Surface Expression, Function, and Pharmacology of Disease-Associated Mutations in the Membrane Domain of the Human GluN2B Subunit

Vojtech Vyklicky 1†, Barbora Krausova 1†, Jiri Cerny 1, Marek Ladislav 1,2, Tereza Smejkalova 1, Bohdan Kysilov 1, Miloslav Korinek 1, Sarka Danacikova 1,2, Martin Horak 1, Hana Chodounsk 3, Eva Kudova 3 and Ladislav Vyklicky *1

1 Department of Cellular Neurophysiology, Institute of Physiology of the Czech Academy of Sciences (CAS), Prague, Czechia, 2 Department of Physiology, Faculty of Science, Charles University, Prague, Czechia, 3 Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences (CAS), Prague, Czechia

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N-methyl-D-aspartate receptors (NMDARs), glutamate-gated ion channels, mediate signaling at the majority of excitatory synapses in the nervous system. Recent sequencing data for neurological and psychiatric patients have indicated numerous mutations in genes encoding for NMDAR subunits. Here, we present surface expression, functional, and pharmacological analysis of 11 de novo missense mutations of the human hGluN2B subunit (P553L; V558I; W607C; N615I; V618G; S628F; E657G; G820E; G820A; M824R; L825V) located in the pre-M1, M1, M2, M3, and M4 membrane regions. These variants were identified in patients with intellectual disability, developmental delay, epileptic symptomatology, and autism spectrum disorder. Immunofluorescence microscopy indicated that the ratio of surface-to-total NMDAR expression was reduced for hGluN1/hGluN2B(S628F) receptors and increased for hGluN1/hGluN2B(G820E) receptors. Electrophysiological recordings revealed that agonist potency was altered in hGluN1/hGluN2B(W607C; N615I; and E657G) receptors and desensitization was increased in hGluN1/hGluN2B(V558L) receptors. The probability of channel opening of hGluN1/hGluN2B (V558I; W607C; V618G; and L825V) receptors was diminished ~10-fold when compared to non-mutated receptors. Finally, the sensitivity of mutant receptors to positive allosteric modulators of the steroid origin showed that glutamate responses induced in hGluN1/hGluN2B(V558I; W607C; V618G; and G820A) receptors were potentiated by 59–96% and 406–685% when recorded in the presence of 20-oxo-pregn-5-en-3β-yl sulfate (PE-S) and androst-5-en-3β-yl hemisuccinate (AND-hSuc), respectively. Surprisingly hGluN1/hGluN2B(L825V) receptors were strongly potentiated, by 197 and 1647%, respectively, by PE-S and AND-hSuc. Synaptic-like responses induced by brief glutamate application were also potentiated and the deactivation decelerated. Further, we have used homology modeling based on the available crystal structures of GluN1/GluN2B NMDA receptor followed by molecular dynamics simulations to try to relate the functional consequences of mutations to structural changes. Overall, these data suggest that de novo missense mutations of the hGluN2B subunit located...
INTRODUCTION

The N-methyl-D-aspartate receptor (NMDAR) is a heteromeric ligand-gated ion channel permeable to Ca\(^{2+}\) that is expressed in neurons and glia. Virtually all brain and spinal cord circuits rely on excitation evoked by transient activation of NMDARs by glutamate, giving rise to NMDAR excitatory postsynaptic currents (EPSCs) to regulate physiological functions. In addition, NMDAR signaling is implicated in various forms of synaptic plasticity (Dingledine et al., 1999; Lynch, 2004; Traynelis et al., 2010; Huganir and Nicoll, 2013).

NMDAR is composed of two obligatory subunits (GluN1) and two variable ones, which can include either GluN2(A-D) or GluN3(A-B) (Traynelis et al., 2010). The GluN1 subunit is expressed throughout the central nervous system, whereas the four subtypes of GluN2(A-D) subunits have differential temporal and spatial expression patterns (Akazawa et al., 1994; Monyer et al., 1994). Expression of the GluN2B subunit (encoded by \textit{GRIN2B} gene) dominates during rapid cortical synaptogenesis in late embryogenesis and early postnatal development, and its expression level starts to decline after birth in most brain regions (Hall et al., 2007). Several lines of evidence indicate that GluN2B subunit plays an important role in brain development, circuit formation, differentiation, and synaptic plasticity (Cohen and Greenberg, 2008). The importance of this subunit is stressed by the neonatal lethality of \textit{GRIN2B} knock-out mice (Kutsuwada et al., 1996).

NMDAR dysfunction is implicated in a variety of nervous system disorders. Early studies indicated that glutamate triggers excitotoxicity (Olney, 1969). This form of neuronal death is dependent on calcium influx through NMDARs (Choi, 1987) and is implicated in neurological disorders such as neurodegenerative diseases, stroke, epilepsy, and traumatic brain injury (Parsons and Raymond, 2014).

Recent advances in high-throughput DNA sequencing have allowed examination of the prevalence of mutations in genes encoding NMDAR subunits among afflicted individuals. While the gene encoding for the GluN2B subunit is the most frequently \textit{de novo} mutated \textit{GRIN} gene in psychiatric and neurodevelopmental disorders, \textit{de novo} mutations of \textit{GRIN1} and \textit{GRIN2A} genes encoding for GluN1 and GluN2A are less frequent and, interestingly, in \textit{GRIN2C}, \textit{GRIN3A}, and \textit{GRIN3B}, only rare truncating mutations affecting both healthy individuals and patients suffering from neurodevelopmental disorders have been reported (Tarabeux et al., 2011; Soto et al., 2014).

\textit{GRIN2B} variants—\textit{de novo} nonsynonymous mutations, missense, nonsense, frame shift, or splice site mutations—were identified in individuals from patient cohorts with defined neurodevelopmental and psychiatric disorders such as intellectual disability, developmental delay, autism spectrum disorder, epileptic encephalopathy, schizophrenia, and to a lesser extent, attention deficit hyperactivity disorder, cerebral visual impairment, and Alzheimer’s disease, as has been recently reviewed (Soto et al., 2014; Burnashev and Szepetowski, 2015; Hu et al., 2016). \textit{GRIN2B} variants were found in the amino-terminal domain (ATD), ligand-binding domain (LBD), transmembrane domain (TMD), and carboxyl-terminal domain (CTD); rare \textit{de novo} mutations in the ATD and CTD, but not the LBD and TMD domains, are present in the exomes of a large control population sample (Lek et al., 2016). This implies that mutations in the LBD and TMD domains may significantly alter the NMDAR channel properties. Indeed, in line with this hypothesis are results of functional examination of disease-causing mutations in the LBD (Adams et al., 2014; Lemke et al., 2014; Swanger et al., 2016) in the case of the TMD, the connections between genetic variation and consequences for the NMDAR channel function and/or trafficking are much less understood.

In this study, our goal was to elucidate the consequences of NMDAR mutations found in individuals diagnosed with neurodevelopmental disorders, in regard to the receptor-channel function and surface expression. We investigated 11 disease-associated variants in the \textit{GRIN2B} gene located in the TMD region of the GluN2B subunit which are found as heterozygous \textit{de novo} missense mutations. We found that some diheteromeric variants of the NMDAR have markedly decreased probability of channel opening and altered receptor surface expression. In addition, the effects of two structurally different neurosteroids, 20-oxo-pregn-5-en-3\(^{β}\)-yl sulfate (PE-S; pregnenolone sulfate) and androst-5-en-3\(^{β}\)-yl hemisuccinate (AND-hSuc), were tested as a pharmacological tool to correct the impaired function of mutated NMDARs.

METHODS

Transfection and Maintenance of Cells

Human embryonic kidney (HEK293T) cells (American Type Culture Collection, ATTC No. CRL1573, Rockville, MD, USA) were cultured in Opti-MEM I (Invitrogen, Carlsbad, CA, USA) with 5% fetal bovine serum (FBS; PAN Biotech, Aidenbach, Germany) at 37\(^{°}\)C in 5% CO\(_2\). One day before transfection, cells were plated in 24-well plates at a density of 2 \times 10\(^{5}\) cells per well. The next day, the cells were transfected with expression vectors containing human wild-type (WT) NMDAR subunits hGluN1-1a (GenBank accession no. NP_015566) and hGluN2B (GenBank accession no. NP_000825) (both genes were generous gifts from Prof. S. Traynelis, Emory University School of Medicine, Atlanta, GA) (Hedegaard et al., 2012) and green

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fluorescent protein (GFP; pQBI 25, Takara, Tokyo, Japan) genes. Briefly, equal amounts (0.2 µg) of cDNAs encoding for hGluN1, hGluN2B and GFP were mixed with 0.6 µL of MATra-A Reagent (IBA, Göttingen, Germany) and added to confluent HEK293T cells cultured in 24-well plates. After trypsinization, the cells were resuspended in Opti-MEM I containing 1% FBS supplemented with 20 mM MgCl₂, 3 mM kynurenic acid, 1 mM D,L-2-amino-5-phosphonovaleric acid and 1 µM ketamine, and plated on 30 mm collagen and poly-L-lysine-coated glass coverslips. Transfected cells were revealed by GFP epifluorescence.

Site-directed mutagenesis of the gene encoding for hGluN2B subunit was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) in accordance with the instructions of the manufacturer using manually designed primers purchased from Sigma-Aldrich. DpnI-treated product of PCR reaction was transformed into ultracompetent XL10-Gold E.coli cells, positive clones were selected, and DNA plasmids were isolated with 20 mM MgCl₂ resuspended in Opti-MEM I containing 1% FBS supplemented with 10% FBS (v/v), as described previously (Kaniakova et al., 2012).

Expression Vectors

The human version of the cDNAs encoding for the full-length GluN1-1a subunit tagged with yellow fluorescent protein (YFP-hGluN1) was generated by the QuikChange II XL site-directed mutagenesis kit according to the manufacturer’s instructions, and the construct was verified by DNA sequencing.

Immunofluorescence Microscopy

COS-7 or HEK293T cells were transfected in 12-well plates with a total of 1.8 µg of cDNAs (in case of co-transfection, equal amounts of cDNAs containing YFP-hGluN1 and hGluN2B subunits were used) and 4 µL Lipofectamine™ 2000 (Thermo Fischer Scientific), as described previously (Kaniakova et al., 2012). COS-7 cells were preferred for microscopy because they attach well to glass coverslips while extensive washing procedures are being performed. HEK293T cells that attach to glass less firmly were used to confirm differences in the surface expression observed on COS-7 cells. After 24–36 h, the cells were washed in PBS, and then incubated on ice for 10 min in blocking solution containing PBS and 10% normal goat serum (v/v). After this blocking step, the cells were labeled with primary rabbit anti-GFP (Merck Millipore; Burlington, MA, USA; 1:1,000) and secondary goat anti-rabbit Alexa Fluor® 555 (Thermo Fischer Scientific; 1:1,000) antibodies. Antibodies were diluted in the blocking solution and the cells were incubated with each antibody for 30 min. The cells were then washed twice in PBS, fixed in 4% paraformaldehyde in PBS (w/v) for 20 min, and mounted using ProLong Antifade reagent (Thermo Fischer Scientific). Images were taken on an Olympus Cell⁴R fluorescence microscope, and the intensity of the surface and total fluorescence signals was analyzed using ImageJ software, as described previously (Lichnerova et al., 2014).

**Quantitative Assay of Surface and Total Expression**

COS-7 cells were prepared as described in the Immunofluorescence Microscopy section. After 38–40 h, COS-7 cells were washed with PBS, fixed for 15 min in 4% paraformaldehyde (w/v) in PBS and incubated for 1 h in PBS containing 0.2% bovine serum albumin (BSA; w/v) without (surface expression) or with (total expression) 0.1% Triton X-100 (TX-100; v/v). Cells were incubated in a primary rabbit anti-GFP antibody (Millipore, Billerica, MA; 1:500 for surface expression and 1:1,000 for total expression) diluted in PBS with 0.2% BSA for 1 h. After being washed in PBS, cells were incubated with a secondary antibody (horseradish peroxidase-conjugated donkey anti-rabbit IgG; GE Healthcare, UK; 1:1,000) for 1 h and washed in PBS. Next, 400 µl of ortho-phenylenediamine (OPD; final concentration 0.4 mg/ml) dissolved in phosphate-citrate buffer containing sodium phosphate (Sigma, St. Louis, MO) was added to cells for 30 min (surface expression) or 15 min (total expression). The color reaction was terminated with 100 µl of 3 M HCl and the optical density was determined at 492 nm using a Personal Densitometer SI (GE Healthcare, Pittsburgh, PA). The average background signal measured from cells transfected with empty vector was subtracted from data obtained from cells transfected with NMDA receptor subunits.

**Electrophysiological Recording Whole-Cell Recordings**

Experiments were performed 18–36 h after the end of transfection on cultured HEK293T cells transfected with vectors containing hGluN1/hGluN2B/GFP. Whole-cell voltage clamp current recordings were performed at room temperature (21–25°C) at a holding potential of ~60 mV using a patch-clamp amplifier (Axopatch 200B; Molecular Devices, Sunnyvale, CA, USA) after a capacitance and series resistance (<10 MΩ) compensation of 80–90%. Data were collected (low-pass filtered at 2 kHz and sampled at 10 kHz) and analyzed using pClamp 10 (Molecular Devices).

Patch pipettes (3–5 MΩ) were filled with an intracellular solution (ICS) containing (in mM): 120 gluconic acid, 15 CsCl, 10 BAPTA, 10 HEPES, 3 MgCl₂, 1 CaCl₂, and 2 ATP-Mg salt (pH-adjusted to 7.2 with CsOH) and extracellular solution (ECS) contained the following (in mM): 160 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, 0.2 EDTA, and 0.7 CaCl₂ (pH-adjusted to 7.3 with NaOH). Glycine (30 µM), an NMDAR co-agonist, was present in the control and test solutions. All steroid solutions were made from freshly prepared 20 mM 20-oxic-pregn-5-en-3β-yl sulfate (PE-S; pregnenolone sulfate), and 5 mM androst-5-en-3β-yl hemisuccinate (AND-hSuc) (Krausova et al., 2018) stock in dimethyl sulfoxide (DMSO). The final dilution to ECS was made.
at 50°C and followed by 1 min sonication (SONOREX DIGITEC GT 100/H, BADELIN electronic, Berlin, Germany). The same concentration of DMSO was added to all extracellular solutions. Drug applications were made with a microprocessor-controlled multibarrel fast-perfusion system. Changes in the glutamate-induced current in HEK293T cells transfected with hGluN1 and hGluN2B subunits were used to estimate the rate of solution exchange around the cell. Standard ECS was switched to the ECS diluted by water (50%) during glutamate application. The osmolarity was adjusted by sucrose to match the standard ECS.

The channel open probability (P) was determined as the ratio of the mean current (I) in the presence of 1 mM glutamate, NMDAR exists with high probability (>99.6% as predicted from calculations using rate constants determined earlier; Cais et al., 2008) only in doubly liganded states with the channel closed (R) or open (O), and/or in a desensitized state (D).

\[ D = \frac{k_o}{k_d} R = \frac{k_o}{k_c} O = B \]

B stands for MK-801 blocked state. The fitting procedure consisted of two steps (Turecek et al., 2004). In the first step, the response induced by 1 mM glutamate in HEK293T cells transfected with the WT or mutated hGluN1/hGluN2B receptors was analyzed for the peak (Ip) and steady state response (IS50), and onset of desensitization was determined by the single exponential function (τD). Desensitization (D) and the kinetic constants describing the onset of desensitization (kD) and resensitization (kR) were determined from Equations 2–4:

\[ D = 1 - (I_{SS}/I_p) \]
\[ k_d = D/\tau_d \]
\[ k_r = (1 - D)/\tau_d \]

In the second step, kD and kR were fixed at values obtained from the first step, and the close rate (kC) at an arbitrary value of 200 s⁻¹. The NMDAR response recorded in the presence of 1 mM glutamate, and 1 μM MK-801 was fitted to the model while the opening rate (kO) was set as a free parameter. MK-801 blocking rate (kB) was set to 25 μM⁻¹ s⁻¹ (Huettner and Bean, 1988; Jahr, 1992; Rosenmund et al., 1995). The binding of MK-801 was virtually irreversible over the time course of the experiment for the WT and the mutated receptors, with the exception of hGluN1/hGluN2B(N615I) (Supplementary Figure S1). In the case of hGluN1/hGluN2B(N615I) receptors, the onset of MK-801 inhibition was fitted to the model where the MK-801 unblocking rate (kU) was set as a free parameter. Microscopic open probability (Po) was calculated as:

\[ P_o = 100 \times k_o/(k_o + k_i) \]

Voltage Dependence of the Mg2⁺ Effect

The I-V relation of the Mg2⁺ inhibitory effect was fitted to the Boltzmann function equation of the form:

\[ I(V) = I_0(V - V_{rev})/(a + [Mg^{2+}]e^{-bV}) \]

in which I0 is the estimated conductance of the NMDAR whole-cell response in the absence of extracellular Mg²⁺, Vrev is the reversal potential of NMDA-induced current, and a, b are parameters with the following interpretation:

\[ a = K_d e^{bV} \]
\[ b = 2δF/(RT) \]

where δ indicates the apparent electrical distance of the Mg²⁺ binding site from the outside of the membrane and F, R, and T have their standard thermodynamic meanings (Abdrachmanova et al., 2002). Unless otherwise noted, recordings were performed at a holding potential of −60 mV (values of the holding potential were corrected for the liquid junction potential, 14 mV).
Analysis of the Steroid Effect

The relative degree of steroid-induced potentiation determined for different steroid doses in individual HEK293T cells was fit to the following logistic equation:

\[ I = \frac{I_{max}}{1 + (EC_{50} /[steroid])^h} \]  

(9)

where \( I_{max} \) is the maximal value of potentiation, \( EC_{50} \) is the concentration of the steroid that produces half-maximal potentiation of glutamate-induced current, \([steroid]\) is the steroid concentration and \( h \) is the apparent Hill coefficient.

**RESULTS**

**Rare Variants in the TMD Reduce Surface NMDAR Expression**

Recently, we elucidated the effects of different NMDAR mutations in an effort to identify sites of action for inhibitory steroids (Vyklicky et al., 2015). A secondary result of these experiments was the finding that mutations in the TMD and adjacent regions often dysregulated the NMDAR function. We therefore screened available databases (https://www.ncbi.nlm.nih.gov/clinvar/) and selected 11 de novo mutations (P553L; V558I; W607C; N615I; V618G; S628F; E657G; G820E; G820A; M824R; L825V) located in the pre-M1, M1, M2, M3, and M4 membrane regions of the hGluN2B subunit (Figure 1, Table 1).

There is no or incomplete information available about the effects of these missense mutations on NMDAR function. We therefore evaluated the amplitude of glutamate-induced responses, relative cell-surface expression, agonist potency, receptor desensitization, relative \( P_o \), and pharmacological properties of each of these variants as an initial step in understanding the potential role NMDAR channelopathy may play in the pathophysiology of neurological diseases. HEK293T cells transfected with WT receptors responded to glutamate (1 mM; applied in the continuous presence of 30 μM glycine) with peak currents of 66 pA/pF. Receptors with mutated hGluN2B subunit either did not respond to glutamate application (currents <5 pA) (P553L; S628F; G820E; M824R) or responded with significantly reduced amplitudes (W607C; N615I; V618G; E657G; G820A). The amplitude of responses was not significantly different for hGluN1/hGluN2B(V558I) and hGluN1/hGluN2B(L825V) receptors (Supplementary Figure S2).

The differences in the peak amplitude normalized with respect to the cell capacitance must be interpreted cautiously since this is influenced by factors that we cannot fully control. We have hypothesized that reduced protein levels and/or altered receptor function may underlie reduced responses to 1 mM glutamate in HEK293T cells expressing mutated NMDARs.

Next, we used immunofluorescence microscopy to determine the surface and total protein levels of the mutated NMDARs. First, we generated a gene encoding for YFP-hGluN1 (see Methods). In the second step, COS-7 cells were transfected with genes encoding for YFP-hGluN1 and WT or mutated hGluN2B subunits. Quantitative analysis of images indicated that the ratio of surface-to-total NMDAR expression was significantly reduced for YFP-hGluN1/hGluN2B(W607C) receptors compared to WT receptors (Figures 2A,B), and the surface expression of YFP-hGluN1/hGluN2B(S628F) receptors (Figure 2C) was quite likely completely lost since the ratio of the surface-to-total receptor levels was the same as that determined for COS-7 cells transfected with YFP-hGluN1 subunit only (Horak et al., 2008; Kaniaкова et al., 2012). In addition, the microscopy assay in cells expressing YFP-hGluN1/hGluN2B(G820E) receptors indicated increased surface expression compared to the WT receptors (Figure 2D). Figure 2E shows relative surface expression of the WT and mutated hGluN2B subunits. YFP-hGluN1 is not surface expressed unless it is co-expressed with the hGluN2B subunit (Okabe et al., 1999; Standley et al., 2009).
In addition, surface-to-total expression of the WT and YFP-hGluN1/hGluN2B(W607C; S628F; and G820E) receptors was assessed using immunofluorescence microscopy on HEK293T cells and a quantitative expression assay in COS-7 cells (see Methods). The data obtained using these methods confirmed a significant decrease in the surface expression of YFP-hGluN1/hGluN2B(S628F) receptors compared to the WT and increased in the surface expression of YFP-hGluN1/hGluN2B(G820E) receptors, however, differences in the surface expression of YFP-hGluN1/hGluN2B(W607C) receptors were significant only for data gained by immunofluorescence microscopy on HEK293T cells (Supplementary Figure S3).

Thus, it is possible to explain the insensitivity of hGluN1/hGluN2B(S628F)-transfected cells to glutamate by virtually no surface expression of the receptors. In contrast, insensitivity or reduced sensitivity of cells transfected with hGluN1 and hGluN2B(P553L; W607C; N615I; V618G; E657G; G820E; G820A; or M824R) subunits contrasts with normal or increased surface expression and indicates impaired receptor function.
Mutations Related to Human Neurological Diseases Decrease Agonist Potency

Seven mutations in the TMD responded to 1 mM glutamate and 30 µM glycine with currents that allowed reliable dose-response analysis of the agonist-induced responses. This analysis indicated that glutamate is 3.2- and 1.7-fold less potent at hGluN1/hGluN2B(W607C; and N615I) receptors and 2.3-fold more potent at hGluN1/hGluN2B(E657G) receptors than at the WT receptors (Figure 3A, Table 2). Similar analysis of glycine dose-response suggested that glycine is 2.0- and 1.8-fold less potent at hGluN1/hGluN2B(W607C; and E657G) receptors than at the WT receptors (Figure 3B, Table 2). Altered glutamate potency in hGluN1/hGluN2B(E657G) receptors and altered glycine potency in hGluN1/hGluN2B(W607C; and E657G) receptors was accompanied by a diminished value of the Hill coefficient indicating on altered degree of cooperativity of the agonists binding to the receptor (Weiss, 1997).

Mutations in the TMD Alter Receptor Desensitization and Decrease Channel Open Probability

To evaluate the effects of mutations in the TMD on NMDAR desensitization, the receptors were activated by a saturating concentration of glutamate (1 mM) in the continuous presence of glycine (30 µM). The analysis of the desensitization—a time-dependent decline of responses (see Equation 2 for definition)—indicated that WT receptors desensitized on average by 16.1% (Figure 4A). The desensitization was significantly increased to 72.9% in hGluN1/hGluN2B(V558I) receptors (Figure 4B) and was not significantly changed in hGluN1/hGluN2B(L825V) receptors (Figure 4C); by contrast, hGluN1/hGluN2B(V618G) receptors desensitized only by 4.1% (Figure 4D).

The time course of the desensitization onset of responses induced in the WT and hGluN1/hGluN2B(V558I; W607C; N615I; and L825V) receptors was next analyzed and desensitization ($k_d$) and $k_r$ resensitization rate constants were determined (see Equations 2-4). The desensitization observed in the WT receptor responses was characterized by $k_d = 0.24 \pm 0.08 \text{ s}^{-1}$ ($n = 10$). The value of $k_d$ was significantly increased in hGluN1/hGluN2B[V558I (3.55 \pm 0.46 \text{ s}^{-1}; n = 7); and W607C (1.59 \pm 0.54 \text{ s}^{-1}; n = 4)] receptor responses (one-way ANOVA, $p < 0.001$; followed by multiple comparisons of the rate constant determined in the mutated receptors vs. the WT; Dunnett’s method, $p < 0.050$). The values of the $k_d$ determined in hGluN1/hGluN2B[N615I (0.48 \pm 0.14 \text{ s}^{-1}; n = 4); and L825V (0.43 \pm 0.16 \text{ s}^{-1}; n = 6)] receptor responses was not significantly different from that determined in WT. WT receptor responses were characterized by $k_r = 1.06 \pm 0.17 \text{ s}^{-1}$ ($n = 10$). The value of the $k_r$ was significantly increased in hGluN1/hGluN2B[W607C (4.79 \pm 1.22 \text{ s}^{-1}; n = 4); and N615I (2.61 \pm 0.51 \text{ s}^{-1}; n = 4)] receptor responses (one-way ANOVA, $p < 0.001$; followed by multiple comparisons of the rate constant determined in the mutated receptors vs. the WT; Dunnett’s method, $p < 0.050$). The values of the $k_r$ determined in hGluN1/hGluN2B[V558I (1.29 \pm 0.28 \text{ s}^{-1}; n = 7); and L825V (1.47 \pm 0.20 \text{ s}^{-1}; n = 6)] receptors was not significantly different from that determined in WT receptors. Low amplitude of the hGluN1/hGluN2B(V618G) receptor responses precluded detailed analysis of the rate constants characterizing receptor desensitization.

To evaluate the effects of mutations in the TMD on $P_o$, the WT and mutated receptors were activated by a saturating concentration of glutamate (1 mM) and the rate of channel block by 1 µM MK-801 was fitted to the kinetic model and the $P_o$ determined (see Methods and Equation 5). Using this approach, the $P_o$ of the human WT receptors was determined to be $10.5 \pm 1.3\%$ ($n = 10$) (Figure 4A). This value is not different from the $P_o$ determined from the analysis of single-channel of activity induced by 1 mM glutamate in cell-attached patches ($P_o = 10.6 \pm 2.9\%$ ($n = 12$); unpaired t-test, $p = 0.977$) (see Supplementary Figure S4) and, in addition, it agrees well with that determined for rat GluN1/GluN2B receptors (Chen et al., 1999).
Superimposed traces indicate that in hGluN1/hGluN2B(V558I; and L825V) receptors, the onset of MK-801 inhibition was decelerated compared to WT receptors (Figures 4A–C). The results of the analysis indicated that the $P_o$ decreased from 10.5% in WT receptors to 0.66% in hGluN1/hGluN2B(V558I) and 1.47% in hGluN1/hGluN2B(L825V) receptors. Similar decrease in $P_o$ was observed in hGluN1/hGluN2B(W607C; and V618G) receptors (Figure 4E). These data indicate that de novo mutations in the TMD alter receptor desensitization and $P_o$.

**Mutations in the Channel Pore Alter Mg$^{2+}$ Inhibition and Single-Channel Amplitude**

Structural data indicate that residues hGluN2B(W607; N615; and V618) are part of the NMDAR channel pore. A functionally important feature of NMDARs is the voltage-dependent inhibition by extracellular Mg$^{2+}$, and several residues located within the ion channel pore have been shown to alter Mg$^{2+}$ sensitivity (Kuner et al., 1996; Kupper et al., 1996; Williams et al., 1998). Fitting the current-voltage relation of WT receptor responses recorded in the presence of 1 mM Mg$^{2+}$ to the
The responses induced in hGluN1/hGluN2B(V618G) receptors were small, precluding detailed analysis of the current-voltage relation. Based on the relative inhibitory effect of 1 mM Mg$^{2+}$ at a holding potential of −60 mV, which decreased from 97 ± 2% (n = 5) in WT to 35 ± 3% (n = 6) in hGluN1/hGluN2B(W607C), we infer that Mg$^{2+}$ binding within these receptor channels is also altered. The responses induced by hGluN1/hGluN2B(V618G) were small, precluding any reliable analysis of the Mg$^{2+}$ effect. These results agree well with reduced Mg$^{2+}$ block observed in mutated receptors (Lemke et al., 2014).

Next, we aimed to characterize the effect of mutations on the ion permeation employing single-channel recording from outside-out patches. Figure 5B shows records of channel activity in outside-out patches recorded in response to the application of 1 mM glutamate in the presence of Mg$^{2+}$-free extracellular solution at a holding potential of −60 mV (−74 mV after junction potential correction). The mean amplitude of the principal single-channel current was decreased from −4.5 ± 0.1 pA (n = 6) in the WT receptors to −2.4 ± 0.1 pA (n = 5) for hGluN1/hGluN2B(W607C) receptor channels (p < 0.001, compared to WT), −1.8 ± 0.1 pA (n = 5) for hGluN1/hGluN2B(N615I) receptor channels (p < 0.001, compared to WT), and −3.6 ± 0.1 pA (n = 5) for hGluN1/hGluN2B(V618G) receptor channels (p < 0.001, compared to WT). These data indicate that mutations in the NMDAR channel pore have opposing effects—an increase in responses due to the reduced Mg$^{2+}$ inhibitory effect and a
FIGURE 4 | Mutations in the TMD alter receptor desensitization and $P_o$. (A-C) Representative currents induced in WT, hGluN1/hGluN2B(V558I); and L825V) receptors by fast application of 1 mM glutamate (in the continuous presence of 30 µM glycine) and their inhibition by MK-801 (1 µM). (Aa-Ca) Show the response to glutamate at an expanded time scale. Desensitization of WT receptors was best described by rate constants $k_d = 0.17 s^{-1}$ and $k_r = 0.69 s^{-1}$; desensitization of hGluN1/hGluN2B(V558I) receptors by $k_d = 4.06 s^{-1}$ and $k_r = 1.12 s^{-1}$, and desensitization of hGluN1/hGluN2B(L825V) receptors by $k_d = 0.11 s^{-1}$ and $k_r = 0.83 s^{-1}$ (indicated by a superimposed red trace). (Ab-Cb) Show the onset of MK-801 inhibition on an expanded time scale. The time course of the onset of MK-801 (gray response in Bb and Cb) inhibition was best described by $P_o = 12.2\%$ for WT receptors, $P_o = 0.61\%$ for hGluN1/hGluN2B(V558I) receptors, and $P_o = 1.16\%$ for hGluN1/hGluN2B(L825V) receptors (indicated by a superimposed red trace) (see Methods section for details of the analysis procedure). Graphs show the summary of mean desensitization (D) and $P_o$ (E) ± SEM (n) for WT and mutated receptors. The differences in the mean values of desensitization and $P_o$ determined for the WT and mutated receptors was statistically significant; Kruskal-Wallis one-way ANOVA on Ranks, $p < 0.001$; subsequent Mann-Whitney Rank Sum Test or unpaired $t$-test was used to assess the significance compared to the WT, *$p = 0.001–0.05$, **$p < 0.001$. 

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FIGURE 5 | Mutations in the channel pore alter ion permeation. (A) Effect of Mg$^{2+}$ on WT and hGluN1/GluN2B(N615I) receptor responses. Whole-cell current responses were induced by 1 mM glutamate in the absence and presence of 1 mM Mg$^{2+}$ at a holding potential of −60 mV. I-V relations without Mg$^{2+}$ were fit by a linear equation and normalized to have a slope of 1. Corresponding responses recorded in the presence of Mg$^{2+}$ were normalized and fitted to the Boltzmann equation (Equations 6–8). (B) Examples of single-channel openings induced in WT, hGluN1/hGluN2B(W607C; N615I; and V618G) receptors by 1 mM glutamate. Dashed lines show where the current levels identified from the fit to the amplitude distribution for the WT would fall. For individual patches the distribution is shown fitted with a single Gaussian component. Glutamate-induced single-channel currents had mean amplitude: −4.7 pA (WT), −2.4 pA [hGluN1/hGluN2B(W607C)], −1.9 pA [hGluN1/hGluN2B(N615I)], and −3.9 pA [hGluN1/hGluN2B(V618G)].

reduction of responses due to the decrease in the single-channel amplitude; the overall effect will be a function of the membrane potential.

**GluN2B(L825V) Mutation Enhances Sensitivity to Endogenous and Synthetic Steroids**

Endogenous steroids are potent allosteric modulators of NMDAR activity (Korinek et al., 2011). The sensitivity of the mutant receptors to extracellular PE-S, a steroid that has a positive allosteric and subunit-dependent effect at NMDAR (with preference for GluN2A/B subunits) (Wu et al., 1991; Horak et al., 2006), was evaluated by generating dose-response curves for the potentiation of responses to glutamate. In addition, we analyzed the potentiating effect for AND-hSuc, a more potent synthetic PE-S analog (Krausova et al., 2018). Since the effect of steroids with a potentiating effect at NMDAR is disuse-dependent (Horak et al., 2004), a low concentration of glutamate (1 µM) was used in these experiments. Figures 6A,B shows that PE-S and AND-hSuc...
potentiate both the WT and mutated receptors, although to a different extent. Analysis revealed that GluN1/GluN2B(L825V) receptors were significantly more potentiated by a nearly saturating concentration of PE-S (100 µM) than responses induced in the WT and hGluN1/hGluN2B(V558I; W607G; V618G; and G820A) receptors (Figure 6C; Kruskal-Wallis one-way ANOVA on Ranks, \( p < 0.001 \); unpaired t-test, \( p < 0.001 \)). Similarly to the effect of PE-S, AND-hSuc (30 µM) potentiated glutamate responses induced in the mutated receptors with a higher potency than in the WT (Figure 6C; Kruskal-Wallis one-way ANOVA on Ranks, \( p < 0.001 \); unpaired t-test, \( p < 0.001 \)). GluN1/GluN2B(L825V) receptors were potentiated robustly (1647%) by AND-hSuc. Receptors containing hGluN1 and hGluN2B(V558I; W607C; V618G; or G820A) subunits were potentiated to an extent not significantly different from that determined for the WT receptors (Figure 6C; \( p = 0.117–0.412 \) for PE-S; \( p = 0.448–0.966 \) for AND-hSuc). Dose-response analysis of the effect of PE-S and AND-hSuc indicated that the increased steroid potentiation of GluN1/GluN2B(L825V) receptors was attributable to the increase in steroid efficacy rather than potency (Figures 6D,E).

The time course of the NMDAR responses following brief glutamate application has been suggested to mimic the time course of the NMDAR component of the EPSCs (Lester et al., 1990). To evaluate the effects of steroids on the deactivation time course, we measured current responses induced by a brief (~5 ms) application of 1 mM glutamate to HEK293T cells expressing WT or hGluN1/hGluN2B(L825V) receptors. In the WT receptors, PE-S (100 µM) significantly increased the amplitude of synaptic-like responses and slowed the deactivation (Figure 6F, Table 3). The deactivation of hGluN1/hGluN2B(L825V) receptors was not significantly different (\( p = 0.696 \)) from that determined for the WT receptors. This agrees well with the prediction made from the simulation of responses to brief glutamate application to NMDARs that have diminished \( P_o \) (Figure 4E, Table 2). In the hGluN1/hGluN2B(L825V) receptors, PE-S (100 µM) significantly increased the amplitude of synaptic-like responses and slowed the deactivation (Figure 6G, Table 3).

Next, we estimated the relative charge transfer recorded for synaptic-like responses in the presence of PE-S. The charge transfer was calculated as a product of the response amplitude and deactivation time course, and its value increased 3.3-fold for the WT and 3.8-fold for hGluN1/hGluN2B(L825V) receptors when synaptic-like responses were recorded in the presence of PE-S (100 µM) (Table 3). Even though this augmentation of synaptic-like responses is prominent, it is not sufficient to compensate for a 7-fold diminution of the synaptic-like response of hGluN1/hGluN2B(L825V) receptors due to their decreased \( P_o \) (Figure 4E). The increase in the charge transfer was much more robust for synaptic-like responses recorded in the presence of AND-hSuc (30 µM)-its value increased 161-fold for the WT and 180-fold for hGluN1/hGluN2B(L825V) receptors (Figures 6F,G, Table 3). Estimates indicate that the augmentation of synaptic-like responses is more than sufficient to compensate for a 7-fold diminution of the synaptic-like response of hGluN1/hGluN2B(L825V) receptors when we take into an account their decreased \( P_o \) (Supplementary Figure S2, Figures 4D,E).

**DISCUSSION**

The development of next-generation DNA sequencing has given rise to unique data linking patient variants in *GRIN1* and *GRIN2* genes to neurological and psychiatric disorders. While the list of rare variants expands rapidly, the consequences for receptor biogenesis, its structure, function, and pharmacology have been only partially revealed (Yuan et al., 2014; Swanger et al., 2016; Chen et al., 2017; Ogden et al., 2017; Platterz et al., 2017). In this study, we investigated 11 disease-associated rare variants in the GluN2B TMD and found that dysregulation of NMDARs involves multiple mechanisms - altered surface expression, agonist potency, receptor desensitization, probability of channel opening, single-channel currents, Mg\(^{2+}\) sensitivity - and their combination. Through functional assessments, we show that loss-of-function variants can be rectified by a pharmacological approach, using PE-S and its synthetic analog AND-hSuc. Impaired surface expression and/or receptor function have important implications that may help to find links connecting genetic variation to disease as well as to understand the role of NMDAR variants in neurodevelopmental disorders (Mcrae et al., 2017).

**Mechanisms of hGluN2B(P553L; and V558I) Dysregulation**

Crystal structures of the GluN1/GluN2B receptor show that the LBD, the pre-M1 helix, and the M1 helix are structurally coupled, indicating a role in receptor gating (Lee et al., 2014). Conserved residues GluN2B(P553; and V558) are part of the pre-M1 and M1 helix, respectively (Sobolevsky et al., 2009; Karakas and Furukawa, 2014; Figure 1A), and despite their physical proximity, the mutation hGluN2B(P553L) rendered the transfected cells virtually glutamate nonresponsive while hGluN2B(V558I) receptors exhibited similar current responses to 1 mM glutamate to the WT, although with a prominent desensitization and a decreased \( P_o \) (Supplementary Figure S2, Figures 4D,E). Surface expression
FIGURE 6 | hGluN1/hGluN2B(L825V) has an enhanced sensitivity to steroids. (A) Representative responses are shown for WT and hGluN1/hGluN2B(L825V) receptors. PE-S (100 µM) (A) and AND-hSuc (30 µM) (B) potentiated the WT receptor responses induced by co-application with 1 µM glutamate in the continuous presence of 30 µM glycine to a different extent (by 85% and 575%, respectively). Both steroids potentiated responses induced in hGluN1/hGluN2B(L825V) receptors (Continued)
of YFP-hGluN1/hGluN2B(P553L) receptors was not altered (Figure 2E; however, see Ogden et al., 2017), who show a moderate reduction (by 33%) indicating that minimal current responses to 1 mM glutamate in hGluN1/hGluN2B(P553L) receptors are not due to the lack of surface expression but rather a functional alteration in the receptor. We may hypothesize that the loss of function in hGluN1/hGluN2B(P553L) receptors may be attributed to receptor desensitization since rat GluN1/GluN2A(P552R) receptors, homologous in position to GluN2B(P553), desensitize profoundly (by 98.5%). It is less likely that it is due to altered agonist potency and P o since rat GluN1/GluN2A(P552R) and rat GluN1/GluN2B(P553R) receptors have the EC 50 value for both glutamate and glycine decreased in combination with an increased P o (Ogden et al., 2017). Augmented desensitization of hGluN1/hGluN2B(V558I) receptors agrees well with results of previous experiments with chimeric receptors containing desensitizing GluN2A and non-desensitizing GluN2C subunit, as well as single-point mutations, which have identified the pre-M1 and M1 region as critical for rapid glycine- and Ca 2+ -independent desensitization of NMDARs (Krupp et al., 1998; Villarroel et al., 1998; Thomas et al., 2006). In addition, this region has been shown to influence P o of the NMDAR channels (Ogden et al., 2017).

We have employed computational methods to infer the structural consequences of de novo missense mutations based on the liganded state with the channel just prior to opening (Vyklicky et al., 2015) as deduced from the available crystal structures of the heterotetrameric GluN1/GluN2B receptor (Furukawa et al., 2005; Karakas and Furukawa, 2014; Lee et al., 2014). In the pre-open liganded state, the pre-M1 helix controls the spacing between M1 and M4 helices. It is also involved in further transmitting the mechanical signal from ligand-induced LBD reorientation through the pore-forming M3 helix to the M1 and M4 helices (Figure 7A). Specifically, the hGluN2B(P553) residue interacts weakly with L650 and F653 of the M3 helix. MD simulation further suggests that the hGluN2B(P553L) mutation leads to a formation of a nearly continuous M1 helix as a result of removing the helix-terminating proline residue (Figure 7B). The hGluN2B(V558) is mediating an interaction with surrounding hydrophobic residues of M3 helix in the liganded state. The MD simulation suggests that the hGluN2B(V558L) mutation induces stronger interaction of these residues, leading to reorientation of the pre-M1 helix from its horizontal position (Figure 7C). As a result, the M1/M4 interaction is weakened.

**Mechanisms of hGluN2B(W607C; N615I; and V618G) Dysregulation**

Cysteine accessibility to sulfhydryl reagents indicates that conserved residues hGluN2B(W607; N615; and V618) are exposed to the lumen of the NMDAR ion channel (Kuner et al., 1996). Our results show that mutations of these residues underline multiple effects: hGluN1/hGluN2B(W607C)

| Table 3 | Summary of the pharmacological properties for synaptic-like responses. |
|--------------------------|--------------|--------------|--------------|
| Receptor | Control PE-S (100 μM) | Control | AND-hSuc (30 μM) |
| WT | Potentiation (%) | 98.7 ± 11.5 (5) | – | 924.5 ± 172.7 (5) |
| hGluN2B(L825V) | Potentiation (%) | 140.8 ± 10.8 (5) | – | 2,323 ± 121 (5) |
| **Deceleration** | **(ms)** | **307 ± 14 (5)** | **349 ± 20 (5)** | **370 ± 35 (5)** |
| **Charge transfer** | **(ms)** | **1.63 ± 0.06 (5)** | – | **16.36 ± 1.44 (5)** |
| **AND-hSuc (30 μM)** | | | | |
| hGluN2B(L825V) | Potentiation (%) | 140.8 ± 10.8 (5) | – | 2,323 ± 121 (5) |
| hGluN2B(L825V) | Potentiation (%) | 140.8 ± 10.8 (5) | – | 2,323 ± 121 (5) |
| **Deceleration** | **(ms)** | **323 ± 9 (5)** | **507 ± 28 (5)** | **335 ± 29 (5)** |
| **Charge transfer** | **(ms)** | **1.57 ± 0.05 (5)** | – | **7.19 ± 0.59 (5)** |

*The data are expressed as mean ± SEM (n) is the number of cells recorded from.*

**p < 0.001 compared to WT, paired t-test.**

**And** determined from the single exponential fit.

**And** determined from the double exponential fit.

**Deceleration is defined as 1/τo.**

**Charge transfer is defined as τ × (potentiation/100) + 1.**

FIGURE 6 | ~2-fold more than in WT receptors. Structure of PE-S (A) and AND-hSuc (B). (C) Graph shows relative PE-S (100 μM) potentiation ± SEM (n = 5–31) plotted vs. that induced by AND-hSuc (30 μM) ± SEM in the WT and in mutated receptors. Data were fit by a linear regression (Correlation coefficient r = 0.928; p = 0.00764). Concentration dependency for PE-S (D) and AND-hSuc (E) induced potentiation of WT and hGluN1/hGluN2B(L825V) receptors activated by 1 μM glutamate in the continuous presence of 30 μM glycine. Parameters of the fit (see Equation 9) were for the WT receptors: PE-S induced relative maximal potentiation I max = 101 ± 9%; EC 50 = 24.3 ± 1.8 μM with h = 1.54 ± 0.08 (n = 7); AND-hSuc I max = 57.5 ± 5.7%; EC 50 = 9.5 ± 1.0 μM; h = 1.50 ± 0.16 (n = 6). In hGluN1/hGluN2B(L825V) receptors: PE-S induced I max = 185 ± 50% (p = 0.030 compared to WT); EC 50 = 24.8 ± 1.2 μM (p = 0.842 compared to WT) with h = 1.52 ± 0.12 (n = 5); AND-hSuc I max = 1492 ± 514% (p = 0.030 compared to WT); EC 50 = 9.6 ± 2.3 μM (p = 0.961 compared to WT) with h = 1.58 ± 0.36 (n = 6). Normalized representative current responses of WT and hGluN1/hGluN2B(L825V) receptors induced by brief (5 ms) application of 1 mM glutamate made in the ECS (Control, gray) and in the presence of PE-S (100 μM) or AND-hSuc (30 μM) following steroid pre-application for (>30 s). The vertical gray bar indicates the amplitude of the control response at the same amplitude scale as that recorded in the presence of steroid. See Table 3 for values and statistics on the steroid effect.
receptors have a normal or moderately decreased surface expression, a decreased agonist potency, and a decreased $P_0$, whereas hGluN1/hGluN2B(V618G) have only a decreased $P_0$ (Figures 2–4). Similarly to hGluN1/hGluN2B(W607C; and V618G) receptors, hGluN1/hGluN2B(N615I) receptors exhibit reduced responses to 1 mM glutamate (by 72%, see Supplementary Figure S2), although without an obvious change in trafficking and/or the functional parameters studied.

A characteristic feature of the hGluN1/hGluN2B(N615I) receptors was an accelerated rate of recovery from MK-801 inhibition (see Supplementary Figure S1), indicating structural differences in the ion channel pathway between WT and mutated receptors. There is compelling evidence that asparagine residues [GluN1(N616), GluN2A(N614), GluN2B(N615)] and adjacent residues including tryptophan [GluN2A(W606) or GluN2B(W607)] control permeability and block by Mg$^{2+}$ (Williams et al., 1998) and, in addition, GluN2B(N615) controls single-channel conductance (for review see Dingledine et al., 1999). We therefore performed additional experiments to analyze Mg$^{2+}$ block and single-channel currents. In agreement with recent data of Mullier et al. (2017), our analysis showed that Mg$^{2+}$ sensitivity was decreased in hGluN1/hGluN2B(N615I; and W607C) receptors. Since the structural determinants of external Mg$^{2+}$ block are similar to those governing Ca$^{2+}$ permeability through NMDA receptor channels, it is possible that hGluN1/hGluN2B(W607C; N615I; and V618G) receptors may also have altered Ca$^{2+}$ permeability. However, this was not possible to assess reliably due to very small current responses (Supplementary Figure S2). Similarly to Mg$^{2+}$ block, the mean amplitude of the single-channel current was decreased for hGluN1/hGluN2B(W607C; N615I; and V618G) receptor channels.

To infer structural changes induced by mutations in the M2 segment and re-entrant loop, we used our previous model of the TMD with the vestibule open (Vyklicky et al., 2015). The ion filter as well as the lower part of M1, M3, and M4 helices are not changed significantly between the model liganded pre-open and the open state. The used model of the WT NMDAR channel is compatible with previous estimates of the minimum cross-section of the channel permeation pathway.
Mechanisms of hGluN2B(S628F) Dysregulation

Reduced responses to 1 mM glutamate in HEK293T cells transfected with hGluN1/hGluN2B(S628F) receptors (Supplementary Figure S2) correlate with their reduced surface expression (Figure 2E, Supplementary Figure S3). hGluN2B(S628) is located at the intracellular segment of the M3 helix and is conserved within the GluN2 family (Figure 1). We have shown earlier that amino acid residues within the M3 helix of GluN2B subunit (W635; S645; Y646; and T647) contribute to the regulation of the surface expression of NMDARs and alter the functional properties of NMDARs (Kaniakova et al., 2012). The quality-control at intracellular points is only poorly understood, and it is a matter of speculation whether the defect in surface expression described here for hGluN1/hGluN2B(S628F) receptors involves a mechanism similar to that of other amino acid mutations in the M3 helix or other TMD regions.

Our MD simulation suggests that in the hGluN1/hGluN2B(S628F) receptors, the mutation of a polar and small serine for a larger nonpolar phenylalanine leads to steric hindrance, with the bottom part of the M1 helix altering the quaternary structure of the TMD. This may be the reason why they are offloaded from their trafficking to the cell surface (Figures 8A,B).

Mechanisms of hGluN2B(E657G) Dysregulation

Reduced responses to 1 mM glutamate in HEK293T cells transfected with hGluN1/hGluN2B(E657G) receptors with normal surface expression imply that the mutation introduced a severe functional defect (Supplementary Figure S2, Figure 2E). Glutamate residue hGluN2B(E657) is conserved within the GluN2, GluA, and GluK family and is part of the linker that connects the LBD and M3 helices. Rearrangement of M3 helices in the activated conformation of the receptor makes the central cavity of the channel accessible to ions, therefore implying a crucial role of the M3-S2 linkers in channel opening (Sobolevsky et al., 2004). Mutations in this region lead to profound alterations of the receptor channel function characterized by diminished P0 and altered transduction of agonist activation to channel opening (Kazi et al., 2014). Missing experimental electron density data in available crystal structures (Karacas and Furukawa, 2014; Lee et al., 2014) does not allow the localization of residues in the linker regions and related structural information is mostly missing, we therefore decided to explore linker structures computationally. Our data indicate that the overall effect of glycine substitution hGluN2B(E657G) on NMDAR gating can be explained by an interaction of specific residues at a site located in the proximity of the M3 helices (Figures 7A, 8C).

Mechanisms of hGluN2B(G820E; G820A; M824R; and L825V) Dysregulation

Reduced responses to 1 mM glutamate observed in HEK293T cells transfected with genes encoding for hGluN1 and hGluN2B(G820A) subunits and a complete loss of responses in
hGluN1/hGluN2B(G820E; and M824R) receptors are not due to altered surface expression since this was normal or even increased (Figure 2E). Small currents in hGluN1/hGluN2B(G820A) receptors precluded detailed functional examination. In contrast, responses to 1 mM glutamate in cells transfected with genes encoding for hGluN1 and hGluN2B(L825V) subunits were not significantly different from those observed in the WT although they had reduced $P_o$ by 86% compared to the WT receptors.

Structurally conserved residues hGluN2B(G820, M824, and L825) (Figure 1) are located in mutual proximity in the M4 helix. Several lines of evidence indicate that M1 and M4 helices are located at the periphery of the TMD and seem to have a modulatory role in channel opening and closing, which is primarily mediated by M3 helices that form the central cavity of the channel in the activated receptor (Villarroel et al., 1998; Sobolevsky et al., 2004; Ogden and Traynelis, 2013; Karakas and Furukawa, 2014). The hGluN2B(L825) residue interacts with the F639 from the M1 helix of hGluN1 in the liganded state (Figure 9A). The hGluN2B(L825V) mutation leads to a weaker interaction with the hGluN1(F639), although structural effects are inconclusive (Figure 9B). MD revealed that hGluN2B(G820) serves as the last residue in the M4 helix, thus allowing the necessary extension of the preceding linker in response to the LBD reorientation. The hGluN2B(G820E; and G820A) mutations remain in the helical context and their side chains interact with the pre-M1 helix, shifting it from its horizontal position and changing the relative positions of M1 and M4 helices (Figures 9C,D). In the liganded state, the hGluN2B(M824) residue is involved in an interaction with hGluN2B(L551) of the pre-M1 helix (Figure 7A). As a consequence of the hGluN2B(M824R) mutation, the pre-M1 interaction is not formed (Figure 9E); this leads to an altered relative position of M1 and M4 helices, which in turn results in inefficient propagation of the mechanical signal from the LBD.

Pharmacology of Mutated Receptors

Therapeutic strategies for the development of new effective drugs for rectifying loss-of-function variants associated with diminution of the receptor $P_o$ may encompass PE-S and its derivatives, since these compounds have a positive allosteric effect that is specifically mediated by the increase in the receptor $P_o$ (Horak et al., 2004; Korinek et al., 2011). Both an endogenously occurring neurosteroid PE-S (Wu et al., 1991;
Bowly, 1993) and its more potent synthetic analog AND-hSuc (Krausova et al., 2018) were approximately as effective at WT as at the hGluN1/hGluN2B(V558I; W607C; V618G; and G820A) receptors. Interestingly, both steroids potentiated the amplitude of tonic and synaptic-like responses more profoundly when studied at hGluN1/hGluN2B(L825V) receptors, indicating their prospective use in personalized therapies. It is likely that the augmented potentiating effect for PE-S is not specifically associated with residues at the TMD, since similar increases have been recently shown for receptors mutated in the LBD of the GluN2A subunit (V685G; and D731N) (Swanger et al., 2016).

Functional defects described here in the NMDAR apply for cases in which both alleles in a patient were mutated at exactly the same site. This is expected to be rare, in contrast to heterozygous individuals, who are much more likely to carry only one mutated allele. In this case, the expected genotypic ratio predicts that each neuron will contain 25% of WT receptors, 50% of receptors composed of one hGluN2B subunit mutated and one non-mutated, and 25% of receptors comprised of both hGluN2B subunits mutated.

To build a conception of the degree of the NMDAR defect, we may use as an example the mutation-induced decrease in the receptor \( P_o \) \( (P_o = 10\% \) for the WT and \( P_o = 1\% \) for receptors with both hGluN2B subunits mutated). Based on the Mendelian laws of inheritance and the law of dominance, we may predict that the overall activity of NMDARs will be diminished, depending on whether the mutation is recessive, independent, or dominant, to 77.5, 55, and 32.5%, respectively, of the activity in the healthy subjects. These calculations indicate that for a full pharmacological rectification of the NMDAR defect, a potentiating effect of 22.5, 45, and 67.5% would be required. Since most neurons express more than one type of GluN2/GluN3 subunits (Monyer et al., 1994), the expected rectifying potentiation effect may be even smaller. However, further studies are required to understand how the degree of NMDAR hypofunction is linked to various clinical symptoms.

**AUTHOR CONTRIBUTIONS**

VV and LV designed the experiments; VV, BKr, BKy, ML, TS, and MK performed electrophysiological experiments; BKr, SD, and MH performed trafficking experiments; VV, BKr, and LV analyzed the data; JC performed molecular modeling; HC and EK synthesized the steroids; LV wrote the paper.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2018.00110/full#supplementary-material

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