K669N was 184%, 95% CI [146, 231], whereas L812M was 492%, 95% CI [386, 625], which is more like the slower NMDA-EPSC decay observed in neurons rescued with GluN2B WT (Fig. 2d). In summary, these rescue experiments broadly support the mutation classification determined by analysis of heterologous expression data and presented in Fig. 1.\textsuperscript{27,58}

**Loss-of-function GluN2A mutants vary in their ability to deliver NMDA receptors to synapses.** To test whether the poor functional incorporation of LOF mutants reflected an absence of the mutant NMDARs at synapses, we adopted a simple assay that takes advantage of the obligatory assembly of GluN1 subunits into NMDARs\textsuperscript{27} and the ER-retention of unassembled GluN1 in neurons.\textsuperscript{66} GFP (or its pH-sensitive variant super ecliptic pHlourin (SEP)) fused to the extracellular (or ER-luminal) amino terminus of GluN1 has previously been used to investigate trafficking competence of different GluN2 variants to synapses.\textsuperscript{27,55,67} Exogenous expression of SEP-GluN1 alone in cultured hippocampal neurons gave largely dendritic shaft fluorescence (Fig. 3a). However, co-expression with WT GluN2A drove GluN1 into the cell membrane within dendritic spines, as evidenced by an almost 2-fold enrichment of SEP-GluN1 at spine heads colocalizing with a postsynaptic density marker, Homer1C-tdTomato. We rationalised that GluN2 mutations that disrupt the normal enrichment of NMDARs at synapses would also be incompetent at driving SEP-GluN1 at spine heads.

Differing levels of GluN1 staining were observed in transfected neurons, with most expressing GluN1 at a similar extent to untransfected neurons (Fig. 3b). To determine whether the decreased SEP-GluN1 staining was caused by a decreased number of spine heads expressing GluN1, or whether the GluN1 that was expressed was less efficient at delivering SEP-GluN1 to the spine, we performed SEP-GluN1 immunofluorescence on double transfected neurons expressing GluN2A and WT GluN2B. We found that GluN2A-C436R and T531M displayed a decreased SEP-GluN1 staining at spine heads, compared to WT GluN2B WT and GluN2A, with the C436R mutation exhibiting the lowest levels of SEP-GluN1 staining at spine heads (Fig. 3c).

We quantified SEP-GluN1 staining at spine heads by measuring the percentage of spine heads that co-stained positively for SEP-GluN1 and Homer1C-tdTomato, and showed that SEP-GluN1 levels were significantly reduced at spine heads in transfected neurons expressing GluN2A-C436R and T531M compared to WT GluN2B (Fig. 3d). In contrast, SEP-GluN1 levels at spine heads were similar in transfected neurons expressing GluN2A-K669N and L812M compared to WT GluN2B (Fig. 3d).

In summary, we provide strong evidence that gain of function GluN2A mutations result in efficient delivery of NMDA receptors to the spine head, whereas loss-of-function GluN2A mutations result in inefficient delivery of NMDA receptors to the spine head, with C436R and T531M displaying the least efficient delivery.
CI [90, 132]. Most striking of all, despite the poor functional rescue we observed earlier for R518H, expression of this mutant restored NMDA receptor levels in spines to 97% that of WT, 95% CI [82, 114]. Together, this suggests that R518H loss of function arises from functional defects while C436R (and to some extent T531M) exhibit defective enrichment at synapses. We note that for the L812M mutant, transfected cell density appeared very low in cell counts and was rescued by incubating the cells after transfection with NMDA receptor blocker memantine, which suggests that overexpressing GluN2A L812M could be neurotoxic (Fig. S2). In summary, there is substantial heterogeneity in the capability of GluN2A mutants to facilitate enrichment of NMDA receptors at synapses.

Both severe gain- and loss-of-function types of GluN2A mutant can prolong the decay of NMDA-EPSCs. The GluN2A mutants examined above were extremely varied in their abilities to contribute functional receptors at synapses. To understand
Fig. 2 Gain-of-function GluN2A mutants are more effective than loss-of-function GluN2A mutants at rescuing NMDAR-mediated EPSCs in GluN2A/B double knockout neurons. a Experimental protocol used to test functional incorporation of GluN2A mutants. Plasmids expressing Cre-GFP and human GRIN2A CDNA were cotransfected into CA1 pyramidal neurons of organotypic hippocampal slices from grin2a^−/− b^+/b^− mice. b Simultaneously recorded untransfected and transfected neurons, are connected by a line. (contrasts based on a priori clustering of the mutations in Fig.1e (Table S1); and in (− variants (wildtype or mutant). Untransfected neurons continue to express native GluN2A and B. The image of the P6 mouse pup was adapted from the JAX Mouse Pup Appearance by Age poster (available from https://jackson.jax.org/) b NMDA-EPSC−/−mV peak amplitudes from grin2a^−/− b^+/b^− (untransfected, -Cre), grin2a^−/− b^+/+ (transfection + Cre, double KO or DKO) neurons, or DKO neurons with aCSF supplemented with selective GluN2C/D antagonist DQP-1105 (10 μM). The residual current in the DKO was effectively blocked by DQP-1105. c NMDA-EPSC−/−mV peak amplitudes in grin2a^−/− b^+/b^− (untransfected) neurons and grin2a^−/− b^−/− neurons rescued with human GluN2A WT, LOF (C436R, T531M or R518H) or GOF (K669N or L812M) mutants (transfected). d NMDA-EPSC−/−mV decay time constant (tau) in grin2a^−/− b^+/b^− (untransfected) neurons, and grin2a^−/− b^−/− neurons rescued with human GluN2A WT, GOF (K669N and L812M) mutants or GluN2B (transfected). d (i). Representative NMDA-EPSCs from transfected neurons (and untransfected neuron in (b) for each experimental group (ii) Data points of measurements made in individual neurons. Matched data points, for simultaneously recorded untransfected and transfected neurons, are connected by a line. (iii) Response ratios (transfected/untransfected) are expressed as a percentage and plotted for each pair of transfected-untransfected neurons. Crossbars in (b) and (d) show the estimated marginal means with 95% confidence intervals backtransformed from the linear mixed models (Fig. S1.1 and 1.2 for (c) and (d), respectively). Hypothesis tests in (cii) are orthogonal contrasts based on a priori clustering of the mutations in Fig. 1e (Table S1); and in (dii) are posthoc pairwise comparisons using Westfall stepwise procedure. Standardised effect sizes (c) for comparisons of each mutant with WT for response ratios of (cii) peak amplitudes were −0.55, −0.46, −0.50, −0.28 and −0.26 for mutants C436R, T531M, R518H, K669N and L812M respectively (N = 93); or (dii) decay taud were 0.60 and 0.88 for mutants K669N and L812M, respectively (N = 52).

Marginal effects of loss-of-function GluN2A mutants on the amplitude of AMPA-EPSCs. NMDARs are key regulators of synaptic strength and genetic deletion of NMDAR subunits has been shown to upregulate AMPAR-mediated synaptic transmission. To assess whether alterations to levels of synaptic AMPAR receptors could occur as a consequence of less synaptic NMDA receptors for some GluN2A mutants, we also measured evoked AMPAR-receptor-mediated EPSCs (AMPA-EPSCs). While we resolved a significant difference for AMPA-EPSC amplitude in the contrast between GOF and LOF mutations (Fig. S4 and S1.7, F (1,119) = 7.23, p = 0.008) the effects were relatively small, and overall the evidence was more in favour there being no effect of mutation on NMDA-EPSCs. Compared to WT (100%), NMDA-EPSC charge transfer was slightly larger for LOF mutations C436R (162%), T531M (177%) and R518H (126%), than it was for GOF mutations K669N (125%) and L812M (114%), 95% CIs [106, 247], [116, 270], [83, 193], [86, 182] and [78, 167], respectively.

Intermediate effects of a heterozygote grin2a loss-of-function allele on NMDA-EPSCs. The data thus far raise two important questions: (1) Why do LOF mutations have the same direction of how the missense mutations are likely to affect synaptic transmission with native GluN2B expression intact, we next assessed the effect of GluN2A mutant rescue in grin2a knockout neurons. Experiments with WT GluN2A showed that rescue of the peak amplitude and decay time constant of NMDA-EPSCs was highly sensitive to the level of GluN2A expression (Fig. S3). The concentration of WT GRIN2A plasmid that effectively rescued peak amplitude and decay time of NMDA-EPSCs in GluN2A KO (3.8 ng/µL, Fig. S3) was used to assess rescue of NMDA-EPSCs for GluN2A mutants (Fig. 4). Despite mutant NMDA-EPSCs being consistently smaller than WT, we could not resolve a significant difference for peak amplitude in the contrast between mutants and WT (Fig. 4a, Fig. S1.4, F (1, 119) = 2.47, p = 0.12), and the evidence was overall strongly in favour of there being no effect among the peak amplitudes across the mutants (Fig. S1.4, BF10 = 0.084). Relative to WT (100%), estimates of the peak NMDA-EPSC amplitudes appeared slightly smaller for LOF mutants (80% for C436R, 80% for T531M and 74% for R518H, 95% CIs [57, 111], [57, 111] and [53, 103], respectively), than for GOF mutants (87% for K669N and 91% for L812M, 95% CIs [65, 116] and [68, 122], respectively).

While examining the NMDA-EPSCs, we noticed that when scaling the NMDA-EPSCs to normalise their amplitudes, the decay kinetics appeared to differ between the genotypes (Fig. 4b). In quantifying and comparing the time constant of the NMDA-EPSCs we found strong support for an effect of GluN2A mutation on NMDA-EPSC decay (Fig. S1.5, F (5, 119) = 12.1, p < 0.001, BF10 = 3.43 × 108). Overall, the decay time constants of the mutants were significantly different from WT (Figs. 4b and S1.5, F (1, 119) = 37.1, p < 0.001) as well as between GOF and LOF mutation types (Figs. 4b and S1.5, F (1, 119) = 28.3, p < 0.001). Relative to WT (100%), the decay time constants were slower for GOF mutants, it being 138% for K669N and 143% for L812M, 95% CIs [111, 171] and [115, 178], respectively. Surprisingly, not only was the direction of effect the same for LOF mutants, but the effect was also larger, with the decay time constants for LOF mutants relative to WT being 201% for C436R, 213% for T531M and 199% for R518H, 95% CIs [158, 256], [167, 272] and [156, 254], respectively.

Since we detected changes in the decay of NMDA-EPSCs, we next assessed whether there would be any effect of GRIN2A mutations on NMDA-EPSC charge transfer by taking the integral of the current traces (Fig. 4c). Significant differences could be resolved in orthogonal contrasts comparing all mutants with WT (Fig. 4c and Fig. S1.6, F (1, 119) = 4.18, p = 0.04), and between the GOF and LOF mutation types (Fig. 4c and Fig. S1.6, F (1, 119) = 4.26, p = 0.04). However, the effects were relatively small and the evidence was more in favour of there being no effect of mutation on NMDA-EPSC charge transfer (Fig. S1.6, BF10 = 0.632). Compared to WT (100%), NMDA-EPSC charge transfer was slightly larger for LOF mutations C436R (162%), T531M (177%) and R518H (126%), than it was for GOF mutations K669N (125%) and L812M (114%), 95% CIs [106, 247], [116, 270], [83, 193], [86, 182] and [78, 167], respectively.

Overall, the effects LOF and GOF GluN2A mutations on NMDA-EPSC decay kinetics were large and in the same direction, but because of the slightly smaller mutant NMDA-EPSC amplitudes, the effect that the slowed kinetics had on NMDA-EPSC charge transfer was much more subtle.
effect on NMDA-EPSC time course as the GOF mutations? and (2) What effect can we expect in heterozygotes for LOF alleles, especially considering that patients with GRIN2A mutations have only one mutant allele? We addressed these questions by examining the effect of homozygous and heterozygous null alleles on evoked NMDA-EPSCs. We achieved this genetic manipulation by transfecting Cre-recombinase in pyramidal neurons in CA1 of organotypic slices prepared from $grin2a^{+/+}$, $grin2a^{+/−}$ and $grin2a^{-/-}$ mouse pups. We found some evidence against differing effects of Cre across the genotypes for NMDA-EPSC charge transfer (Fig. S1.8, $p = 0.30$, $BF_{10} = 0.277$), and even the apparent upward trend was not statistically significant for increasing doses of mutant allele in a linear polynomial contrast (Figs. 4a and S1.8, $F(1,96) = 2.39$, $p = 0.13$). However, a differing effect of Cre across the genotypes was strongly supported for NMDA-EPSC peak amplitude, decay time constant and rise-time.

**Fig. 3 Loss-of-function GluN2A mutants vary in their ability to deliver NMDA receptors to synapses.**

**a** Representative images of cultured neurons overexpressing GluN2A, SEP-GluN1 (green) and Homer1c-tdTomato (red). The latter was used as a synaptic marker. Arrow heads point to examples of synapses that have high synaptic levels of SEP-GluN1. Circles highlight examples of synapses that exhibit low-to-moderate levels of SEP-GluN1. **b** Levels of synaptic enrichment of NMDA receptors and Homer1c in cultured hippocampal neurons transfected with or without human GluN2A WT, LOF (C436R, T531M or R518H) or GOF (K669N or L812M) mutants. Each data point represents mean fluorescence intensity of SEP-GluN1 (green) or Homer1c-tdTomato (red) across all visible putative synaptic regions-of-interest (ROIs) in a neuron. **bi** Data points for SEP-GluN1 (green) and Homer1c-tdTomato (red) fluorescence measured from the same neuron are connected by a line. **bii** Response ratios (SEP-GluN1 / Homer1c-tdTomato) are expressed as a percentage and plotted as a point for each neuron. Crossbars in (i) and (ii) show the estimated marginal means with 95% confidence intervals backtransformed from the fitted linear mixed models (Fig. S1.3). Hypothesis tests in (bii) are orthogonal contrasts based on a priori clustering of the mutations in Figs. 1e (Table S1). Standardised effect sizes ($r$) for comparisons of each mutant with WT were $−0.39$, $−0.26$, $−0.03$, $0.06$ and $−0.20$ for mutants C436R, T531M, R518H, K669N and L812M respectively ($N = 191$).
Significant differences could be resolved for all pairwise comparisons between grin2a+/+, grin2a−/− and grin2a−/−, except for the comparison of peak amplitudes between grin2a+/+ and grin2a−/+ (Fig. 5c, d, and S1.9-1.11). Consistent with the NMDA-EPSCs observed in the rescue experiments of the LOF GluN2A mutants, the decay time constant was 207% slower for grin2a−/− (95% CI [183, 233]) relative to WT grin2a+/+ (100%). Together with the negligible NMDA-ESPC in the double knockout (grin2a−/− grin2b−/−, Fig. 2b), this suggests that the residual current is mediated by GluN2B, which has slower deactivation kinetics and could explain

**Fig. 4** Gain- and loss-of-function LBD mutants are both associated with more prolonged NMDAR-mediated EPSCs. NMDA-EPSCs at 20 mV peak amplitudes (a), decay time constants (b), or charge transfer (c) in grin2a+/+ (untransfected) neurons, and grin2a−/− neurons rescued with human GluN2A WT, LOF (C436R, T531M or R518H) or GOF (K669N or L812M) mutants (transfected). a–c(i): Representative NMDA-EPSCs from transfected neurons for each experimental group. (ii) Data points of measurements made in individual neurons. Matched data points, for simultaneously recorded untransfected and transfected neurons, are connected by a line. (iii) Response ratios (transfected/untransfected) are expressed as a percentage and plotted for each pair of transfected-untransfected neurons. Crossbars in (ii) and (iii) show the estimated marginal means with 95% confidence intervals backtransformed from the linear mixed models (Fig. S1.4–S1.6). Hypothesis tests are orthogonal contrasts based on a priori clustering of the mutations in Fig. 1e (and Table S1). Standardised effect sizes (r) for comparisons of each mutant with WT for response ratios of: (a) peak amplitudes were −0.12, −0.12, −0.16, −0.09 and −0.06; (b) decay taus were 0.46, 0.49, 0.46, 0.26 and 0.28; and (c) charge transfer were 0.20, 0.24, 0.10, 0.11 and 0.06, for mutants C436R, T531M, R518H, K669N and L812M respectively (N = 125).
the more prolonged NMDA-EPSCs observed also for LOF GluN2A mutants (Fig. 4b). The effect observed for NMDA-EPSC decays in grin2a−/− neurons (i.e., heterozygotes) was 120% (95% CI [106, 137]), it being intermediate between wildtype and homozygous cases. Overall, these results illustrate that even a single null allele of grin2a is sufficient to cause defects in synaptic transmission.

Mutation-associated changes in NMDA-EPSCs can shape synaptic excitability and calcium dynamics. The results raise questions about how the changes in NMDA-EPSCs by the mutations will ultimately affect excitability. In particular, how could smaller and slower NMDA-EPSCs alter excitability when the amount of charge transfer is effectively unchanged? Using a computational model of a CA1 pyramidal neuron, complete with a wide range of active conductances and synaptic AMPA and NMDA receptors (Fig. 6ai), we simulated the effects of slowing the kinetics of NMDA-EPSCs to 150% and 200% compared to WT (100%), which roughly correspond to the effects observed for GOF and LOF mutants respectively. In each case the synaptic weight (a.k.a peak amplitude) was also scaled to equalise charge transfer for single EPSCs (Fig. 6aii and iii). We then simulated short trains of synchronous synaptic activation and compared spine head currents and intracellular calcium levels at activated synapses between the different NMDA-EPSC profiles.

**Fig. 5 Dose-dependent effects of grin2a null alleles on NMDA-EPSC kinetics.** NMDA-EPSC−20 mV charge transfer (a), peak amplitude (b), decay time constant (c), or 20–80% rise-time (d) in grin2a+/+, grin2a+/− or grin2a−/− (untransfected) neurons and grin2a+/+, grin2a+/− or grin2a−/− neurons (transfected, with Cre-GFP). a–d (i). Representative NMDA-EPSCs from transfected neurons for each experimental group. (ii) Data points of measurements made in individual neurons. Matched data points, for simultaneously recorded untransfected and transfected neurons, are connected by a line. (iii) Response ratios (transfected/untransfected) are expressed as a percentage and plotted for each pair of transfected-untransfected neurons. Crossbars in (i) and (ii) show the estimated marginal means with 95% confidence intervals backtransformed from the fitted linear mixed models (Figs. S1.8–S1.11). Hypothesis tests are posthoc pairwise comparisons using Westfall stepwise procedure. Standardised effect sizes (r) for comparisons of each genotype with WT for response ratios of: (a) charge transfer was 0.06 and 0.16; (b) peaks amplitudes were 0.00 and −0.35; (c) decay taws were 0.28 and 0.77; and (d) rise-time were 0.22 and 0.68, for heterozygous and homozygous genotypes respectively (N = 99).
Trains of 40 randomly positioned synapses, simultaneously activated over a range of frequencies (20, 40, 80 and 200 Hz) were simulated and the membrane potential (in soma and spine heads) and inward currents, outward currents and calcium concentrations in spine heads were stored, averaged across spines and summarised as current scapes\textsuperscript{71} (Fig. 6b). The simulation was repeated with 10 different random seeds for each NMDA-EPSC profile and stimulation protocol. While an individual EPSP burst was subthreshold across the three groups, WT-like (100%) EPSP trains drove neuronal spiking by the 4th or 5th EPSP during short high frequency (200 Hz) trains, and the action potentials backpropagated to spine heads (Fig. 6b, ci). The voltage changes...
in spine heads were associated with robust activation of NMDARs, R-type Ca$^{2+}$ channels (CaR) and Ca$^{2+}$-activated K$^+$ channels (KCa) (Fig. 6b, cii) and large increases in Ca$^{2+}$ reaching a peak of >10 μM, and an integral of 1.3 mmol.ms/L (Fig. 6b, ciii). At the same frequency, neuronal spiking was either lower or absent for the GOF-like (150%) or LOF-like (200%) NMDA-EPSP condition respectively (Fig. 6b, cii). These changes in spiking were also associated with less activation of NMDARs, CaR and KCa, and less accumulation of Ca$^{2+}$, in spines for mutant-like NMDA-EPSP profiles (Fig. 6b, ciii). Summation of EPSPs at slower frequencies did not result in any spiking and the differences between NMDA-EPSP profiles for depolarisation and intracellular Ca$^{2+}$ became smaller (Fig. 6ciii).

The full set of simulations above were repeated with 200 synchronous synaptic inputs to evaluate the effect of NMDA-EPSP profiles on the summation of suprathreshold EPSPs. WT-like (100%) (but not mutant-like) EPSP trains at 80 Hz and above triggered plateau potentials that were associated with robust activation of spine NMDARs, CaR and KCa (Fig. 6d). Interestingly, the spiking output was very frequency dependent, with there being greater spiking in mutant-like NMDA-EPSPs at 80 Hz, but less spiking at 40 Hz and similar spiking at the more extreme train frequencies (Fig. 6d, ei). Examination of the voltage traces at 80 Hz revealed that spiking was strongly attenuated during the plateau potential, likely due to Na-channel inactivation. Overall spine Ca$^{2+}$ accumulation reached up to 2–3 times the levels compared to 40 active synaptic inputs, and the mutant-like NMDA-EPSP profiles still lead to less accumulation of spine Ca$^{2+}$ (Fig. 6ciii).

Overall, these results indicate that even in the absence of changes in charge transferred during synaptic activation, mutant NMDAR kinetics could still effectively modulate synaptic excitability and spine calcium dynamics, with the effects being very frequency dependent.

**Discussion**

Our investigation revealed how mutations from patients with GRIN2A-related epilepsy disorders result in defective synaptic transmission. The robust differences in the ability of mutant GluN2 subunits to rescue NMDA-EPSCs in GluN2-null (grin2a$^{-/-}$, b$^{-/-}$) neurons (Fig. 2) were largely consistent with the classification of mutations, as severe GOF and LOF, based on analysis of data obtained in heterologous expression systems by others (Fig. 1). Remarkably though, replacing only the native GluN2A with the mutant receptor subunit gave rise to NMDA-EPSCs with very similar defects for LOF and GOF mutations: NMDA-EPSCs that were slower in time course but similar in charge transfer (Fig. 4). In general, mutations phenocopied the GluN2A knockout (Fig. 5), but the mechanism underlying the defect varied, with some mutants being associated with poor trafficking to synapses (Figs. 3 and 7b) while others reached the synapse but were either functionally silent (Figs. 2, 3, 7c) or contributed directly to the NMDA-EPSCs through altered functional properties of the mutant receptor (Figs. 2, 7d). In the case of the LOF mutations, the changes in the NMDA-EPSC time course likely reflect the contribution of natively expressed GluN2B (Figs. 5, 7b, c).

The ability of GluN2A mutants to incorporate into synaptic NMDARs varied considerably (Figs. 2, 3). The LOF mutation C436R gave similarly low levels of synaptic enrichment of NMDARs as the control without GluN2A (Fig. 3). The C436R mutation disrupts the formation of a disulfide bridge, which is likely key to the folding and stability of the GluN2A protein structure (Fig. S5). In contrast, the LOF R518H gave similar levels of NMDARs at synapses as wildtype GluN2A (Fig. 3), and the lack of functional incorporation of R518H (Fig. 2c) may be explained by the effect of the mutation on lowering glutamate potency (Fig. 1c–e) by breaking coulombic interactions between GluN2A and the glutamate agonist (Fig. S5). Some mutations, like the T531M mutation, may even show a combination of these mechanisms (Figs. 2, 3 and S5). It is important to point out that some mutations may have effects not represented by this sample, the LOF mutants of which were all from the LBD. It is conceivable that mutations in other parts of the receptor could give receptors that traffic to the synapse but whose functional defects lead to distinct synaptic phenotypes. Nonetheless, our results show that mutations with severe and contrasting LOF and GOF effects on GluN2A-containing receptors can lead to effects on synaptic function that while abnormal, are in the same direction.

A current question in the field of GRIN2A-related disorders is whether expression or synaptic delivery of the native GluN2B subunit is specifically upregulated and compensates for GluN2A loss of function. The smaller NMDA-EPSCs in neurons that are homozygous for the null GluN2A allele indicate that compensation by GluN2B could be incomplete (Figs. 5a, 2d). However, in neurons heterozygous for a null allele, the slower, GluN2B-like time course of NMDA-EPSCs in the absence of changes in amplitude suggests that there could be some, albeit small, compensation. The mechanism of this compensation may simply reflect a greater propensity for existing GluN2B-containing NMDARs to accumulate at synapses when synaptic content of GluN2A is reduced. This means of compensation would still be consistent with GluN2A deletion not having any effect on either GluN2B total protein or the amplitude of ifenprodil-sensitive whole-cell currents. It should be mentioned though that since GluN2A and GluN2B subunits couple to distinct downstream signalling pathways through their distinct intracellular C-tails, changes in the GluN2A/B subunit composition at synapses could be expected to have an impact on long-term regulation of synaptic function (e.g. synaptic plasticity). As such, more complex aspects of synapse phenotype between GOF and LOF mutants may indeed differ in more than ways we can
the overall effects of GluN2A mutations on the properties of the NMDA-EPSCs raised some interesting questions about how they may modify synaptic excitability. In particular, the slower time course and smaller amplitudes of the NMDA-EPSCs in grin2a−/− neurons rescued with GluN2A mutants resulted in charge transfer through the NMDAR channels being similar to wildtype. This prompted us to model the effect of the mutations on NMDA-EPSCs and simulate the depolarisations and spine calcium dynamics during trains of concerted synaptic activity onto CA1 neurons (Fig. 6). These simulations serve to illustrate that the slower time course of NMDA-EPSCs mediated by GluN2A mutants can affect EPSP summation even when charge transfer is the same. However, there may be other effects of the GluN2A that we have not measured or modelled (e.g., pre- synaptic or extrasynaptic receptors, synaptic plasticity) that contribute to synaptic excitability and neuronal signalling. In addition, GluN2A is expressed in other neuronal cell types (including inhibitory neurons75) and how the mutation effects in all cells sum together to affect excitatory-inhibitory balance and network activity is unknown. Together, understanding how NMDAR mutations affect excitability and network stability warrants further investigation and may ultimately help in designing therapeutic strategies to treating GRIN2A-related disorders.

In summary, severe loss- and gain-of-function GRIN2A mutations associated with epilepsy-aphasia spectrum disorders lead to similar dysfunctional NMDAR-mediated synaptic currents in CA1 pyramidal neurons when expressed in cultured mouse hippocampal slices, albeit via different mechanisms. Modelling suggests that mutant-like NMDA-EPSCs can lead to abnormal temporal summation and spine calcium dynamics.

Methods
Mutant classification. The functional consequences of 20 GRIN2A mutations were collected from publications and databases45,47,51–58. The functional properties were Glutamate EC50 and Glycine EC50 (in oocytes), and deactivation time constant. 

**Fig. 7 Model summarising how different molecular defects can converge on similar NMDAR-mediated EPSCs.**

- **a** WT GluN2A and GluN2B mostly contribute to NMDA-EPSCs in CA1 pyramidal neurons. GluN2B-containing NMDARs have more prolonged time course and cation influx as illustrated by the bold purple arrows.
- **b** GluN2A mutants with defects culminating in low levels of GluN2A at synapses result in NMDA-EPSCs that are dominated by residual GluN2B-containing NMDARs.
- **c** GluN2A mutants that traffic to the synapse but are functionally defective result in NMDA-EPSCs that are dominated by residual GluN2B-containing NMDARs.
- **d** GluN2A mutants that traffic to the synapse but have slower deactivation kinetics directly contribute to prolonging NMDA-EPSCs.

The effect of mutations may result in a combination of these different mechanisms.
293T cells). Potency for R518H was approximated from the fold change in potency for R518C documented in the CFERV database. Potency for T531M was approximated by scaling the R518H potency according to the R518H/T531M ratio for single-channel opening frequency correct for the differences in surface expression. To handle other remaining instances of missing data, single imputation was achieved by linear interpolation following orthogonal regression on the rank-transformed data using principal component analysis (PCA) by the alternating least-squares algorithm in Matlab (Mathworks). The complete dataset was then normalised to the wildtype (WT) GluN2A measurement for each property and transformed to the log scale. The dataset had a Kaiser–Meyer–Olkin test statistic of >0.5 and was considered suitable for PCA. More than 85% of the variance was explained by the first two principal components, which were then extracted from the loadings matrix and subject to oblique (Varimax) rotation. The resulting pattern matrix was used to calculate rotated component scores. The number of clusters chosen for K-means clustering of the scores was determined from the elbow in a plot of the sum-of-squares error against the cluster number.

Molecular biology. Mutagenesis was performed on the cDNA of human GRIN2A in the pCI-Neo vector (pcMV GluN2A). GluN2A mutations with respect to the amino acid numbering in NP_000824.1 were C436R, T531M, R518H, K669N, EK609N, and L812M. Oligonucleotide primers (Integrated DNA Technologies) were designed to incorporate single-base substitutions using the GeneArt® Site-Directed Mutagenesis Kit (ThermoFisher Scientific) (Table S3). The presence of each mutation was confirmed by DNA sequencing (Eurofins Genomics) and the whole coding sequence was also examined to ensure that no other mutations were introduced by polymerase chain reaction (PCR) errors. Plasmid DNA was amplified and purified using EZNző endo-free plasmid maxi kit (VWR International).

Animals. C57Bl/6j mice and Sprague Dawley rats were purchased from Charles River Laboratories. Rodents were maintained under conventional housing conditions and a 12-12 light-dark cycle. Heterozygous grin2a–/– and grin2b–/– mice were a gift from Kenji Sakumura (Niigata University, Japan). Generation of these mice has been described previously. Homozygous grin2a–/– and grin2b–/– mice were generated from the respective heterozygous mice and the colonies were subsequently maintained as homozygous lines taking care to avoid brother-sister matings. Double-flox (grin2afl/–/fl and grin2bfl/–/fl) mice were generated by crossing the grin2afl/–/fl and grin2bfl/–/fl mouse lines. Ear biopsies taken for identification were genotyped by polymerase chain reaction (PCR) using previously published primers for grin2a (1745395) and grin2b (1745397). Ear biopsies were removed in dissection medium composed of HBSS (14155048, ThermoFisher Scientific), B27 supplement (17504044, Gibco), 25 mM HEPES, 3 mML-glutamine, 1 mM CaCl2, 1 mM MgSO4, 0.25 mM ascorbic acid (in mM): CaCl2 (0.5), KCl (2.5), MgCl2 (1), glucose (10), Na-pyruvate (1), NaHCO3 (1), MgCl2 (0.53 nM (3 ng/ml)) were added dropwise to 30 µl 2x HEPS buffered saline (120 mM NaCl, 30 mM NaHCO3, 30 mM HEPES, 1.8 mM CaCl2, 1.3 mM MgCl2, 0.53 nM (3 ng/ml)) in intracellular solution containing (in mM): CH3SO3H (135), KOH (135), MgCl2 (12), MgSO4 (0.28), NaCl (30), NaHCO3 (0.85), glucose (25), NaHCO3 (2.7), succrose (175) and HEPS (2) (pH 7.3, 300 mOsm). Transfected neurons were illuminated with a white background to stimulate the Schaffer collateral pathway. 2 × 20 Hz for 0.25 s with a photodiode array (isoFlex, A.M.P.I.) triggered from ACQ4 software (v0.9.3) through a USB-SCXI-RGB digital I/O card (USB-6343, National Instruments, Austin, TX). The patch pipette was positioned in the slice under visual guidance using a mechanical manipulator (PatchStar, Scientif i c Instruments). Cell recordings were performed with a multiclamp 700B (Molecular devices). Neurons in organotypic slice cultures were transfected by single-cell electroporation. At 6-8 days in vitro (DIV) CA1 pyramidal neurons were transfected using the calcium phosphate method. DNA constructs cotransfected (μg/coverslip) were pCMVII–/a-Homer1c-ttdTomato (1), empty pcDNA3.1 (+), and pcMV constructs driving expression of SEP-GluN1 (1.5) (Addgene plasmid #23999) and GluN2A (1.5). Per well of a 12-well plate, a mixture of plasmid DNA and calcium chloride (50 µl, 2.5 M) was added dropwise to 30 µl 2x HEPS buffered saline and rotated 20 min at room temperature. Coverslips containing neurons were transfected to a fresh 12-well plate containing 500 µl prewarmed culture medium supplemented with 1 mM kynurenic acid and followed by the addition of DNA/CaCl2–HEPS solution. After a 90-minute incubation at 37 °C with 5% CO2, the coverslips were transferred to a new 12-well plate containing conditioned medium and incubated at 37 °C with 5% CO2 until the day of imaging.

Electrophysiology. The electrophysiology method utilised throughout was current measurements in whole-cell patch-clamp configuration. Hippocampal slices had a cut made between the CA3 and CA1 regions to reduce recurrent excitation during superfusion of the recording chamber (6 ml/min). Neurons in organotypic slice cultures were transfected by single-cell electroporation. Neurons in organotypic slice cultures were transfected by single-cell electroporation. Neurons in organotypic slice cultures were transfected by single-cell electroporation. Neurons in organotypic slice cultures were transfected by single-cell electroporation.
EGTA (0.2 (pH 7.25). Osmolarity of intracellular and extracellular solutions was checked and adjusted on the day of each experiment and adjusted by addition of water to the major salt so that the aCSF osmolarity was 10–15 mMol/kg greater than the intracellular solution. To account for inter-slice variability in our recordings, two CA1 neurons were patched simultaneously—one transfected neuron identified by the Cre-GFP fluorescence (pE-300 white, CoolLED; 49018 EGFP long-pass, Chroma) and one neighbouring untransfected neuron. The intensity and constant of constant voltage (50 µs duration) was adjusted (IsoFlex, A.M.P.I.) to obtain reliable NMDA-EPSCs (in untransfected neurons) that were <250 pA (typically 10 V). The configuration of constant voltage stimulus enabled us to induce rapid changes in the potential difference across the electodes with minimal influence of stray capacitance. Stimuli were delivered at an inter-sweep interval of 10 s. Signals were acquired with a MultiClamp 700B ( Molecular Devices), low-pass filtered (1 kHz, 4-pole Bessel) and digitised (25 kHz) directly to hard disk using a USB-X Series Multifunctional DAQ interface (NI USB-6363, National Instruments). Hardware was controlled using Python-based ACQ4 software (v0.9.3)17. The series resistance was left uncompensated during data acquisition (but corrected offline, see below). Pairs of neurons were discarded during data collection if the difference in series resistance varied by more than ±8 Ω.

For molecular replacement experiments (Figs. 2, 4 and 54), 10 evoked mixed AMPAR- and NMDA-EPSCs were recorded at a range of voltages (−100 to +20 mV in 20 mV increments). These holding potentials accounted for the liquid-liquid junction potential, which we calculated to be +10 mV for our solutions (PowerPatch Tools Igor Pro Tool collection). The aCSF included 50 mM picrotoxin (ab120315, Abcam) and 10 µM CaCl2 (Fig. 5), the aCSF included 50 µM picrotoxin (ab120315, Abcam) and 10 µM CaCl2 (pH 7.25). Osmolarity of intracellular and extracellular solutions was water or the major salt so that the aCSF osmolarity was 10

For experiments where the NMDAR current was isolated pharmacologically for experiments where the NMDAR current was isolated pharmacologically (Fig. 5), the aCSF included 50 mM picrotoxin, 10 µM gabazine as above, and 2 µM GPP243243 to inhibit GABAδ receptors. The intracellular solution was supplemented with 3 mM QX-314-Cl to attenuate currents generated by activated sodium and calcium channels. For each voltage, 10 evoked NMDA-EPSCs were recorded per neuron at an inter-sweep interval of 10 s. Transsections and recordings for GluN2A mutant molecular replacements were randomised and performed double blind. All molecular replacement experiments were carried out in slice cultures prepared from the left hemisphere since the injection parameters for AMPA- and NMDA-EPSCs differed and tended to show less variance compared to those in neurons of slices from the right hemisphere. The mutants selected for recordings in this study are known to have no more than 2.5-fold changes in magnesium potency25,26. We thus separated AMPAR- and NMDAR-components of evoked mixed EPSCs during the analysis using the following protocol: (1) The current traces at each holding potential were converted to conductance using Ohm’s law; (2) The AMPAR-mediated conductance at −100 mV was subtracted from the conductance trace at +20 mV. The resulting conductance traces thus reflect AMPA-EPSCs (−100 mV) and NMDA-EPSCs (+20 mV).

For experiments where the NMDAR current was isolated pharmacologically (Fig. 5), the aCSF included 50 mM picrotoxin (ab120315, Abcam) and 10 µM gabazine (SR95531, Abcam) to block ionotropic gamma-aminobutyric acid receptors (GABAδRs) and 10 µM NBQX (1044, Tocris Biosciences) to inhibit α-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAδRs). The membrane potential was raised from −75 mV to −20 mV at a rate of 0.5 mV per second to provide time for the Cs+ to block potassium (leak) conductances. For each neuron, 30 evoked AMPA-EPSCs were recorded. Since the current–voltage relationship for AMPA-EPSCs 100 mV and NMDA-EPSCs 20 mV relative to the reversal potential (approximately 0) can be assumed linear, we made no attempt to correct for series resistance59. The stimulus artefact was interpolated and the series resistance (Rs) and cell membrane capacitance (that were estimated from the test pulse in each recording) were used to perform offline series resistance compensation. For consistency, final, uncompensated current in each recording (R uncompensated) was set to a constant 3 Mohm, where 10% of compensation applied to each recording was calculated using the following equation.

\[
R_{\text{comp}}(\%) = 100 \times \max \left[ 1 - \frac{R_{\text{uncompensated}}}{R_s}, 0 \right]
\]

Series resistance compensated recording traces were analysed using a custom python module (https://github.com/acp29/penn) within Stimfit software (v0.13 or 0.15.8)30 or custom scripts in Matlab (https://github.com/acp29/Elmasri_GRIN2A). Decay of the EPSC currents was fit by a two-component exponential decay with offset initially using the Chebyshev algorithm. The weighted exponential time constant was calculated as the sum of component time constants multiplied by their fractional amplitude. Where the two-component fits failed, a single exponential was found to fit well. 

Imaging. Transfected cultures of dissociated hippocampal neurons were imaged at DIV11. Neurons were imaged using an inverted microscope (AsioVert.A1, Zeiss), a 40 x 0.55 NA objective and a complementary metal-oxide-semiconductor (CMOS) camera (ProRes GRYPHAX Arturk 8 Mpix, Jenoptik). Images were acquired using GRYPHAX software (v2.2.0, Jenoptik). Coverslips were placed in ASCF composed of (in mM): NaCl (140), KCl (3), MgCl2 (4), CaCl2 (4), glucose (10), Na-pyruvate (1), HEPES (4) and HEPES buffer, pH 7.4. Images were acquired at the GluN1-SEP signal at (LED λ= 670 nm) and the Homer1c-tTomato signal at (LED λ= 530 nm). Five images (8-bit) were taken for each.

Neuron simulations. Simulations were conducted in NEURON 7.5 software83. Simulations were carried out using a multiparametrical model of a CA1 pyramidal neuron developed by Graham and colleagues69. The model includes spatially distributed ion channels responsible for fast sodium (Na) currents, delayed rectifier potassium (K) currents, A-type potassium (K) currents, h-current, HVA R-type calcium (Ca2+) currents, and Ca2+-activated medium-duration after-hyperpolarization (mAH) potassium (K) currents. In all cases, simulations were conducted with a time step of 25 µs. Stimuli were delivered at a frequency of 0.1 s.

Statistics. Data were collected until the number of cell pairs reached between a total of about 15–30 per mutation (or genotype). The experiment was repeated in 3–6 animals. The choice of which slices/animals to allocate to which mutation or genotype was random. The experimenter was blind to the mutation or genotype of the sample. Since it is possible that the occasional neuron we patched was not representative of the population (e.g. an interneuron, or had poor co-transfection with GRIN2A or CDNA), we performed outlier detection prior to data analysis.

Whole-cell properties and measurements of NMDA-EPSCs in matched transfected and untransfected where expressed as log, response ratios and included in multivariate outlier detection by robust principal component analysis (ROBPCA) using the LIBRA Matlab library52 in Matlab 9.2 (R2017a, Mathworks). ROBPCA was used to detect principal components (PCs) that are representative of the trend of the majority of the data with lowest influence from extreme values. The number of PCs used was the number that explained 90% of the variance. Samples that were extreme orthogonal outliers, bad leverage points and were beyond the cut-off value of \(\sqrt{\lambda - \lambda_{975}}\) were excluded from all subsequent analysis. Typically, about 2–3 principal components (ranged from 9 to 4) were excluded per group of data (e.g. per mutation). A summary of the sample sizes after multivariate outlier detection is presented in Table S4.

Statistical analysis was carried out in R (v 4.1.0) and RStudio (v 1.4). Each response datum from the pitch-clip recordings (in Figs. 2, 4, 5 and 54) was collected along with its assignment to (levels of) the following factors: mutation (WT, C436R, T531M, R518H, K669N, L812M), protein (GFP-GluN1 or Homer1c-tdTomato), region of interest (ROI) and experimental repeat (with unique identifier). ANOVA was performed on each pairwise comparison of the data with an equal number of observations using the lmer function in the R package lme4. Model fitting was set to optimise the restricted maximum likelihood (REML) criterion. The lmer formula for each random intercept model was reported in Figs. S1–S11 and follows the described convention for mixed models86. Where hypothesis testing was based on a priori clustering of the data (as in Fig. 1e), orthogonal contrasts (Table S1) were setup and applied before model fitting. Where hypothesis testing was based on testing the significance of trends across ordered (categorical) factors, polynomial contrasts were setup and applied before model fitting.

Model assumptions were evaluated by graphing plots of the residuals. Homoscedasticity was assessed by examining the scatter of the standardised model
residuals against the fitted values. Normality of the model residuals was assessed by 
(1) superimposing a histogram and kernel density estimate of the standardised model residuals with a normal standard distribution; and by (2) creating a
quantile–quantile (Q–Q) plot of sample quantiles against normal quantiles together with
95% tail-sensitive confidence bands. Model outliers were assessed from the above
plots of the standardised model residuals and the degree to which they were
influential was assessed using a stem-and-leaf plot of the Cook’s distance calculated
using the influence function value from the lm() function in R. Cook’s distances were all <0.15, far below the threshold for being considered
influential (1.0). Graphs were created using functions from the ggplot2 package.

The LMM results for the fixed effects were summarised as ANOVA tables using
Type III Wald F-tests and with degrees of freedom calculated by the Kenward-Roger
method using the Anova function from the car package. To summarise the random
effects structure, the variance of each group level was used to calculate its respective
classic intraclass correlation coefficient (ICC) using the icc function from the
performance package. When posthoc tests were requested, they were pairwise
comparisons with type I error rate controlled by the Westfall stepdown procedure
and were calculated using the multcomp R package. Parameter estimates and effect
sizes are reported in the main text and figures as response ratios (expressed as %)
with 95% confidence intervals calculated using functions from the emmeans
package. Effect sizes were standardised by conversion to correlation coefficients
using the r_to_r function from the effsize package and reported in the figure
legends. Details of LMM results and diagnostics are provided in the supplement
(Fig S1). All summaries of statistics annotated in graphs have used the following
anovaBF and bayesFactor_inclusion functions from the BayesFactor
package. BF0g indicates the likelihood ratio of evidence for (BF0g > 1), or against (BF0g < 1), matched (null) models. Default
(Cauchy) priors for fixed and random effects had the usual scales of 0.5 (‘medium’)
and 1 (‘nuisance’) respectively.

Confidence intervals and p-values for Kendall’s tau-b (τb) (non-parametric)
correlation coefficients were calculated in Matlab using ibool59, now part of the
OCTAVE statistics-bootstrap package (https://github.com/ octave.github.io/packages-
statistic/bootstrap). Central coverage of bias corrected and accelerated (BCa)
intervals was calibrated to reduce small sample bias using a double bootstrap
procedure with 20,000 and 200 resamples for the first and second bootstrap
eventually.

Structural modelling. Structural modelling was performed on GluN2A in
a pseudo full-length human model of a triheteromeric NMDA receptor. The
coordinates of the triheteromeric structure of Xenopus laevis GluN1/2A/2B (4.5 Å, Suow)
were downloaded from the Orientations of Proteins in Membranes (OPM)
database60. GalaxyFill was used to fill in missing main chain and sidechain atoms and
loops of each protomer89. The protomers then the reassembled and steric
clashes removed by brief energy minimisation using steepest decent. This struc-
ture had a root mean squared deviation of CA atoms (CA-RMSD) of 0.08 Å (from
Suow) and was used as scaffold for modelling the ligand-bound human tri-
eteromeric receptor in Modeller (v9.19)90 using multiple high-resolution templates:
4nf8, 5fxh, 5fxi, 5i57, 5tpw, 5tpz and 5un1. The

code availability

Code used for analysis and modelling is deposited at the following repositories:
https://github.com/acp29/penn , https://github.com/acp29/Elmasri_GRIN2A , and
https://github.com/acp29/pymage/tree/master/Penn.

Received: 3 September 2020; Accepted: 31 January 2022; Published online: 28 February 2022

Resided: 3 September 2020; Accepted: 31 January 2022; Published online: 28 February 2022

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The authors gratefully acknowledge the following kind gifts: pCMV Cre-GFP from Kasper Hansen, Homer1c-tdTomato from Daniel Choquet (IINS, Bordeaux, France). The PhD studentship of M.E. was funded on a University of Sussex Life Sciences School studentship. A.C.P. was funded on MRC Career Development Award (MR/M02746/1), in which W.A. and M.E. were funded as post-docs.

**Author contributions**

M.E. performed cloning and electrophysiology experiments and analysis related to Figs. 2, 4 and 5, and S3, 4. D.M.V.D, D.W.H. and E.E.B. performed imaging experiments and analysis related to Fig. 3 and S2. G.W. and E.E.B. performed simulations related to Fig. 6. W.A. performed some electrophysiology experiments related to Fig. 5. E.K. performed cloning experiments related to Figs 2, 3 and 4. K.S. provided all genetically-altered mice. A.C.P. obtained funding, designed the experiments, analysed data related to all figures, performed molecular modelling and analysis related to Fig. 1 and S5, performed all statistical analysis and wrote the paper. Design of experiments was discussed with all authors.

**Competing interests**

The authors declare no competing interests.

**Additional information**

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s42003-022-03115-3.

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**Peer review information** Communications Biology thanks the anonymous reviewers for their contribution to the peer review of this work. Primary Handling Editors: Eliana Scemes, Karli Montague-Cardoso and Brooke LaFlamme. Peer reviewer reports are available.

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