Effects of GluN2A and GluN2B gain-of-function epilepsy mutations on synaptic currents mediated by diheteromeric and triheteromeric NMDA receptors

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ABSTRACT

Mutations in synaptic NMDA receptors (NMDARs) are associated with epilepsy and neurodevelopmental disorders. The effects of several such mutations have been investigated in recombinantly-expressed NMDARs under conditions of steady-state activation. Such experiments provide only limited insight into how mutations affect NMDAR-mediated excitatory synaptic currents (EPSCs). The present study aimed to characterize the effects of the GluN2AN615K, GluN2BN615I and GluN2BV618G gain-of-function mutations on EPSCs mediated by diheteromeric GluN1/2A and GluN1/2B receptors and triheteromeric GluN1/2A/2B receptors, as these are the most abundant synaptic NMDARs in vivo. Subunit composition was controlled by studying ‘artificial’ synapses formed between cultured neurons (which provide presynaptic terminals) and HEK293 cells that express the NMDAR subunits of interest plus the synapse-promoting molecule, neuroligin-1B. When incorporated into diheteromeric receptors, all three mutations ablated voltage-dependent Mg2+ block of EPSCs, as previously shown. In addition, we were surprised to find that increasing external Mg2+ from 0 to 1 mM strongly enhanced the magnitude of EPSCs mediated by mutant diheteromers. In contrast, triheteromeric receptors exhibited normal voltage-dependent Mg2+ block. The GluN2AN615K mutation also slowed the decay of GluN1/2A/2B- but not GluN1/2A-mediated EPSCs. The GluN2BN615I mutation enhanced the magnitude of both GluN1/2B- and GluN1/2A/2B-mediated EPSCs. The GluN2BV618G mutation enhanced the magnitude of both GluN1/2B- and GluN1/2A/2B-mediated EPSCs, although these effects were partly compensated by a faster EPSC decay rate. The mutations also diminished the potency of the anti-epileptic pore-blocker, memantine, thus explaining the lack of memantine efficacy in patients with GluN2BN615I or GluN2BV618G mutations. Given these effects, the three mutations would be expected to enhance the cation influx rate and thereby contribute to epilepsy phenotypes.

1. Introduction

Epilepsy is a spectrum of neurological disorders that involves recurrent seizures (Fisher et al., 2014). Around 30% of patients are refractory to pharmacological treatments (Luciano and Shorvon, 2007; Regesta and Tanganelli, 1999) partly because the underlying causes of many epilepsies are unknown (Berg et al., 2010; Merwick et al., 2012). With the development of next-generation DNA sequencing, the rate of identification epilepsy susceptibility genes has increased considerably (Merwick et al., 2012). Many of these genes encode synaptic proteins, including excitatory N-methyl-D-aspartic acid receptor (NMDAR) subunits (Endele et al., 2010; Lemke et al., 2013; Lesca et al., 2013). Individuals harboring mutations in NMDAR subunits present with epilepsies ranging in severity from benign focal epilepsy to epileptic encephalopathies, with the latter often associated with impaired speech development, intellectual disability, autism and psychiatric disorders (Endele et al., 2010; Lemke et al., 2013; Lesca et al., 2013; Kenny et al., 2014; Lesca et al., 2012). It is essential to resolve the mechanisms by which these mutations affect NMDAR-mediated excitatory postsynaptic currents (EPSCs) to both understand epileptogenic mechanisms and...
design optimal therapies for particular epilepsy genotypes.

NMDARs are glutamate-gated cation channels that mediate excitatory neurotransmission in the brain. Seven NMDAR subunits have been identified: GluN1, GluN2A-D, GluN3-A-B. NMDARs are tetrameric oligomers that typically comprise two GluN1 subunits with either two GluN2 subunits or a mixture of GluN2 and GluN3 subunits. The most prevalent stoichiometries in native synapses are the diheteromeric GluN1-GluN2A (GluN1/2A) and GluN1-GluN2B (GluN1/2B) isoforms and the triheteromeric GluN1-GluN2A-GluN2B (GluN1/2A/2B) isoform (Traynelis et al., 2010). These isoforms exhibit distinct spatial and temporal expression patterns, kinetics and pharmacological profiles. For example, GluN1/2B-containing receptors predominate extrasynaptically in immature neurons where they exhibit longer channel open times and high sensitivity to the inhibitor, ifenprodil (Traynelis et al., 2010). By adulthood these receptors have largely been replaced by GluN1/2A-containing receptors that are insensitive to ifenprodil and mediate fast-decaying EPSCs.

A variety of mutations in the GRIN2A and GRIN2B genes have been linked to epilepsy (Endele et al., 2010; Lemke et al., 2013; Lesca et al., 2013; Lesca et al., 2012; Carvill et al., 2013; Reutlinger et al., 2010). Although many mutations are loss-of-function or uncharacterised, three mutations in the pore-lining M2 domain have been characterized as gain-of-function. These include N615K in GRIN2A and N615I and V615I in GRIN2B. All three mutations lie adjacent to the pore Mg2+ binding site (Wollmuth et al., 1998) where they have been shown to reduce voltage-sensitive Mg2+ block (Lemke et al., 2014; Marwick et al., 2013; Carvill et al., 2013; Reutlinger et al., 2010). Here we sought to understand how the GluN2A N615K, GluN2B N615I and GluN2B V615I mutations affect the physiological and pharmacological properties of NMDAR-mediated EPSCs. Although expressing the relevant NMDAR subunits in recombinant expression systems permit the study of defined NMDAR isoforms, it is difficult to replicate the fast glutamate dynamics that exist in the synapse. Hence, to date re-combinantly-expressed NMDARs have only been investigated under steady-state glutamate activation conditions (Lemke et al., 2014; Marwick et al., 2015; Fedele et al., 2018; Mullier et al., 2017; Vyklicky et al., 2018). An alternate method is to overexpress GluN2B N615I and GluN2B V615I mutant subunits in neurons and characterize changes in NMDAR-mediated EPSCs (Fedele et al., 2018). The problem with this approach is that the stoichiometries of the investigated synaptic NMDARs may be variable and cannot be ascertained. We circumvented both problems by using an engineered ‘artificial’ synapse preparation (Biederer and Scheiffele, 2007; Zhang et al., 2015). This technique involves co-culturing cortical neurons with HEK293 cells that re-combinantly express the NMDA subunits of interest plus neurologin-1B, a synapse-promoting molecule. The cortical neurons efficiently form presynaptic contacts onto the HEK293 cells. EPSCs mediated by defined NMDAR isoforms can then be recorded from HEK293 cells. Here we use this approach to evaluate the effects of the three mutations on EPSCs mediated by NMDARs formed by diheteromeric and triheteromeric combinations of GluN1, GluN2A and GluN2B subunits.

2. Methods

2.1. Primary neuronal cultures

Cortical neurons were harvested from Wistar rat embryos of both sexes at embryonic day 18 (University of Queensland, Institutional Breeding Colony). Euthanasia of timed-pregnant rats was performed via CO2 inhalation. All experiments were performed in accordance with relevant guidelines and regulations as approved by the University of Queensland Animal Ethics Committee (approval number: QBI/142/16/NHMRC/ARC).

2.2. HEK293 cell culture

HEK293 cells were obtained from ATCC (#CRL-1573). Cells were cultured at 37 °C with 5% CO2 in Dulbecco’s modified Eagles medium (DMEM) with 10% fetal bovine serum. Cells were transiently transfected via a calcium phosphate precipitation protocol. Cells were passaged once or twice a week and were used until the 30th passage.

2.3. cDNA site-directed mutagenesis, cell culture and transfection

The human NMDAR GluN1 (isoform GluN1-4b), GluN2A and GluN2B cDNAs were cloned into the pRK5 expression vector (with CMV promoter) with optimized Kozak sequences. Site-directed mutagenesis using the QuikChange Lightning kit (Stratagene, Agilent Technologies) was used to generate GluN2A and GluN2B mutants, which were confirmed by Sanger DNA sequencing. Methods for preparing neurons and HEK293 cells for artificial synapse recordings have previously been described in detail (Dixon et al., 2015). Briefly, HEK293 cells were transfected with cDNAs encoding human GluN1, GluN2A, GluN2B subunits (all in the pRK5 expression vector) plus empty pEGFP and mouse neurologin 1B (Addgene #15261; in the pCAGGS expression vector) in a ratio of 1:1:0.5:1, using a calcium-phosphate co-precipitation protocol. Euthanasia of timed-pregnant rats was performed via CO2 inhalation. The cerebral cortices of e18 rat embryos were dissected out, triturated and plated on poly-l-lysine coated coverslips at a density of ~100 × 103 cells per coverslip. The cells were plated into DMEM with 10% fetal bovine serum and this was replaced after 24 h with Neurobasal medium, including 2% B27 and 1% glutamax. After one week, half of this medium was replaced with fresh medium. Neurons were allowed to grow for 3–4 wk. before freshly transfected HEK293 cells were plated onto the neurons. Artificial synaptic connections typically formed spontaneously within 24 h and EPSCs in HEK293 cells were recorded by whole-cell patch clamp between 2 and 5 d later. Spontaneous EPSCs were recorded from transfected HEK293 cells identified by their strong green fluorescence. The voltage-step experiments described in Fig. 1 were performed on isolated HEK293 cells that were not in co-culture with neurons.

2.4. Electrophysiology

All electrophysiological experiments were performed at room temperature (22 ± 2 °C). Artificial synaptic recordings were performed in the whole-cell patch clamp recording configuration at a holding potential of −70 mV unless otherwise stated. Patch pipettes were fabricated from borosilicate hematoctrit tubing (Harvard Apparatus, Germany) and had tip resistances of 2–5 MΩ when filled with the intracellular solution which contained (in mM): 145 CsCl, 2 CaCl2, 2 MgCl2, 10 HEPS, and 10 EGTA, adjusted to pH 7.4 with CsOH. Cells were perfused with extracellular solution, which contained (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 10 HEPS and 10 d-glucose, adjusted to pH 7.4 with NaOH. Synaptic currents were filtered (-3 dB, 4-pole Bessel) at 4 kHz and sampled at 10 kHz and recorded using a Multiclamp 700B amplifier and pClamp 10 software (Molecular Devices, Sunnyvale, CA, USA). Recordings with series resistance above 20 MΩ were discarded. Capacitance of the HEK293 cells was typically 20 pF, resulting in a typical corner frequency of 398 Hz. Because this was satisfactory for our experiments, series resistance compensation was not applied. Where drugs were applied, the events from the wash phase were averaged with the pre-drug period to control for any rundown or run-up. Ifenprodil (+)-tartrate salt was dissolved in water as a 10 mM stock solution. Memantine hydrochloride dissolved in dimethylsulfoxide as a 100 mM stock solution. All the chemicals employed in the study were obtained from Sigma-Aldrich. Sliding templates in Axograph X were used to identify synaptic events. 10–90% rise times were recorded and mono-exponential simplex fits applied to the decay period of individual events, which were then averaged for each.
cell.

Conventional whole-cell recordings of glutamate- and glycine-gated currents in transfected HEK293 cells were performed using intracellular and extracellular solutions as described in the previous paragraph. In these experiments, currents were filtered at 1 kHz and digitized at 2 kHz. Solutions containing defined concentrations of glutamate, glycine, Mg²⁺, ifenprodil and memantine were applied to cells via gravity induced perfusion through parallel microtubules. Whole-cell current-voltage (I-V) experiments were performed by holding the membrane potential at 0 mV, then stepping the voltage from −100 to +100 mV for 500 ms in 20 mV increments. Leak currents recorded in the absence of both glutamate and glycine were digitally subtracted to produce the displayed recordings.

Fig. 1. Effects of Mg²⁺ on I-V relationships of whole cell currents mediated by WT and mutant diheteromeric receptors in HEK293 cells. In all experiments, currents were activated by 300 μM glutamate plus 100 μM glycine. The ‘0 Mg²⁺’ solution was nominally Mg²⁺-free. A. Left panel: I-V relationships of glutamate-gated currents mediated by GluN1/2A receptors recorded in the absence and presence of 1 mM Mg²⁺ in the same cell. The holding voltage was −70 mV. Cells were initially stepped to 0 mV and then to voltages from −100 to +100 mV for 500 ms in 20 mV increments. Leak currents recorded in the absence of glutamate and glycine were digitally subtracted to produce the displayed recordings. Right panel: averaged, leak-subtracted I-V relationships in the absence and presence of 1 mM Mg²⁺. The 0 Mg²⁺ and 1 Mg²⁺ I-Vs were recorded from the same 6 cells and all data points are normalized to the +40 mV data point in 0 Mg²⁺. In this and all subsequent panels, error bars are shown when larger than symbol size. B-E. As for A but with currents recorded from HEK293 cells expressing the diheteromeric receptors as indicated. All data points were averaged from at least 6 cells.
produce the resultant I-V relationship. All currents were normalized to the +40 mV current in 0 Mg\(^{2+}\) recorded from the same cell.

2.5. Statistical analysis

Analyses of IPSC amplitude, 10–90% rise time, and decay time constant (single-exponential) were performed using Axograph X (Axograph Scientific). Only cells with a stable series resistance of < 25 MΩ throughout the recording period were included in the analysis. Single peak IPSCs with amplitudes of at least three times above the background noise were detected using a semiautomated sliding template. Each detected event was visually inspected and only well-separated IPSCs with no inferences in the rising or decay phases were included. All selected events from a single cell were digitally averaged. Parameters derived from these digitally averaged waveforms were then pooled with those from other cells to obtain group data. These averages from multiple recording days were then pooled to obtain group data. Statistical analysis and plotting were performed with Prism 7 (GraphPad Software). All data are presented as mean ± SEM. Data sets were first tested for normal distribution prior to using one-way ANOVA (followed by Bonferroni post-hoc test) or paired t-test as indicated below to determine statistical significance. In all experimental analyses, \(p < .05\) was taken as the significance threshold, with ** and *** representing significance levels of \(p \leq .01\) and \(p \leq .001\), respectively. The tests were conducted with SigmaPlot 14.0 software. All data are presented as mean ± SEM, with n values (representing the total number of cells or patches) presented within the bars of bar plots. Graphs were rendered in Sigmaplot 14.0 and figures were assembled using Adobe Illustrator.

3. Results

3.1. Effects of mutations on the Mg\(^{2+}\) sensitivity of NMDARs

GluN2A\(^{N615K}\), GluN2B\(^{N615S}\) and GluN2B\(^{V618G}\) mutations lie close to the pore-lining Mg\(^{2+}\) binding site and previous studies have demonstrated that they reduce voltage-dependent Mg\(^{2+}\) block in diheteromeric NMDAR isoforms (Lemke et al., 2014; Marwick et al., 2015; Fedele et al., 2018). We sought to repeat these experiments: to 1) quantify Mg\(^{2+}\) potentiation by recording current-voltage (I-V) relations in both the absence and presence of Mg\(^{2+}\) in the same cell, and 2) to ensure the ionic conditions of these experiments were identical to the synaptic current experiments described below. Thus, we analysed Mg\(^{2+}\) block and potentiation on the two wild-type (WT) and three mutant diheteromeric NMDAR isoforms. Currents were activated by applying saturating concentrations of glutamate (300 μM) and glycine (100 μM) to HEK293 cells expressing GluN1/2A, GluN1/2A\(^{N615K}\), GluN1/2B, GluN1/2B\(^{N615S}\) and GluN1/2B\(^{V618G}\) receptors, in both 0 and 1 mM Mg\(^{2+}\). Note that the '0 Mg\(^{2+}\)' solution was nominally Mg\(^{2+}\)-free (i.e., it contained no divalent cation chelator). Fig. 1A-E shows sample currents in response to voltage-step experiments recorded sequentially in 1 and 0 mM extracellular Mg\(^{2+}\) from the same cell. Averaged I-V relationships, measured from \(n \geq 7\) cells each, are presented in the right-hand panels of Figs. 1A-E. All data points recorded from a given cell have been normalized to the +40 mV value recorded in 0 Mg\(^{2+}\) in that cell.

As expected, GluN1/2A receptors exhibited voltage-dependent Mg\(^{2+}\) block at negative potentials that was abolished in 0 Mg\(^{2+}\) (Fig. 1A). In addition, 1 mM Mg\(^{2+}\) also potentiated the saturating current. This effect was evident at positive potentials where it was not confounded by voltage-dependent Mg\(^{2+}\) block. This potentiating action of Mg\(^{2+}\) has previously been described (Paolelli et al., 1995; Wang and MacDonald, 1995). By contrast, GluN1/2A\(^{N615K}\) receptors exhibited a linear I-V relationship in 0 Mg\(^{2+}\), which was enhanced in a voltage-independent manner by 1 mM Mg\(^{2+}\) (Fig. 1B). Thus, the N615K mutation ablates voltage-dependent Mg\(^{2+}\) block but not the potentiating effect of Mg\(^{2+}\). This lack of voltage-dependence implies a discrete positive modulatory Mg\(^{2+}\) site lying outside the membrane electrical field. The linearized I-V was not surprising; it has been previously shown that mutations to N615 in the GluN2A subunit pore-lining domain eliminate voltage-dependent Mg\(^{2+}\) block (Wollmuth et al., 1998).

GluN1/2B receptors also exhibited the expected voltage-dependent Mg\(^{2+}\) block at negative potentials (Fig. 1C). However, 1 mM Mg\(^{2+}\) did not elicit the potentiating effect that was observed with GluN1/2A receptors (compare Fig. 1A and C). The GluN1/2B\(^{N615S}\) and GluN1/2B\(^{V618G}\) receptors also exhibited near linear I-V relationships in 0 Mg\(^{2+}\), which were both enhanced in a voltage-independent manner by 1 mM Mg\(^{2+}\) (Fig. 1D, E). This potentiating effect of extracellular Mg\(^{2+}\) on mutant GluN1/2B receptors is surprising given it was not observed in the corresponding WT receptor (Fig. 1C). Thus, we conclude that the three epilepsy mutations abolish voltage-dependent Mg\(^{2+}\) block but also reveal a voltage-independent potentiating effect of Mg\(^{2+}\) that is particularly evident at positive potentials.

3.2. Properties of EPSCs mediated by WT NMDARs

We next sought to determine the physiological and pharmacological properties of spontaneous EPSCs mediated by WT diheteromeric GluN1/2A and GluN1/2B NMDARs and WT triheteromeric GluN1/2A/2B NMDARs in artificial synapses. We sought to form triheteromers by expressing all three subunits simultaneously. Assuming they recombined randomly, binomial theory predicts a receptor distribution of 25% GluN1/2A, 25% GluN1/2B and 50% GluN1/2A/2B. However, there is now abundant evidence for the preferential formation of triheteromeric NMDARs in neurons and HEK293 cells (Rauner and Kohr, 2011; Tovar et al., 2013; Bhattacharyya et al., 2018). For the purpose of brevity, in Results we describe the receptors formed following the transfection of all three subunits as 'presumptive triheteromers' and consider the evidence for triheteromer formation in the Discussion. Sample recordings of spontaneous EPSCs mediated by each receptor isoform, revealing Mg\(^{2+}\) block at −70 mV, are presented in Fig. 2A-C. From each cell we collected and digitally averaged all well-isolated EPSCs recorded in the absence and presence of 1 mM Mg\(^{2+}\), separately. These averaged currents were normalized to peak amplitude and overlaid (Fig. 2A-C, right panels). The results suggest that the EPSCs mediated by each isoform were not greatly affected by changes in external Mg\(^{2+}\) concentration. Fig. 2D displays the same six waveforms grouped according whether they were recorded in 0 or 1 mM Mg\(^{2+}\). The mean amplitudes, 10–90% rise times and decay time constants of EPSCs derived from the analysis of these digitally averaged waveforms in these and all mutant constructs are summarized in Table S1 and presented as bar plots in subsequent figures. The respective mean amplitudes of EPSCs recorded from cells expressing GluN1/2A, GluN1/2B and presumptive triheteromeric GluN1/2A/2B receptors in the absence and presence of 1 mM Mg\(^{2+}\) are displayed in Fig. 2E, confirming strong block by Mg\(^{2+}\) at −70 mV in each case. Mean rise times of EPSCs mediated by GluN1/2B and presumptive GluN1/2B/2A receptors were found to be significantly slower than those mediated by GluN1/2A receptors (Fig. 2F), but were not significantly Mg\(^{2+}\)-dependent. The EPSC decay time constants were not significantly Mg\(^{2+}\)-dependent for GluN1/2A and GluN1/2B receptors although 1 mM Mg\(^{2+}\) induced a significant slowing in the EPSC decay rate for presumptive GluN1/2A/2B receptors (Fig. 2G). Furthermore, as suggested by the sample traces in Fig. 2D, the mean decay time constant of EPSCs mediated by presumptive GluN1/2A/2B receptors (89 ± 8 ms in 0 Mg\(^{2+}\)) was intermediate between those mediated by GluN1/2A receptors (43 ± 5 ms in 0 Mg\(^{2+}\)) and GluN1/2B receptors (204 ± 11 ms in 0 Mg\(^{2+}\)) (Fig. 2G). The decay rates for GluN1/2A and GluN1/2B receptors correspond well with those reported in a previous artificial synapse study (50 and 340 ms, respectively) (Fu et al., 2003). These results are also in close agreement with data from native hippocampal neurons. For example, Rauner and Kohr reported that EPSCs mediated by GluN2A-
GluN2B-containing receptors decayed with time constants of 34 and 306 ms, respectively (Rauner and Kohr, 2011), whereas Tovar et al. observed respective decay time constants of 23 and 315 ms (Tovar et al., 2013). The mean decay time constants of EPSCs mediated by triheteromeric GluN1/2A/2B receptors have previously been found to lie between those mediated by GluN1/2A and GluN1/2B receptors in hippocampal neurons (Rauner and Kohr, 2011; Tovar et al., 2013). A similar observation applies to the rank order of deactivation rates of the three receptors when expressed in recombinant expression systems (Stroebel et al., 2014; Hansen et al., 2014). However, as with previous studies investigating the properties of triheteromeric NMDARs (Rauner and Kohr, 2011; Tovar et al., 2013; Stroebel et al., 2014; Hansen et al., 2014; Bhattacharya et al., 2018), we sought to strengthen the case for the existence of triheteromeric GluN1/2A/2B receptors via a pharmacological analysis. For this purpose we employed ifenprodil, an inhibitor that is specific for GluN1/2B over GluN1/2A and GluN1/2A/2B receptors (Stroebel et al., 2014; Hansen et al., 2014).

As a control, we first applied 1 μM ifenprodil to EPSCs mediated by WT GluN1/2B NMDARs and observed that it reduced their mean magnitude to 57 ± 6% of control (n = 10, p < .001 by paired t-test; Fig. 2H). In contrast, EPSCs mediated by cells transfected simultaneously with GluN1, GluN2A and GluN2B subunits were not significantly affected by 1 μM ifenprodil (Fig. 2I). The mean effects of 1 μM ifenprodil, normalized to the pre-ifenprodil control values from the same cell, are presented in Fig. 2J and confirm that the amplitude of EPSCs mediated by GluN1/2B diheteromers were significantly inhibited by ifenprodil whereas those mediated by presumptive GluN1/2A/2B triheteromers were not affected (Fig. 2J, left panel). However,
ifenprodil had no significant effects on EPSC rise or decay times at either receptor isoform (Fig. 2J, middle and right panels). The lack of effect of ifenprodil on presumptive GluN1/2A/2B triheteromers is consistent with previous studies showing that it exerted only a weak inhibitory effect on tonically-activated recombinant GluN1/2A/2B receptors (Stroebel et al., 2014; Hansen et al., 2014). Thus, two lines of evidence (i.e., the intermediate decay rate of EPSCs and the Mg$^{2+}$-sensitivity of EPSC decay) are consistent with the formation of a distinct population of triheteromeric GluN1/2A/2B receptors. However, our data do not allow us to disprove the null hypothesis, which predicts 25% GluN1/2A, 25% GluN1/2B and 50% GluN1/2A/2B receptors.

### 3.3. Properties of EPSCs mediated by NMDARs containing the GluN2A$^{N615K}$ mutation

A similar set of experiments was carried out on cells transfected with the GluN1, GluN2B and mutant GluN2A$^{N615K}$ subunits in diheteromeric and triheteromeric combinations. A sample recording of
EPSCs from cells expressing diheteromeric GluN1/2A615K receptors is shown in Fig. 3A. A surprising result is that these EPSCs exhibited a reverse Mg²⁺ sensitivity compared to WT receptors. The averaged data confirmed that 1 mM Mg²⁺ strongly enhanced rather than reduced EPSC peak amplitudes (Fig. 3B). We next compared the amplitudes, rise times and decay time constants of EPSCs mediated by diheteromeric GluN1/2A615K receptors with those mediated by WT GluN1/2A receptors. In 1 mM Mg²⁺, the GluN1/2A615K receptor mediated EPSCs with significantly larger peak amplitudes than to the corresponding WT receptor (Fig. 3C, left panel), as expected given its reverse Mg²⁺ sensitivity. The EPSC rise times in the presence of 1 mM Mg²⁺ were significantly slower for GluN1/2A615K versus GluN1/2A receptors, but no difference was observed in the EPSC decay rates (Fig. 3C, middle and right panels).

We next investigated the effects of Mg²⁺ and ifenprodil on EPSCs mediated by receptors formed following the transfection of GluN1, GluN2A615K, and GluN2B subunits. A sample recording of EPSCs mediated by presumptive GluN1/2A615K/2B receptors (Fig. 3D) reveals strong block by 1 mM Mg²⁺. Fig. 3E shows an overlay of normalized, digitally averaged EPSCs from presumptive GluN1/2A615K/2B and GluN1/2A615K/2B receptors in both 0 and 1 mM Mg²⁺, suggesting strong Mg²⁺-dependence of the mutant receptor EPSC decay rate. Mean data from many averaged traces are presented in Fig. 3F and G. Fig. 3F provides a comparison of EPSC amplitudes, rise times and decay rates for presumptive GluN1/2A/2B and GluN1/2A615K/2B receptors in 0 mM Mg²⁺. The amplitudes and rise times were not significantly different, although the decay rates of presumptive GluN1/2A615K/2B receptors were dramatically slower in 1 mM Mg²⁺ (see also Fig. 3E). In Fig. 3G we demonstrate that 1 mM Mg²⁺ inhibited the amplitudes and accelerated the decay rates of EPSCs mediated by the two receptors to a similar extent. A full comparison of EPSC amplitudes, rise times and decay time constants for all WT and mutant triheteromeric receptors in 0 and 1 mM Mg²⁺ is provided in Fig. 5I.

Finally, we observed no detectable ifenprodil block in cells transfected with GluN1, GluN2A615K and GluN2B subunits (Fig. 3H). The pronounced inhibitory effect of Mg²⁺ on the receptor population thus formed indicates the virtual complete absence of diheteromeric GluN1/2A615K receptors. It is notable that 1 mM Mg²⁺ caused a significant acceleration of the decay rate of EPSCs mediated by WT- and GluN2A615K-containing triheteromeric receptors (Fig. 3G, middle panel), because this effect is not observed at EPSCs mediated by either GluN1/2A or GluN1/2B diheteromeric receptors (Fig. 2). Together, these observations provide strong evidence for the predominant expression of the triheteromeric GluN1/2A615K/2B receptor in artificial synapses following transfection with the three component subunits.

### 3.4. Properties of EPSCs mediated by NMDARs containing the GluN2B8615I and GluN2B8618G mutations

We next investigated EPSCs mediated by NMDARs containing the GluN2B8615I and GluN2B8618G mutations in diheteromeric and triheteromeric subunit combinations. Sample recordings of EPSCs from cells expressing diheteromeric GluN1/2B8615I (Fig. 4A) and GluN1/2B8618G (Fig. 4B) receptors suggest that these GluN2B mutations also reverse the sensitivity to extracellular Mg²⁺ when compared to the WT GluN1/2B receptors. The averaged results confirm that 1 mM Mg²⁺ strongly increased the mean EPSC current amplitude in both mutant NMDARs (Fig. 4C). We next compared the amplitudes, rise and decay times of EPSCs mediated by the GluN1/2B8615I and GluN1/2B8618G receptors with those mediated by WT GluN1/2B receptors. In 1 mM Mg²⁺, both diheteromeric receptors mediated EPSCs with significantly larger amplitudes relative to WT diheteromers (Fig. 4D), as expected due to their reverse Mg²⁺ sensitivity. The EPSC rise times in the presence of 1 mM Mg²⁺ were not significantly different from WT diheteromer values (Fig. 4E). However, the GluN1/2B8618G receptor mediated EPSCs with a significantly accelerated decay rate relative to those mediated by GluN1/2B and GluN1/2B8615I receptors (Fig. 4F).

Next, we analysed EPSCs recorded from HEK293 cells transfected with GluN1, GluN2A and either GluN2B8615I or GluN2B8618G subunits. Sample artificial synapse recordings suggest strong block by 1 mM Mg²⁺ of presumptive GluN1/2A/2B8615I receptors but no apparent block of presumptive GluN1/2A/2B8618G receptors (Fig. 4G, H). This effect, quantified in Fig. 4I, confirms the Mg²⁺-resistance of presumptive GluN1/2A/2B8618G receptors. We next compared the amplitudes, rise times and decay time constants of EPSCs mediated by all three triheteromers in 0 and 1 mM Mg²⁺. Both datasets are displayed in Table S1 although only the 0 Mg²⁺ data are plotted in Fig. 4J-L. We found that EPSC decay time constants did not vary significantly among the three presumptive triheteromeric receptors in either 0 or 1 mM Mg²⁺. However, amplitudes of EPSCs in 0 Mg²⁺ were significantly larger for presumptive GluN1/2A/2B8615I receptors than for either GluN1/2A/2B or GluN1/2A/2B8618G receptors (Fig. 4J). Rise times of EPSCs in 0 Mg²⁺ were significantly slower for presumptive GluN1/2A/2B8618G receptors than for either GluN1/2A/2B or GluN1/2A/2B8615I receptors (Fig. 4K).

If HEK293 cells transfected with GluN1, GluN2A and GluN2B8615I subunits formed predominantly diheteromeric GluN1/2A and GluN1/2B8615I NMDARs then we would expect little net effect of 1 mM Mg²⁺, given that the mean amplitudes of EPSCs mediated by these receptors should be inhibited and enhanced by 1 mM Mg²⁺, respectively. Thus, our finding that 1 mM Mg²⁺ produced strong inhibition implies the dominant expression of triheteromeric GluN1/2A/2B8615I and/or diheteromeric GluN1/2A receptors. However, memantine block data (see below) is consistent only with the dominant expression of GluN1/2A/2B8615I receptors.

The Mg²⁺-resistance of EPSCs in cells transfected with GluN1, GluN2A and GluN2B8618G subunits also implies a high expression level of triheteromeric GluN1/2A/2B8618G receptors because the otherwise substantial expression of GluN1/2A receptors should have resulted in detectable Mg²⁺ block.

### 3.5. Effects of memantine on EPSCs mediated by WT and mutant NMDARs

Memantine exhibits anticonvulsant properties in animal models of epilepsy (Ghasemi and Schachter, 2011), and is employed as an adjunct therapy in some human forms of epilepsy, notably those caused by gain-of-function NMDAR mutations (Li et al., 2016; Pierson et al., 2014). Memantine has been shown to bind in the NMDAR channel pore where it interacts directly with GluN1N616, with H-bonding between GluN1 and GluN2B615 stabilising memantine coordination (Fedele et al., 2018; Limapichat et al., 2013). It has also recently been demonstrated that the GluN2B61615I mutation reduces, whereas GluN2B615I enhances memantine block of steady-state whole-cell currents activated by saturating glutamate and glycine concentrations in Mg²⁺-free solution (Fedele et al., 2018). Here we sought to investigate the inhibitory potency of 10 μM memantine on EPSCs mediated by the WT and three mutant subunits in diheteromers and triheteromers. To maximize EPSC magnitudes, we employed 0 Mg²⁺ containing external solution for WT receptors and 1 mM Mg²⁺-containing external solution for mutant receptors. Fig. 5A-E displays sample recordings of EPSCs mediated by diheteromeric NMDAR combinations suggesting strong inhibition by memantine of all isoforms except GluN1/2B8618G, consistent with an earlier study (Fedele et al., 2018). The averaged data (Fig. 5F) confirm this effect and show that the other mutant diheteromers exhibit a similar memantine sensitivity to GluN1/2A or GluN1/2B receptors.

Since the effects of memantine on mutant triheteromeric receptors have not previously been investigated, we examined the effect of 10 μM memantine on the mean amplitudes, rise times and decay time constants of EPSCs mediated by WT and three mutant triheteromeric NMDARs (containing GluN2A615K, GluN2B615I or GluN2B8618G; Fig. 5G–H). EPSC amplitudes mediated by all four triheteromeric...
Fig. 4. Properties of EPSCs mediated by dimeric and trimolecular receptors incorporating GluN2B<sup>N615I</sup> or GluN2B<sup>V618G</sup> mutant subunits. A, B. Sample recordings of spontaneous EPSCs recorded from HEK293 cells transfected with GluN1/2B<sup>N615I</sup> or GluN1/2B<sup>V618G</sup> subunits, respectively, in 0 and 1 mM Mg<sup>2+</sup>-containing external solutions. C. Mean amplitude of EPSCs mediated by both mutant heteromers in 0 and 1 mM Mg<sup>2+</sup>, ***p ≤ .001 by paired t-test. D-F. Comparison of mean EPSC amplitudes (D), rise times (E) and decay time constants (F) for dimeric GluN1/2B, GluN1/2B<sup>N615I</sup> and GluN1/2B<sup>V618G</sup> receptors in 1 mM Mg<sup>2+</sup>, ***p ≤ .001 by one way ANOVA between receptor types. G, H. Sample recordings of spontaneous EPSCs recorded from HEK293 cells transfected with trimolecular GluN1/2A/2B, GluN1/2A/2B<sup>N615I</sup> or GluN1/2A/2B<sup>V618G</sup> receptors, respectively, in 0 and 1 mM Mg<sup>2+</sup>-containing external solutions. I. Comparison of the effect of 1 mM Mg<sup>2+</sup> on the amplitudes of EPSCs recorded from cells expressing trimolecular GluN1/2A<sup>N615I</sup>, GluN1/2A/2B<sup>N615I</sup> or GluN1/2A/2B<sup>V618G</sup> receptors. All data were normalized to the corresponding 0 Mg<sup>2+</sup> control values in the same cell. ***p ≤ .001 by paired t-test. J-L. Comparison of mean EPSC amplitudes (J), rise times (K) and decay time constants (L) for EPSCs mediated by trimolecular GluN1/2A/2B, GluN1/2A/2B<sup>N615I</sup> and GluN1/2A/2B<sup>V618G</sup> receptors in 0 mM Mg<sup>2+</sup>, ***p ≤ .01, ***p ≤ .001 by one way ANOVA between receptor types.
receptors were significantly inhibited by memantine (Fig. 5G). However, with the exception of GluN2B<sup>V618G</sup>-containing receptors, the mean inhibitory efficacy of memantine at each triheteromeric receptor was dramatically less than for the corresponding diheteromeric receptor (Fig. S2). These experiments provide an additional line of evidence for the formation of triheteromeric GluN1/2A/2B, GluN1/2AN<sup>N615K</sup>/2B and GluN1/2A/2BN<sup>N615I</sup> receptors. In contrast, the EPSC rise times were not affected by memantine (Fig. 5H). However, the decay time constants of EPSCs mediated by GluN1/2A/2B receptors, but not by mutant receptors, were significantly accelerated by memantine (Fig. 5I).

4. Discussion

4.1. Validation of artificial synapses for the study of NMDAR epilepsy mutants

A unique advantage of the artificial synapse system is that it permits the investigation of EPSCs mediated by defined NMDAR isotypes. It has long been known (Fu et al., 2003) that the decay time constants of GluN1/2A- and GluN1/2B-mediated EPSCs in artificial synapses correspond closely to those mediated by the same isoforms in neurons (Traynelis et al., 2010; Rauner and Kohr, 2011; Tovar et al., 2013) and the results of the present study are consistent with this. The present study also provides evidence for triheteromeric GluN1/2A/2B NMDARs in artificial synapses. Similar to previous studies conducted in neurons (Traynelis et al., 2010; Rauner and Kohr, 2011; Tovar et al., 2013) or recombinant expression systems (Stroebel et al., 2014; Hansen et al., 2014), we found that the decay rate of EPSCs mediated by expression of the three subunits was intermediate between those mediated by GluN1/2A and GluN1/2B receptors. We also found that transfection of the three subunits produced EPSCs that were resistant to ifenprodil inhibition. This fits well with recent studies showing that ifenprodil exhibits only low efficacy inhibition of steady-state glutamate-gated currents mediated by recombinant GluN1/2A/2B receptors (Stroebel et al., 2014; Hansen et al., 2014). We also found that only GluN1/2A/2B receptors mediated EPSCs that decayed more slowly when Mg<sup>2+</sup> was removed (Fig. 2G), consistent with a study that reported a similar effect on EPSCs mediated by GluN1/2B receptors in neurons (Rauner and Kohr, 2011). Finally, EPSCs mediated by transfection of three subunits were less memantine-sensitive than those mediated by either the GluN1/2A or GluN1/2B diheteromers. Taken together, these results
indicate that the artificial synapse faithfully recapitulates EPSC properties as mediated by the corresponding NMDAR isoforms in neurons.

4.2. Evidence for the formation of triheteromeric receptors

There is abundant evidence for the preferential formation of triheteromeric GluN1/2A/2B receptors in HEK293 cells (Bhattacharya et al., 2018) and neuronal synapses (Rauner and Kohr, 2011; Tovar et al., 2013). Here we took as the null hypothesis a relative abundance of isoforms according to the binomial theorem: i.e., 25% GluN1/2A, 25% GluN1/2B and 50% GluN1/2A/2B. Although three lines of evidence (i.e., the intermediate decay rate of EPSCs, the Mg$^{2+}$-sensitivity of EPSC decay and the reduced memantine sensitivity) are consistent with the formation of a distinct population of WT triheteromeric GluN1/2A/2B receptors, our data do not allow us to disprove the null hypothesis that 50% of WT receptors were triheteromeric. However, the case for preferential triheteromer formation is stronger for receptors incorporating mutations. For example, expression of GluN1, GluN2A and GluN2B$^{N615K}$ subunits produced EPSCs that were strongly inhibited by Mg$^{2+}$. Since diheteromeric GluN1/2A$^{N615K}$ receptors were only observed in the presence of Mg$^{2+}$, we can rule out significant contamination by these receptors. Furthermore, GluN1/2A$^{N615K}$ dimers formed efficiently in the absence of GluN2B subunits, we infer that triheteromeric GluN1/2A$^{N615K}$/2B receptors were the major isoform formed when all three subunits were transfected. The same argument applies to receptors containing GluN2B$^{N615I}$ subunits. Finally, the Mg$^{2+}$-resistance of receptors formed following expression of GluN1, GluN2A and GluN2B$^{V618G}$ subunits also implies a high expression level of triheteromeric GluN1/2A/2B$^{N615K}$/2B receptors because the otherwise substantial expression of GluN1/2A receptors should have resulted in detectable Mg$^{2+}$ block. Thus, we conclude that the mutations uncover a high level of expression of triheteromeric NMDARs in our experiments.

4.3. Functional properties of NMDARs containing GluN2A$^{N615K}$

The GluN2A$^{N615K}$ variant is associated with early-onset epileptic encephalopathy and severe developmental delay (Endele et al., 2010). This mutation was shown to reduce Mg$^{2+}$ block of steady-state activated GluN1/2A receptors expressed in oocytes (Marwick et al., 2015), although its effect on NMDAR-mediated EPSCs has not previously been characterized. Here we demonstrated that GluN1/2A$^{N615K}$-mediated EPSCs recorded at -70 mV exhibit a novel characteristic whereby increasing Mg$^{2+}$ from 0 to 1 mM greatly enhances EPSC magnitude. This would enhance neuronal excitability under physiological conditions by increasing the net cation influx per synaptic event.

EPSCs mediated by predominantly triheteromeric GluN1/2A$^{N615K}$/2B receptors exhibited WT-like voltage-dependent Mg$^{2+}$ block at -70 mV (Fig. 3D, G), although the EPSC decay time constants were dramatically slower than those mediated by WT GluN1/2A/2B receptors (Fig. 3F). This effect will also enhance neuronal excitability by increasing the amount of cation influx during each synaptic event.

It should also be mentioned that in neurons expressing unmutated NMDARs, glutamatergic excitation is mediated at resting potentials (e.g., -70 mV) by AMPA receptors due to voltage-dependent Mg$^{2+}$ block of NMDARs. However, in neurons expressing any of the mutant NMDARs investigated here, both NMDARs and AMPARs would be activated at -70 mV, leading to longer-lasting and larger EPSPs. The increased rate of Na$^{+}$ and Ca$^{2+}$ influx would result in stronger neuronal excitation and would thus promote epileptogenesis.

A preliminary study, conducted on NMDARs activated under steady-state conditions in Xenopus oocytes, reported reduced block by 10 μM memantine of GluN1/2A$^{N615K}$ relative to GluN1/2A receptors (Marwick et al., 2015). However, we found no significant impairment in memantine block of EPSCs mediated by mutant GluN1/2A$^{N615K}$ receptors relative to their WT counterparts (Fig. 5). On the other hand, whereas 10 μM memantine significantly enhanced the EPSC decay rate of GluN1/2A/2B receptors, it had no effect on mutant GluN1/2A$^{N615K}$/2B receptors (Fig. 5I). The GluN2A$^{N615K}$ mutation thus produces a degree of memantine resistance when incorporated into triheteromeric receptors.

4.4. Functional properties of NMDARs containing GluN2B$^{V618G}$

This mutation is associated with West syndrome and severe developmental delay (Lemke et al., 2014) and was originally shown to reduce Mg$^{2+}$ block of GluN1/2B$^{N615I}$ receptors activated under steady-state conditions in Xenopus oocytes (Lemke et al., 2014). A subsequent study confirmed the loss of Mg$^{2+}$ block but also demonstrated that the mutation reduced the Ca$^{2+}$ influx rate and enhanced memantine block of steady-state activated GluN1/2B$^{N615I}$ relative to GluN1/2B receptors (Fedele et al., 2018). Here we demonstrated that GluN1/2B$^{N615I}$, mediated EPSCs also exhibit an unusual characteristic whereby increasing Mg$^{2+}$ from 0 to 1 mM greatly enhances EPSC magnitudes. Again, even allowing for a reduced Ca$^{2+}$ influx rate, this effect will increase the amount of cation influx during each synaptic event and thus increase neuronal excitability. The mutation did not affect rise or decay times of EPSCs mediated by diheteromeric receptors. EPSCs mediated by predominantly triheteromeric GluN1/2A$^{N615K}$/2B$^{V618G}$ receptors exhibited WT-like voltage-dependent Mg$^{2+}$ block at -70 mV (Fig. 4G, I). In addition, the peak magnitudes of EPSCs mediated by GluN1/2A/2B$^{N615I}$ receptors were significantly larger than those mediated by GluN1/2A/2B receptors in 1 mM Mg$^{2+}$ (Fig. 4J). This will also enhance neuronal excitability by increasing the amount of cation influx during each synaptic event. Despite a previous study reporting enhanced block by 10 μM memantine of steady-state glutamate-activated currents in GluN1/2B$^{N615I}$ receptors (Fedele et al., 2018), we found no significant change in memantine block of EPSCs mediated by synaptic mutant GluN1/2B$^{V618G}$ receptors relative to WT diheteromers (Fig. 5). On the other hand, whereas 10 μM memantine significantly enhanced the EPSC decay rate of GluN1/2A/2B receptors, it had no effect on mutant GluN1/2A/2B$^{V618G}$ receptors (Fig. 5I). Thus, when incorporated into triheteromeric NMDARs, the GluN2B$^{N615I}$ mutation enhances receptor insensitivity to memantine.

In contrast to our results, when the GluN2A$^{N615K}$ mutant subunit was overexpressed in hippocampal neurons, the resulting EPSCs exhibited an accelerated decay rate, a modestly reduced sensitivity to block by 1.2 mM Mg$^{2+}$, and an unchanged memantine sensitivity (Fedele et al., 2018). One possible explanation is that the EPSCs were largely mediated by NMDAR isoforms not investigated here.

4.5. Functional properties of NMDARs containing GluN2B$^{V618G}$

This mutation also results in West syndrome and severe developmental delay (Lemke et al., 2014) and eliminates Mg$^{2+}$ block of steady-state activated GluN1/2B$^{V618G}$ receptors (Lemke et al., 2014; Fedele et al., 2018). Although GluN2B$^{V618G}$ has little effect on the Ca$^{2+}$ influx rate, it does render steady-state activated GluN1/2B receptors highly permeant to Mg$^{2+}$ while dramatically impairing memantine block (Fedele et al., 2018). Here, we demonstrated that GluN1/2B$^{V618G}$, mediated EPSCs at -70 mV are also increased in amplitude by increasing Mg$^{2+}$ from 0 to 1 mM. EPSCs mediated by GluN1/2B$^{V618G}$ receptors also exhibited dramatically faster decay rates than those mediated by GluN1/2B receptors. As with the other mutations investigated here, EPSCs mediated by predominantly triheteromeric GluN1/2A/2B$^{V618G}$ receptors exhibited WT-like voltage-dependent Mg$^{2+}$ block at -70 mV (Fig. 4G, I), although their respective EPSC decay time constants were not significantly different (Fig. 4L). This will enhance neuronal excitability by increasing the amount of cation influx during each synaptic event. Lastly, we found that memantine did not significantly block EPSCs mediated by GluN1/2B$^{V618G}$ receptors (Fig. 5F). Whilst 10 μM memantine significantly enhanced the EPSC decay rate of GluN1/2A/2B receptors, it had no effect on mutant...
GluN1/2A/2B V618G receptors (Fig. 5I). The GluN2B V618G mutation therefore results in a relatively high degree of memantine resistance. When overexpressed in hippocampal neurons, the GluN2A V618G mutant subunit resulted in EPSCs that exhibited no change in the decay rate, a modestly reduced sensitivity to block by 1.2 mM Mg²⁺, and an unchanged memantine sensitivity (Fedele et al., 2018). These results would be expected if the dominant synaptic subtype were the triheteromeric GluN1/2A/2B receptors.

5. Conclusion

This study provides a characterisation of EPSCs mediated by pure populations of GluN1/2A and GluN1/2B receptors and by a pre-dominantly triheteromeric population of receptors formed by transfection of GluN1/2A/2B subunits. The EPSC characteristics correspond well with previous studies on the same receptors expressed in neuronal synapses and thus validate artificial synapses as a model system for evaluating the effects of mutations on synaptically-activated NMDARs. When incorporated into diheteromeric receptors, each of the three mutations ablated voltage-dependent Mg²⁺ block. We further demonstrated that increasing external Mg²⁺ from 0 to 1 mM strongly enhanced EPSC magnitude, predominantly via a voltage-independent potentiating effect of Mg²⁺. The GluN2A N615I mutation resulted in a higher rate of cation influx at negative potentials by enhancing the magnitude of GluN1/2A-mediated EPSCs and slowing the decay rate of GluN1/2A/2B-mediated EPSCs. The GluN2B N615I mutation enhanced the magnitude of both GluN1/2B-mediated and GluN1/2A/2B-mediated EPSCs. The GluN2B V618G mutation also enhanced the magnitude of both GluN1/2B-mediated and GluN1/2A/2B-mediated EPSCs, although these effects were partly compensated by a faster EPSC decay rate. The mutations would thereby each result in an enhanced cation influx rate which would dramatically increase neuronal excitability and thus contribute directly to the epilepsy phenotype. Importantly, the mutations also reduced the potency of the use-dependent blocker, memantine, with the GluN2B V618G mutation having the largest deleterious effect by virtue of its complete ablation of memantine inhibitory efficacy in the GluN1/2B V618G receptor. Our data explain the lack of beneficial treatment response in patients with GluN2B N615I or GluN2B V618G mutations where memantine was added as an adjunct therapy (Platzer et al., 2017). Our findings explain the effects of three epilepsy mutations on the major synaptic NMDAR isoforms, and suggest that other subtype-selective NMDAR antagonists that bind outside the channel pore should be explored as candidates for personalized therapeutics.

Author contributions

XC, RJH, AK and JWL conceived the project and designed the experiments. RJH cloned human NMDAR subunit cDNAs, designed expression constructs and conducted mutagenesis. XC performed and analysed heterosynapse experiments, All authors interpreted data, wrote and edited the manuscript.

Declaration of Competing Interest

The authors declare no competing financial and non-financial interests.

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Appendix A. Supplementary data

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