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The study of functional and pharmacological properties of glutamate receptors

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Abstract

N-methyl-D-Aspartate receptors (NMDAR) are ionotropic glutamate receptors that are involved in the regulation of nearly every process in the brain. Therefore, even a subtle disturbance in NMDAR function may result in severe pathological consequences. Loss-of-function mutations in the NMDAR-encoding genes have been implicated in numerous neuropsychiatric disorders, including intellectual disability, developmental delay, schizophrenia, autism spectrum disorders, epilepsy, and movement disorders. Insufficient NMDAR function can be rectified by positive allosteric modulators, including neurosteroids; however, the mechanism underlying the potentiating effect of steroids is not well understood.

By employing patch-clamp electrophysiology we assessed the effect of newly synthesized neurosteroid-like pregnane analogues on recombinant GluN1/GluN2B receptors. We demonstrated that compounds with short C3 residues, such as pregnanolone acetate (PA-Ace) and pregnanolone carboxylate (PA-Car), are negative modulators of NMDAR, whereas compounds with longer C3 residues, such as pregnanolone butyrate (PA-But) and epipregnanolone butyrate (EPA-But), are positive modulators of NMDARs. Furthermore, we revealed that EPA-But has a disuse-dependent positive allosteric effect, being similar in that regard to endogenous neurosteroid pregnenolone sulfate (PE-S).

Combining electrophysiology, molecular biology, and computational modelling, we identified the PE-S and EPA-But binding sites at the transmembrane domain of the GluN1/GluN2B receptor. Our results indicate that EPA-But binds the NMDAR at the GluN1(M4)/GluN2B(M1), GluN2B(M4)/GluN1(M1), and GluN2B(M1/M4) interfaces. In contrast, PE-S binds the receptor only at the GluN2B(M1/M4) interface. Moreover, we proposed the mechanisms by which the steroids potentiate NMDAR function.

Next, we characterized the effect of ten *de novo* disease-associated mutations in the hGluN2B subunit on the receptor functional properties and surface expression. In addition, we evaluated the effect of EPA-But and PE-S at NMDARs harbouring disease-associated mutations in hGluN1 and hGluN2B subunits. Our results uncovered the potential of EPA-But and PE-S in compensation for the effect of loss-of-function mutations.

In this study, we revealed structural principles underlying the potentiating effect of steroids. Our results open up new possibilities for developing new steroid-based drugs for treating disorders associated with the hypofunction of NMDAR.

Abstrakt

N-methyl-D-aspartátové receptory (NMDAR) jsou ionotropní glutamátové receptory, které se podílejí na regulaci téměř všech procesů v mozku. Proto i nepatrná porucha funkce NMDAR může vést k závažným patologickým důsledkům. Mutace v genech kódujících NMDAR, které vedou ke snížení jejich funkce, se podílejí na mnoha neuropsychiatrických poruchách, jako jsou například mentální retardace, schizofrenie, poruchy autistického spektra, epilepsie a poruchy pohybu. Nedostatečnou funkci NMDAR lze korigovat pozitivními alosterickými modulátory, včetně neurosteroidů. Mechanismus který je základem potenciačního účinku steroidů však není dosud dostatečně objasněn.

Pomocí elektrofyziologické metody terčíkového zámku jsme změřili účinek nově syntetizovaných analogů pregnanů, které jsou podobné neurosteroidům, na rekombinantní NMDAR s podjednotkovým složením GluN1/GluN2B. Prokázali jsme, že sloučeniny s krátkými residui na uhlíku C3, jako je pregnanolon acetát (PA-Ace) a pregnanolon karboxylát (PA-Car), jsou negativními modulátory NMDAR, zatímco sloučeniny s delšími residui na C3, jako je pregnanolon butyrát (PA-But) a epipregnanolon butyrát (EPA-But), jsou pozitivními modulátory NMDAR. Dále jsme odhalili, že EPA-But má "disuse-dependentní" pozitivní alosterický účinek, přičemž je v tomto ohledu podobný endogennímu neurosteroidu pregnenolon sulfátu (PE-S).

Kombinací elektrofyziologie, molekulární biologie a počítačového modelování jsme identifikovali vazebná místa PE-S a EPA-But v transmembránové doméně receptoru GluN1/GluN2B. Naše výsledky ukazují, že EPA-But se váže na NMDAR na rozhraní transmembránových domén M4 a M1 sousedících podjednotek GluN1(M4)/GluN2B(M1), GluN2B(M4)/GluN1(M1) a dále na toto rozhraní v jedné podjednotce GluN2B(M1/M4). Naproti tomu PE-S se váže na receptor pouze na rozhraní GluN2B(M1/M4). Zde jsme navrhli mechanismy, kterými steroidy potencují funkci NMDAR.

Dále jsme provedli charakterizaci vlivu více než deseti *de novo* mutací asociovaných s neuropsychiatrickými onemocněními v lidské variantě podjednotek hGluN2B a hGluN1 na funkční vlastnosti a povrchovou expresi NMDAR. Zkoumali jsme účinek EPA-But a PE-S u těchto mutovaných NMDAR a naše výsledky ukázaly potenciál EPA-But a PE-S kompenzovat hypofunkci NMDAR, které nesou tyto mutace.

V této studii jsme odhalili strukturální principy, které jsou základem potenciačního účinku steroidů. Naše výsledky otevírají nové možnosti vývoje léčiv na bázi steroidů, které budou určeny pro léčbu poruch spojených s hypofunkcí NMDAR.

1 Introduction

NMDARs play a key role in excitatory synaptic transmission and synaptic plasticity, and even a slight disturbance in the NMDAR function may have severe pathophysiological consequences. Excessive NMDAR activation may result in excitotoxicity, a pathological process that leads to neuronal death. NMDAR-initiated excitotoxicity is involved in the pathophysiology of ischemic neuronal injury and neurodegenerative disorders, such as Alzheimer's disease, Huntington's disease, and Parkinson's disease (Dong et al., 2009). On the other hand, NMDAR hypofunction has been implicated in schizophrenia, intellectual disability, developmental delay, and autism-spectrum disorders (Hansen et al., 2021; Lee et al., 2015). More than 700 *de novo* mutations in the GRIN genes were found in patients suffering from various neuropsychiatric disorders but were absent in healthy individuals (Hansen et al., 2021).

At present, there is considerable interest in developing new drugs that can potentiate NMDAR function as a tool to treat neuropsychiatric diseases. The activity of NMDAR can be positively or negatively modulated by numerous compounds, including neurosteroids. As steroid compounds, neurosteroids have a specific core structure of four fused carbon rings. Neurosteroids that can modulate the NMDAR function share the same structural feature – the presence of a negatively-charged group at the carbon C3 at the core structure (Park-Chung et al., 1994)

2 Objectives of the study

2.1 Functional and pharmacological properties of disease-associated *de novo* mutations in hGluN2B subunit

Hypothesis: Numerous mutations in the transmembrane domain (TMD) of hGluN2B subunit have been identified in patients with various neuropsychiatric disorders, including intellectual disability, epilepsy, autism spectrum disorder, and schizophrenia (Awadalla et al., 2010; Hamdan et al., 2011, 2014; Platzer et al., 2017). However, the effect of many of these mutations on NMDAR function is unknown.

Objectives: 1) To perform complex characterization of the effect of selected disease-associated mutations in the TMD of the hGluN2B subunit on the surface expression and functional characteristics of NMDARs by the combination of electrophysiological and immunofluorescence techniques. 2) To evaluate the pharmacological potential of neuroactive steroids for compensation for mutation-induced impairments in the receptor.

2.2 Identification of the site of action for pregnenolone sulfate at the NMDAR

Hypothesis: Endogenous neurosteroid PE-S is widely-known as positive allosteric modulator (PAM) of GluN2A and GluN2B subunit-containing NMDARs (Malayev et al., 2002; Wu et al., 1991). However, the location of the PE-S site of action at the NMDAR and the mechanism of PE-S-induced positive allosteric modulation are still unknown.

Objectives: 1) To identify the site of action for PE-S at GluN1/GluN2B receptor using a combination of the patch-clamp technique, alanine-scanning mutagenesis, and in *silico* modelling. 2) To uncover the mechanism by which PE-S positively modulates NMDAR activity.

2.3 Structure requirements for potentiating neuroactive steroids

Hypothesis: Neurosteroids are well-known allosteric modulators of NMDAR. Depending on the spatial organization of chiral carbons C3 and C5, the shape of the neurosteroid molecule can be delineated as either "planar" or "bent". Several studies indicated that "planar" neurosteroids, such as PE-S, potentiate the NMDAR activity, whereas "bent" neurosteroids, such as pregnanolone sulfate (PA-S), inhibit the NMDAR activity (Park-Chung et al., 1994; Weaver et al., 2000; Wu et al., 1991). However, the structural determinants for positive and negative modulatory neurosteroid effects at NMDAR are not well understood and require further investigation.
Objectives: 1) To perform structure/activity relationship screening for newly synthesized pregnane analogues in which the ester bond was replaced with the C-C bond (ω5β-pregnan-3β-yl derivatives of carboxylic acids) at recombinant NMDARs using the patch-clamp technique. By performing the structure/activity relationship screening for these novel pregnane-based steroids, we expect to uncover the structural requirements for positive and negative modulatory effects of neurosteroids. 2) To characterize the mechanism of action and identify the site of action for EPA-But, a representative compound selected from the pregnane derivatives mentioned above.

3 Materials and methods

3.1 Site-directed mutagenesis

Site-directed mutagenesis was introduced using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, USA) according to the manufacturer's instructions. Plasmid cDNA was isolated using a High-Speed Plasmid Mini Kit (Geneaid Biotech, New Taipei City, Taiwan) according to the manufacturer's instructions. All mutations were verified by DNA sequencing (Eurofins Genomics).

3.2 Cell culture and transfection

COS-7 and HEK293T cells (Human Embryonic kidney cells) were used as an expression system for recombinant NMDAR for electrophysiology and immunofluorescence experiments, respectively. The transfection of HEK293T cells was performed with the Magnet Assisted Transfection (MATra) technique. The cells were transfected with 8 µl of MATra transfection reagent (IBA Lifesciences, Gottingen, Germany) and 0.9 µg of cDNA (0.3 µg of GluN1, 0.3 µg of GluN2, and 0.3 µg of eGFP) according to the manufacturer's instructions. The transfection of COS-7 cells was performed with Lipofectamine 2000 reagent

(Life Technologies, Carlsbad, USA). The cells were transfected with 4 µl of Lipofectamine 2000 and 1.8 µg of cDNA, as described (Kaniakova et al., 2012).

3.3 Immunofluorescence microscopy

Analysis of the surface expression of NMDAR in COS-7 cells was performed in 24-36 hours after transfection, as described (Horak et al., 2008). Fluorescent images were taken with the Olympus Cell-R system(Olympus, Tokyo, Japan). Analysis of microscopy images was performed using ImageJ software (NIH, Bethesda, MD, USA).

3.4 Electrophysiology

Electrophysiological experiments were conducted on HEK293T cells 24-48 hours after transfection. All electrophysiological measurements were performed using the patch-clamp technique in a voltage-clamp mode in whole-cell or outside-out configurations. If not mentioned otherwise, the holding potential was kept at -60 mV. NMDAR currents were recorded using an Axon Axopatch 200B Microelectrode Amplifier (Molecular Devices, San Jose, USA) after compensation of series resistance (<10MΩ) and capacitance by 80-90%. The analogue signal from the amplifier was filtered at 2 kHz by a low-pass Bessel filter and digitized with Axon Digidata 1550B Low-Noise Data Acquisition System (Molecular Devices) at a sampling rate of 10 kHz. Recording of digitized data was implemented by software pClamp 10.6 (Molecular Devices). Glass microelectrodes 4-6 MΩ were filled with the intracellular solution (ICS). Guidance for accurate microelectrode placement was conducted by a motorized micromanipulator MP-225 (Sutter Instrument).

3.5 Data analysis

Statistical analysis was performed with Sigma Plot 14.0 software package (Systat Software Inc., Palo Alto, USA). Data distribution was evaluated by the Shapiro-Wilk normality test and the equality of group variances was assessed by the Brown–Forsythe test. Parametric statistics (Student's *t*-test, paired *t*-test, or ANOVA) was used for data sets with normal distribution whereas nonparametric statistics (Wilcoxon-signed rank test, Mann–Whitney rank-sum test, or Kruskal–Wallis one-way ANOVA on ranks) was used for data sets without normal distribution. At multiple group comparisons, posthoc tests were performed when a significant difference had been found. Correlation between data sets was assessed with the Pearson product-moment correlation coefficient.

4 Results

4.1 Functional and pharmacological properties of disease-associated *de novo* mutations in hGluN2B subunit

In this study, we evaluated the functional characteristics and surface expression of 10 *de novo* disease-associated mutations (P553L; V558I; W607C; V618G; S628F; E657G; G820E; G820A; M824R; L825V) in the transmembrane domain (TMD) of hGluN2B subunit (Fig.1**)**.

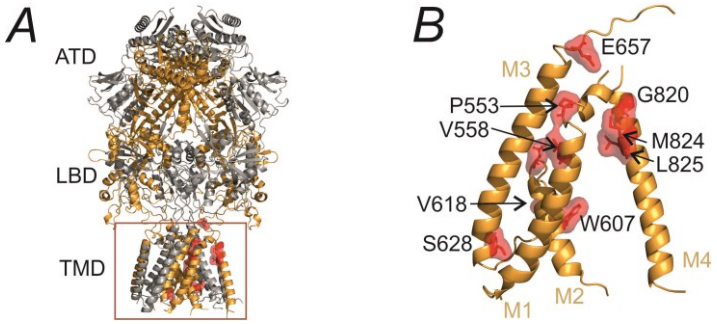

Figure 1. Location of the mutated residues in the NMDAR. **(*A,B*)** Ribbon structure of hGluN1/hGluN2B receptor (ATD – amino-terminal domain; LBD – ligand-binding domain; TMD – transmembrane domain). Mutated residues that have been found in individuals with neuropsychiatric disorders are highlighted in red.

The WT and mutated hGluN1/hGluN2B receptors were expressed in HEK293T cells and the amplitude of the receptor response to 1 mM glutamate was evaluated by the patch-clamp technique. hGluN1/hGluN2B(W607C; V618G; E657G; G820A) receptors exhibited significantly diminished current density (Fig.2*A***)**. The hGluN1/hGluN2B(P553L; S628F; G820E; M824R) receptors showed virtually no response to glutamate (Fig.2*A***)**.

Next, we assessed the surface expression of WT and mutated receptors expressed in COS-7 cells using immunofluorescence microscopy. The surface expression of the hGluN1/hGluN2B(W607C; S628F) receptors was significantly lower than that of WT receptors (Fig.2*B***)**. In contrast, the hGluN1/hGluN2B(G820E) receptors exhibited increased surface expression (Fig.2*B***)**.

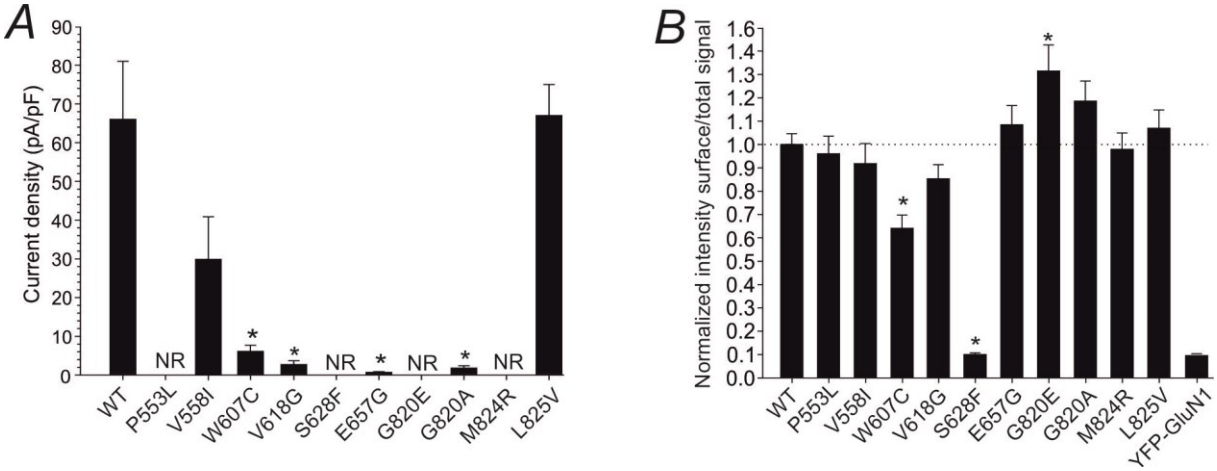

Figure 2. The effect of disease-associated mutation on the current density and surface expression of NMDARs. ***(A)*** Graph shows the average current density of WT and mutated GluN1/hGluN2B receptors. Mutations that showed no glutamate-induced currents are indicated as NR (non-responding); * $p < 0.05$; $n = 5$-23. ***(B)*** Graph shows the relative surface-to-total expression levels of WT and mutated hGluN1/hGluN2B receptors and YFP-hGluN1 subunit in COS-7 cells ($n = 35$); * $p < 0.05$.

For desensitization analysis, WT and mutated NMDARs were activated by saturating concentrations of glutamate and glycine (1 mM and 30 μM, respectively). WT receptors were desensitized by 16%, whereas the hGluN1/hGluN2B(V618G) receptors were desensitized by only 4% (Fig. 3*A,B*). In contrast, the degree of desensitization of the hGluN1/hGluN2B(V558I) receptors was increased to 73% (Fig. 3*A,B*).

The probability of channel opening (P_o) of WT and mutated receptors was assessed by analysing the kinetics of the MK-801 blockade. The receptors were activated by 1 mM glutamate and then the glutamate-induced currents were inhibited by the application of 1 μM MK-801. The rate of MK-801 blockade was fitted to the kinetic model. The results of these experiments indicated that the P_o of the hGluN1/hGluN2B(V558I), hGluN1/hGluN2B(W607C), hGluN1/hGluN2B(V618G), and hGluN1/hGluN2B(L825V) receptors were substantially lower than that of WT receptors (Fig. 3*A,C*).

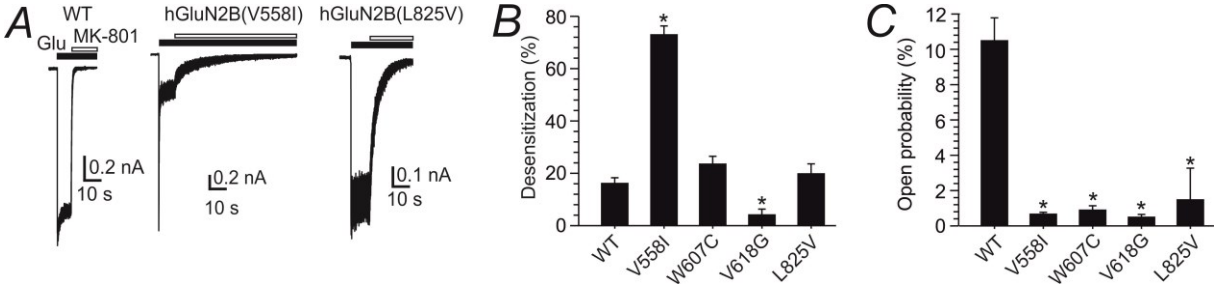

Figure 3. Disease-associated mutations affect the desensitization and the P_o of the receptor. ***(A)*** WT, hGluN1/hGluN2B(V558I), and hGluN1/hGluN2B(L825V) receptor responses to 1 mM glutamate and their inhibition by 1 μM MK-801 in the WT. ***(D-E)*** Graphs represent the desensitization ***(D)*** and open probability ***(E)*** of WT and mutated receptors. Mann-Whitney Rank Sum Test was used to check the statistical difference between the WT and mutated receptors. Error bars indicate SEM; n = 4-19; * $p < 0.05$.

Next, we evaluated the effect of the hGluN2B(V558I; W607C; V618G; L825V) mutations on the receptor sensitivity to naturally-occurring potentiating neurosteroid pregnenolone sulphate (PE-S) and its more potent synthetic analogue androst-5-en-3β-yl hemisuccinate (AND-hSuc). The effect of the steroids was assessed during their co-application with glutamate (1 μM). At hGluN1/hGluN2B(L825V) receptors, both PE-S (100 μM) and AND-hSuc (30 μM) induced significantly greater potentiation of glutamate-induced responses than that at WT receptors ($p < 0.05$, unpaired t-test) (Table 1). In contrast, the responses of hGluN1/hGluN2B(V558I; W607C; V618G) receptors were potentiated to the same extent as the responses of WT receptors (Table 1).

Table 1. Potentiating effect of PE-S and AE-hSuc in WT and mutated receptors

Receptor	PE-S (100 μM) (%)	AND-hSuc (30 μM) (%)
WT	85 ± 4 (n = 31)	575 ± 69 (n = 18)
hGluN2B(V558I)	59 ± 8 (n = 5); p = 0.117	685 ± 67 (n = 6); p = 0.448
hGluN2B(W607C)	73 ± 16 (n = 8); p = 0.412	406 ± 49 (n = 8); p = 0.489
hGluN2B(V618G)	96 ± 10 (n = 8); p = 0.542	509 ± 61 (n = 5); p = 0.966
hGluN2B(L825V)	197 ± 36 (n = 9); $p < 0.001$	1647 ± 425 (n = 7); $p < 0.001$

Statistical analysis was performed by one-way ANOVA ($p < 0.001$) followed by an unpaired t-test for single comparisons vsWT.

In the next step, we evaluated the effect of mutation on the agonist affinity of receptors. The hGluN1/hGluN2B(W607C) receptors demonstrated a 3.2-fold lower glutamate affinity in comparison to that of WT receptors. In contrast, the hGluN1/hGluN2B(E657G) receptors

showed 2.3 times higher glutamate affinity than the WT receptors. The glycine affinity of mutated hGluN1/hGluN2B(W607C) and hGluN1/hGluN2B(E657G) receptors was significantly lower (2.0- and 1.8- fold, respectively) than that of WT receptors.

4.2 Identification of the site of action for pregnenolone sulfate at the NMDAR

Endogenous neurosteroid pregnenolone sulfate (PE-S) is well known for its disuse-dependent potentiating effect on the NMDARs (Horak et al., 2004; Malayev et al., 2002; Wu et al., 1991). However, the molecular mechanism of PE-S modulation and the binding site for PE-S at the NMDAR are not known. In this study, we used a combination of electrophysiology, molecular biology, and computational modelling to characterize the interaction of PE-S with the NMDAR and identify the PE-S binding site.

To test whether PE-S action is associated with interaction with the intracellular domain of the receptor, we employed methyl-γ-cyclodextrin (γCDX), a cyclic oligosaccharide that is able to accommodate steroids, therefore decreasing their free membrane concentration (Ohtani et al., 1989; Szejtli, 1998). To complex intracellularly available PE-S, GluN1/GluN2B receptors expressing HEK293T cells were subjected to intracellular dialysis with the ICS containing 10 mM γCDX via a patch pipette. No significant differences were observed between the degree of potentiation of glutamate-evoked responses of GluN1/GluN2B receptors expressed in HEK293T cells dialysed with control and γCDX-containing ICS (Fig 4*A*). Further, we evaluated the effect of intracellularly dialysed PE-S on the capability of extracellularly applied PE-S to potentiate GluN1/GluN2B receptor responses. To accelerate the process of intracellular dialysis, an outside-out patch-clamp approach was used instead of a whole-cell patch-clamp. Outside-out patches were pulled from HEK293T cells expressing GluN1/GluN2B receptors and dialyzed with control or PE-S-containing (100 µM) ICS. In patches dialyzed with the PE-S-containing ICS, a 10 s pre-application of PE-S (100 µM) potentiated the responses to 1 mM glutamate to the same extent as in patches dialyzed with control ICS (Fig 4*B*). These findings oppose the location of the PE-S binding site within the intracellular domain of NMDAR.

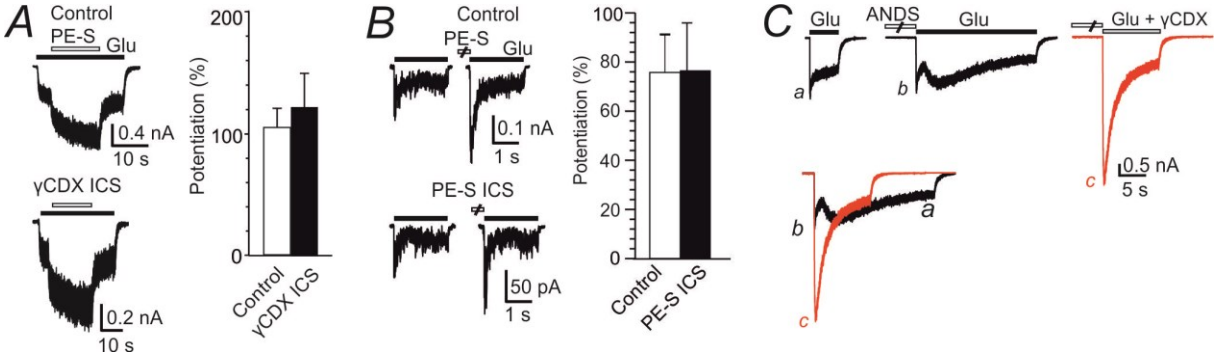

Figure 4. Potentiating effect of PE-S is mediated by the interaction with the TMD. ***(A)***Th effect of PE-S (100 µM) on 1 µM glutamate-induced responses of NMDARs from HEK293T cells dialyzed with control (top) and γCDX-containing ICS (bottom).Graph shows the degree of PE-S-induced potentiation measured in cells dialyzed with the control and γCDX-dialyzed ICS. ***(B)*** Responses of NMDARs to glutamate (1 mM) obtained from outside-out patches isolated from HEK293T cells before and following PE-S (100 µM) pre-application for 10 s. Patches were dialyzed with control ICS (top) and ICS containing 100 µM PE-S (bottom). Graph shows the degree of PE-S-induced potentiation in patches dialyzed with control and PE-S-containing ICS. ***(C)*** NMDAR responses to glutamate (1 mM) recorded before (*a*) and following (*b*) ANDS pre-application for 15 s. (*c*) shows the response to co-application of glutamate (1 mM) and γCDX (10 mM) made following ANDS pre-application.

Afterwards, we elucidated whether the potentiating steroids act at the extracellular domain of the NMDAR similarly to the steroids with inhibitory action (Vyklicky et al., 2015). We hypothesized that if a potentiating and an inhibitory effect of steroids is mediated by binding to the extracellular domain, then the extracellular γCDX application will similarly affect both effects. For the next experiments, we have selected 5α-androstan-3β-yl-sulfate (ANDS), a steroid that demonstrated a dual (positive and negative) effect at the NMDAR. After a 15 s pre-application, ANDS (100 μM) potentiated GluN1/GluN2B receptor responses to 1 mM glutamate by 17 ± 6% (n = 5) and displayed complex kinetics due to a dual effect of the steroid **(**Fig 4*C*)**.** In contrast, NMDAR responses to the co-application of glutamate and 10 mM γCDX were potentiated by ANDS pre-application significantly more (by 172 ± 18%; n = 5 (Fig 4*C*). We reasoned that γCDX precluded the inhibitory effect of ANDS by preventing access to the inhibitory site of action at the extracellular domain, therefore, unmasking the potentiating effect of ANDS. In turn, the fact that the ANDS potentiation effect was not abolished by γCDX indicates that the potentiating site of action for ANDS is located out of the extracellular domain.

The results of these experiments suggest that the potentiating effect of steroids is mediated by the interaction with the TMD. For the investigation of the PE-S binding site, we analysed the effect of mutations in the TMD of the GluN1 and GluN2B subunits on PE-S potentiation. For this purpose, we performed alanine-scanning mutagenesis of the following amino-acid residues in the M1 and M4 helices: GluN1(Q559-V572; T809-V825) and GluN2B(S555-I568; D814-A830). The assessment of the PE-S (100 μM) modulatory effect on WT and mutated receptors were performed upon its co-application with glutamate (0.08-3.20 μM). The degree of PE-S-induced potentiation at the GluN1(Q559A; W563A; L564A; N812A; F817A)/GluN2B and GluN1/GluN2B(M562A; V564A; L567A; M818A; G820A; F822A; L825A; M829A; A830T) receptors was significantly lower than in the WT receptors ($p < 0.05$, one-way ANOVA). At the GluN1/GluN2B(D816A; Y823A; M824A) receptors, PE-S application led to the inhibition of glutamate-induced responses

To determine the steroid site of action, the homology model of the GluN1/GluN2B receptor (Ladislav et al., 2018) was used for the identification of residues located within ~15 Å from the GluN2B(Y823 and M824), the M4 helix residues whose mutations diminished the PE-S potentiation the most. As a result, the GluN1 M3 helix residues G638, I642, and S646 and the GluN2B M1 helix residue W559 were selected for further experiments (Fig 5*A*). PE-S (100 μM) inhibited the responses of GluN1/GluN2B(W559L) receptors to 1 μM glutamate by 15 ± 2% (n = 7) (Fig 5*B,C*). In the GluN1(G638A)/GluN2B and GluN1(I642A)/GluN2B receptors, PE-S (100 μM) potentiated the responses to glutamate (1 μM) significantly less than in WT receptors (Fig 5*B,C*). The responses of GluN1(S646A)/GluN2B receptors were potentiated to the same extent as the WT receptor responses (Fig 5*B,C*). The glutamate EC_{50} values for GluN1(G638A)/GluN2B, GluN1(I642A)/GluN2B, and GluN1/GluN2B(W559L) receptors were similar to those for the WT receptors. These results suggest an interface formed by GluN1(G638 and I642) and GluN2B(W559, Y823, and M824) residues as a possible binding site for the steroid.

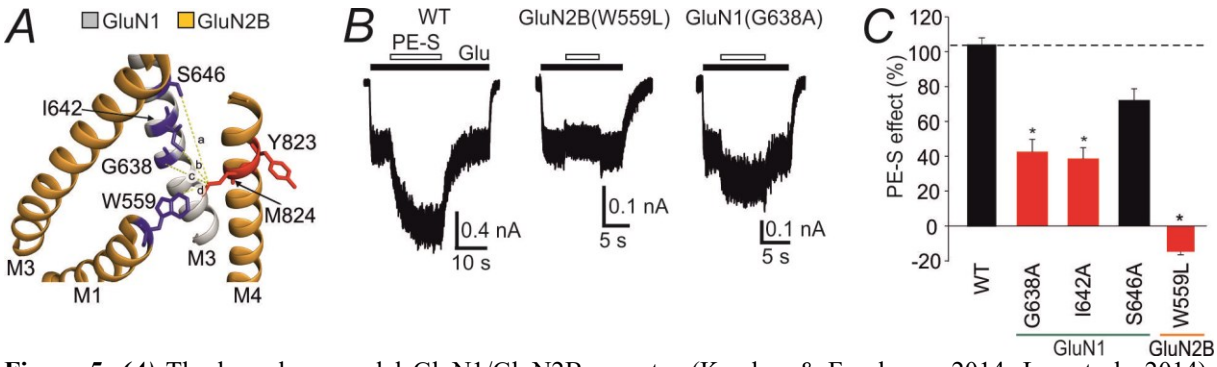

Figure 5. ***(A)*** The homology model GluN1/GluN2B receptor (Karakas & Furukawa, 2014; Lee et al., 2014). GluN1(S646, I642, G638), and GluN2B(W824) residues (blue) are located within 12, 6, 9, and 5 Å from the M824 residue (red). ***(B)*** The effect of PE-S (100 μM) on glutamate(1 μM) responses of WT, GluN1/GluN2B(W559L), and GluN1(G638)/GluN2B receptors. ***(C)*** Graph shows the mean effect of PE-S (100 μM) on the WT and mutated NMDARs. * indicates a significant difference from the WT(one-way ANOVA; ($p < 0.001$) followed by an unpaired t-test for single comparisons versus WT ($p < 0.050$)).

For *in silico* analysis of the steroid binding, we employed a recently developed model of the unliganded GluN1/GluN2B receptor (Černý et al., 2019). The analysis of the steroid-receptor interaction was performed in two steps. First, PE-S was docked in the TMD of the model GluN1/GluN2B receptor. As a result, two pairs of preferential interaction sites were identified: a pair of homologous sites at the GluN1(M1/M4) interface and a pair of sites at the GluN1(M1/M4) interface. In the next step, we used molecular dynamics (MD) to simulate PE-S interaction with the docking-predicted sites in the model lipid environment. The MD simulation of PE-S interaction with the binding site at the GluN1(M1/M4) interface indicated the binding free energy of ∼−15 kcal mol^{-1}. The GluN1(W563; L819) residues were found to possess the greatest contribution to this binding energy by forming van der Waals contacts with PE-S (Fig. 6*A*). For the steroid interaction with the GluN2B(M1/M4) site, the MD simulation indicated the binding free energy of −19.5 kcal mol^{-1}. The GluN2B(W559; M562; Y823) residues were shown to contribute the most to the free binding energy and form van der Waals contacts with the PE-S molecule (Fig. 6*A*).

The MD simulation of the steroid-receptor interaction indicated that PE-S binding facilitates the GluN2B M4 helix rotation and tightens the contact between the GluN2B(M824; I642) residues of the pore-lining M3 helix (Fig. 6*B*). In the absence of PE-S, the GluN2B(M824) residue forms contact with the GluN2B(W559) residue and therefore obstructs the entry to the cavity (Fig. 6*B*).

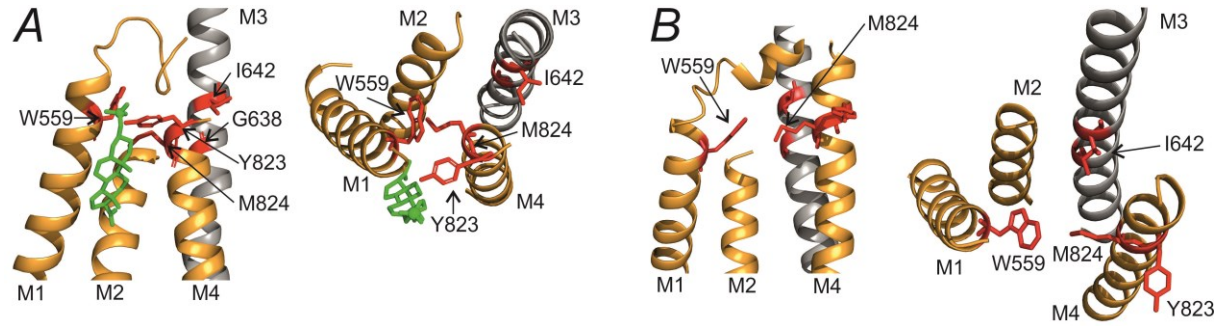

Figure 6. In silico modelling of PE-S binding. ***(A)***Location of the PE-S binding site the TMD of GluN1/GluN2B receptor. Residues which were identified as essential for steroid potentiation are labelled with red. ***(E)*** Model of the receptor in the closed state was simulated without the PE-S molecule bound within the TMD.

4.3 Structure requirements for potentiating neuroactive steroids

In the current study, we aimed to explore the structural requirement for the steroid modulation of NMDAR function. For this purpose, we prepared a set of pregnane derivatives in which the ester bond was replaced with the C-C bond (Fig. 7*A*) and assessed their effect on GluN1/GluN2B receptors expressed in HEK293T cells using the patch-clamp technique.

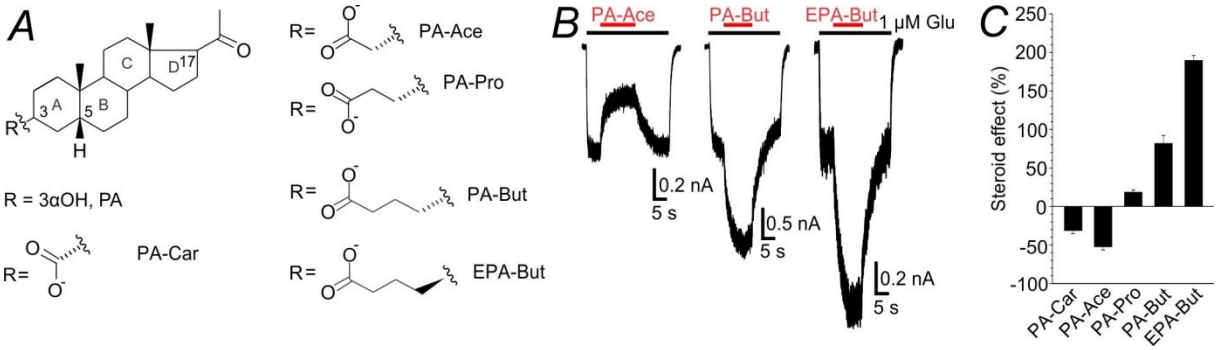

Figure 7. The effect of pregnane analogues on NMDAR. ***(A)*** Structure of 3α-hydroxy-5β-pregnan-20-one (PA) and residues used as substituents at the C3 carbon: PA-carboxylate (PA-Car), PA-acetate (PA-Ace), PA-propionate (PA-Pro), PA-butyrate (PA-But), and EPA-butyrate (EPA-But). ***(B)*** Representative recordings show the effect of PA-Ace (150 μM), PA-Pro (10 μM), PA-But (15 μM), and EPA-But (15 μM) on NMDAR responses to 1 μM glutamate. ***(C)*** Graph shows the mean degree of steroid-induced effect at NMDAR (n = 6-7).

Pregnane analogues with short residues at the C3 carbon at the core structure, such as PA-acetate (PA-Ace; 150 μM) and PA-carboxylate (PA-Car; 150 μM) inhibited the NMDAR responses to 1 μM glutamate by 31.6 ± 3.7% (n = 7) and 51.8 ± 3.7% (n = 7), respectively (Fig. 7*B,C*). However, the analogues with elongated residues at the C3, such as PA-propionate (PA-Pro; 10 μM) and PA-butyrate (PA-But; 15 μM) potentiated the glutamate-induced responses by 18.8 ± 6.5% (n = 6) and 82.2 ± 9.8% (n = 7), respectively (Fig. 7*B,C*). The potentiating effect of PA-Pro and PA-But was surprising because steroids with a "bent" A/B ring junction had been considered to inhibit the NMDAR responses (Korinek et al., 2011; Weaver et al., 2000). To characterize the role of stereo-configuration at the C3 chiral carbon, we have prepared the 3β-isomer of PA-But: 4-(20-oxo-5β-pregnan-3β-yl) butanoic acid (EPA-But; Fig. 7*B,C*). EPA-But (15 μM) also produced a strong potentiation of the GluN1/GluN2B receptor response to 1 μM glutamate (190 ± 6 %; n = 7; Fig. 7*B,C*). These results indicate that steroids with a "bent" molecular geometry, in addition to their well-known inhibitory action, are able to potentiate the NMDAR; the positive modulatory effect is observed for "bent" steroids with elongated aliphatic chain at the C3 carbon.

In the following experiments, EPA-But was ued as a representative potentiating steroid with a "bent" structure. For the dose-response analysis, the GluN1/GluN2B receptor response to 1 μM glutamate were potentiated by the co-application of 0.3-15 μM EPA-But. The obtained data were analysed by fitting the logistic equation with the following parameters: E_{max} = 221 ± 23%; EC_{50} = 6.1 ± 0.8 μM; h = 1.4 ± 0.1 (n = 7) (Fig. 8*A,B*). Next, we evaluated the effect of EPA-But (15 μM) on the GluN1/GluN2B receptor responses induced by 0.03-1000 μM glutamate. Fig. 8*C* demonstrates the negative relationship between the concentration of glutamate co-applied with EPA-But (15 μM) and the degree of EPA-But potentiation. Moreover, the potentiating effect of EPA-But is influenced by the timing of the steroid and glutamate application. Thus, upon co-application with EPA-But (15 μM), GluN1/GluN2B

receptor responses to glutamate (1 mM) were potentiated by only 35 ± 5% (n = 5) (Fig. *8C,D*). In contrast, 30 s pre-application of EPA-But (15 µM) potentiated the responses to subsequent glutamate (1 mM) application by 287 ± 65%, similar to the degree of potentiation observed when the EPA-But (15 µM) pre-application was followed by co-application of glutamate (1 mM) and the steroid (263 ± 65%; n = 6) (Fig. *8C,D*). These results indicate that EPA-But is a disuse-dependent PAM of NMDAR.

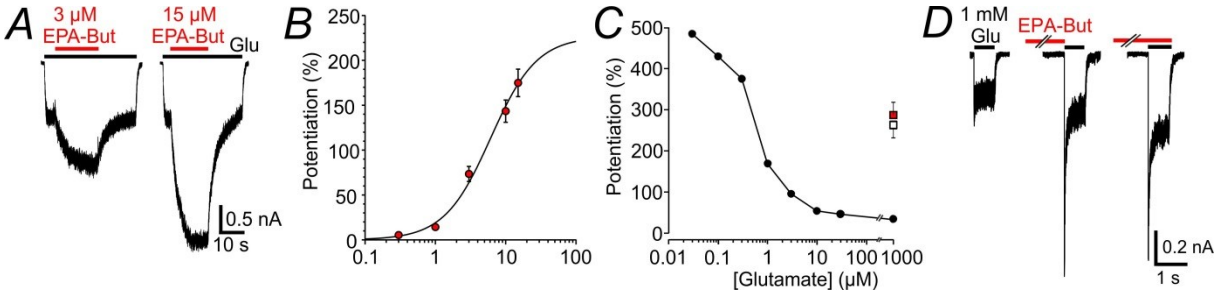

Figure 8. The EPA-But effect is disuse-dependent. ***(A)*** Representative recordings of NMDAR responses to 1 µM glutamate and before and in the presence of 3 µM and 15 µM EPA-But. ***(B)*** Concentration-response curve for the EPA-But effect. The EPA-But effect was evaluated during the steroid co-application with 1 µM glutamate ***(C)*** The plot of the mean EPA-But potentiation vs glutamate concentration. EPA-But (15 µM) was co-applied with glutamate (0.03-1000 µM) (filled circles);. For comparison, the mean potentiation of the responses to 1 mM glutamate after 30 s pre-application of EPA-But (15 µM) (red square) and the mean potentiation of the responses to co-application of glutamate (1 mM) and EPA-But (15 µM) after 30 s EPA-But (15 µM) pre-application (white square) are included. ***(D)*** Representative recordings of NMDAR responses to glutamate (1 µM) in the absence and presence of EPA-But (15 µM) recorded before and after 30 s EPA-But (15 µM) pre-application.

Using alanine scanning mutagenesis, we explored the role of the outer segment of the TMD in the potentiation of the GluN1/GluN2B receptor by EPA-But. Alanine scanning analysis was performed on the same set of residues as during the identification of the site of action for PE-S, i.e. GluN1(Q559-V572; G638; I642; S646; F810-V825) and GluN2B(S555-I568; D814-A830. At mutated receptors whose EC_{50} for glutamate was not significantly different from this for the WT, the EPA-But (15 µM) effect was assessed during its co-application with 1 µM glutamate. At mutated receptors whose EC_{50} to glutamate was significantly smaller or bigger than in WT, the EPA-But (15 µM) effect was evaluated at a glutamate concentration that activates the receptors to the same level as 1 µM glutamate at WT receptors (38% of the WT responses to 1 mM glutamate). This experiment showed that the substitution of the GluN1(G567; S569; V570; G638; I642; E811; N812; M813; F817) and the GluN2B(D557;V558, W559; M561; M562; V564; L566; D816; M818; G820, F822; M824; L825; A830) residues resulted in a significantly decreased EPA-But potentiation of GluN1/GluN2B receptors (one-way ANOVA, $p < 0.001$; followed by t-test). These results indicate the GluN1(M4)/GluN2B(M1), GluN2B(M4)/GluN1(M1), and GluN2B(M1/M4) interfaces as potential sites of action for EPA-But.

To delineate the EPA-But site of action at the NMDAR, we employed a two-step molecular modelling procedure that had been previously developed to identify the site of action for PE-S. The open-state model of the GluN1/GluN2B receptor (Černý et al., 2019) was used for the analysis of the interaction between the receptor and EPA-But. The initial molecular docking-based analysis of the steroid-receptor interaction indicated four pairs of preferential interaction sites which were located within the GluN1(M1/M4),

GluN2B(M1/M4), GluN1(M4)/GluN2B(M1), and GluN2B(M1/M4) interfaces. Subsequent analysis of the inactions between docking-predicted sites and the EPA-But molecule using the MD simulation suggested the presence of three pairs of stable interaction sites within the TMD. The indicated interaction sites were formed by GluN1(M4)/GluN2B(M1), GluN2B(M4)/GluN1(M1), and GluN2B(M1/M4) interfaces (Fig. 9).

The MD simulation indicated that the binding of EPA-But to the GluN2B(M1/M4) interface tightens the interaction between the GluN2B(M1, M4) helices and GluN1 M3 helix (Fig. 9*B*), stabilizing the open state of the channel. In addition, the simulation suggested that EPA-But binding at the GluN1(M4)/GluN2B(M1) and GluN2B(M4)/GluN1(M1) interfaces result in the altered orientation of the steroid-binding residues. Thus, the side chains of these residues are oriented toward the channel pore in the absence of EPA-But. Upon EPA-But binding, these residues form van der Waals contacts with the steroid, thereby reorienting their side chains toward the steroid molecule (Fig. 9*A,C*). Overall, the EPA-But accommodation within the TMD leads to the stabilization of the open state and expansion of the diameter of the ion channel (Fig. 9).

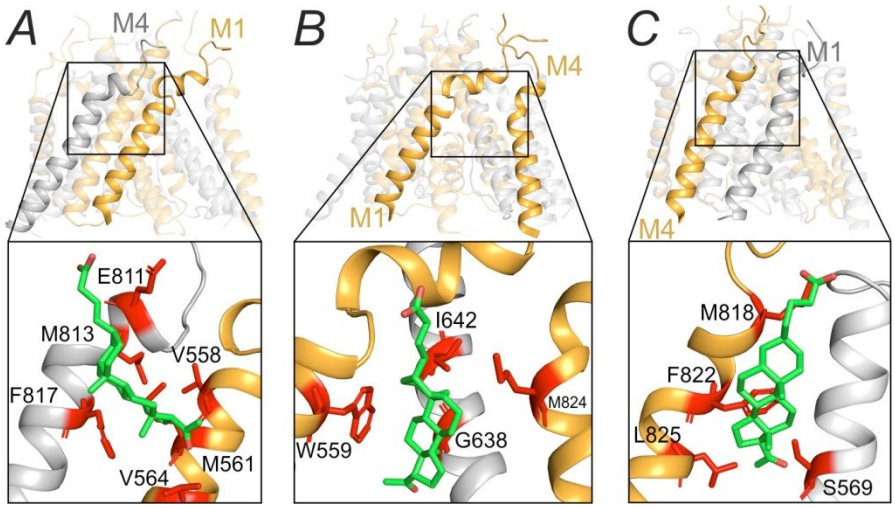

Figure 9. The effect of EPA-But binding on the TMD arrangement. Arrangement of the GluN1(M4)/GluN2B(M1) ***(A)***, GluN2B(M1/M4) ***(B)***, and GluN2B(M4)/GluN1(M1) ***(C)*** interfaces of GluN1/GluN2B receptor in the presence of the EPA-But molecule .

5 Discussion

5.1 Functional and pharmacological properties of disease-associated *de novo* mutations in the hGluN2B subunit

In this study, we assessed the effect of 10 disease-associated *de novo* mutations in the hGRIN2B subunit at functional characteristics and surface expression of recombinant hGluN1/hGluN2B receptors. We explored several mechanisms underlying the dysregulation of mutated receptors, including changes in receptor surface expression, agonist sensitivity, desensitization, and P_o. The investigation of the effect of disease-associated mutations on the functional characteristics of the receptor is crucial for the understanding of the role of these mutations in the pathology of neuropsychiatric disorders.

In addition, we evaluated the effect of potentiating steroids PE-S and AND-hSuc at the mutated receptors. The hGluN1/hGluN2B(L825V) receptors displayed a remarkably

enhanced sensitivity to both steroids. These results indicate the potential of neuroactive steroids to rectify the negative consequences of disease-associated mutations.

For the full manifestation of mutation-induced impairments in receptor function, patients must be homozygous for the mutation. This situation is highly unlikely for *de novo* mutations, so the majority of patients are heterozygous for the given mutation. If only one allele is mutated, 50% of all expressed GluN2B subunits will be mutated; in these circumstances 25% of hGluN1/hGluN2B receptors will be unmutated, 50% of hGluN1/hGluN2B receptors will harbour a mutation in one hGluN2B subunit, and 25% of hGluN1/hGluN2B receptors will harbour a mutation in both GluN2B subunits.

To estimate the effect of a mutation on receptor activity, we may use as an example a GluN2 mutation that reduces the receptor P_o from 10% (in WT receptors) to 1% (in the receptors with both GluN2 subunits mutated). Using Mendel's law of dominance and uniformity, we may approximate that the overall activity of receptors will be reduced to 77.5, 55.0, and 32.5%, respectively. These results indicate that for the full compensation for the mutation-induced impairments, a pharmacological agent may potentiate the receptor responses 1.3-fold, 1.8-fold, and 3.1-fold, respectively. However, these are only rough approximations of the effect of mutations on receptor function, which do not take into account the fact that a significant portion of NMDARs in the brain are triheteromers composed of different types of GluN2 or/and GluN3 subunits (Monyer et al., 1994; Pérez-Otaño et al., 2016; Stroebel et al., 2018). Therefore, further studies are needed for a better understanding of the association between mutations in the NMDAR and their clinical outcomes.

5.2 Identification of the site of action for pregnenolone sulfate at the NMDAR

In this study, we identified a group of residues within the TMD of the GluN1/GluN2B receptor that comprise the PE-S positive allosteric modulatory site.

The suggested steroid-binding site within the cavity enclosed by the GluN1(M3) and GluN2B(M1; M4) helices are subjected to conformational rearrangements upon channel opening. The MD simulation indicated that the steroid binding affects these conformational rearrangements facilitating the GluN2B M4 helix rotation during the receptor activation (Fig. 6). In addition, the interaction with the PE-S molecule leads to a tightening of the interaction between the GluN1(I642) and GluN2B(W559) residues (Fig. 6). Since the GluN1(I642) residue is located within the pore-lining M3 helix, it seems reasonable to assume that this interaction underlies the mechanism by which PE-S increases the NMDAR P_o (Černý et al., 2019; Horak et al., 2004). Upon the steroid unbinding, the GluN2B M4 helix returns to its ordinary position moving the GluN2B(M824) residue closer to the GluN2B(W559) residue (Fig. 6). As a result, these residues form a van der Waals contact with each other therefore impeding proper accommodation of the steroid in the binding pocket. This mechanism sheds light on the disuse-dependent potentiating effect of PE-S (Horak et al., 2004).

Several lines of evidence indicate that the negative allosteric modulatory site is likely to be distinct from the site characterized above. Thus, the potentiating and inhibitory effects of PE-S differ in their mechanisms: whereas PE-S potentiation is disuse-dependent, PE-S inhibition is use-dependent (Horak et al., 2004). Moreover, alanine substitution of

GluN2B(Y823; M824) residues abolishes the potentiating but not the inhibitory effect of PE-S. The results of a recent alanine-scanning study indicated that the GluN1 M4 helix is a potential negative allosteric modulatory site for PE-S (Langer et al., 2021). In addition, the previously identified binding site for inhibitory steroids at the extracellular vestibule of the ion channel is also likely to be a negative allosteric modulatory site for PE-S (Vyklicky et al., 2015). However, additional experiments may be required to clarify the site(s) and mechanisms of NAM action of PE-S.

The identification of the PE-S binding site and better understanding of mechanisms underlying the potentiating effect of the steroid is pivotal for further design of novel potentiating neurosteroid-like compounds to treat disorders associated with reduced NMDAR function.

5.3 Structure requirements for potentiating neuroactive steroids

In this study, we demonstrated that C3-substituted derivatives of pregnanolone exhibit potentiating effect at NMDAR. The results of alanine screening mutagenesis and *in silico* modelling indicated three interaction interfaces for EPA-But at the GluN1/GluN2B receptor. These interaction interfaces are formed by residues at the upper parts of the GluN1(M4)/GluN2B(M1), GluN2B(M4)/GluN1(M1), and GluN2B(M1/M4) helices.

Although the structural principles determining the modulatory effect of steroids at the NMDAR are not fully understood, several studies indicated that steroids with a “planar” molecule potentiate the NMDAR function, whereas steroids with a “bent” molecule inhibit the NMDAR function (Borovska et al., 2012; Korinek et al., 2011; Kudova et al., 2015; Park-Chung et al., 1994; Weaver et al., 2000; Wu et al., 1991). However, our results demonstrated that derivatives of the endogenous inhibitory steroid PA-S, such as PA-But and EPA-But, can potentiate NMDAR function despite the “bent” shape of the molecule (Wu et al., 1991).

Our findings indicated that the NMDAR harbours several separate sites of action for different steroids. The site of action for inhibitory steroids, such as PA-S, is within the outer vestibule of the ion channel (Vyklicky et al., 2015), whereas the site of action for PE-S is located at the interface formed by transmembrane GluN2B(M1/M4) helices (Hrcka Krausova et al., 2020). The results of mutagenesis experiments together with MD simulation indicated that in addition to the site of action at the GluN2B(M1/M4) interface, the TMD contains two extra pairs of sites of action for EPA-But, which are distinct from those for PE-S.

Discovering the principles underlying steroid-receptor interaction is pivotal for understanding the mechanisms of NMDAR modulation by steroids and the development of neurosteroid-like drugs to treat neuropsychiatric disorders associated with abnormal NMDAR function. Further systematic research is needed to enable predictions of the steroid effects on certain disease-associated NMDAR variants and allow the rational design of novel neurosteroid-like drugs specific for these receptor variants.

6 Conclusion

Hypofunction of the NMDAR has been implicated in the pathogenesis of multiple neuropsychiatric diseases, including epilepsy, schizophrenia, intellectual disability, and autism spectrum disorders. Because of this, there is considerable interest in developing new compounds that can potentiate NMDARs as a pharmacological tool to treat neuropsychiatric diseases. When designing new NMDAR-enhancing drugs, it should be taken into account that exaggerated NMDAR activation can lead to excitotoxic effects. In this regard, special attention should be paid to NMDAR PAMs. In contrast to agonists, PAMs are unable to activate NMDARs but only enhance the responses of receptors activated by naturally released glutamate; therefore, PAMs demonstrate reduced risk of NMDAR overstimulation (Yao & Zhou, 2017). Among the NMDAR PAMs are compounds of different classes, including neuroactive steroids; however, the mechanisms underlying steroid potentiation are not well understood.

This dissertation focuses on the mechanisms of interaction between NMDARs and potentiating neuroactive steroids. In this study, we identified novel sites of action for naturally occurring and synthetic potentiating steroids at the TMD of NMDARs and suggested the mechanisms by which steroids enhance NMDAR function. Moreover, we uncovered the structural determinants for the positive modulatory effect of neurosteroids on NMDARs. In addition, this study explores the functional consequences of selected *de novo* disease-associated mutations in NMDAR subunits and indicates the potential of potentiating steroids as pharmacological tools to compensate for the effects of these mutations.

The results given in this dissertation contribute to a deeper understanding of the mechanisms underlying allosteric modulation of NMDARs and offer new possibilities for *in silico* development of new neurosteroid-like drugs for the treatment of neuropsychiatric disorders associated with NMDAR hypofunction.

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8 List of publications

This dissertation is based on the following publications:

1) Kysilov, B., Hrcka Krausova, B., Vyklicky, V., Smejkalova, T., Korinek, M., Horak, M., Chodounska, H., Kudova, E., Cerny, J., & Vyklicky, L. (2022). Pregnane-based steroids are novel positive NMDA receptor modulators that may compensate for the effect of loss-of-function disease-associated GRIN mutations. *British Journal of Pharmacology*, *179*(15), 3970–3990. https://doi.org/10.1111/bph.15841\. IF = 9.473 (2021).
2) Hrcka Krausova, B., Kysilov, B., Cerny, J., Vyklicky, V., Smejkalova, T., Ladislav, M., Balik, A., Korinek, M., Chodounska, H., Kudova, E., & Vyklicky, L. (2020). Site of Action of Brain Neurosteroid Pregnenolone Sulfate at the N-Methyl-D-Aspartate Receptor. *The Journal of Neuroscience*, 5922–5936. https://doi.org/10.1523/JNEUROSCI.3010-19.2020. IF = 6.167 (2020).
3) Vyklicky, V., Krausova, B., Cerny, J., Ladislav, M., Smejkalova, T., Kysilov, B., Korinek, M., Danacikova, S., Horak, M., Chodounska, H., Kudova, E., & Vyklicky, L. (2018). Surface Expression, Function, and Pharmacology of Disease-Associated Mutations in the Membrane Domain of the Human GluN2B Subunit. *Frontiers in Molecular Neuroscience*, *11*, 110. https://doi.org/10.3389/fnmol.2018.00110. IF = 3.72 (2018).

Other scientific publications:

1) Štefková-Mazochová, K., Danda, H., Dehaen, W., Jurásek, B., Šíchová, K., Pinterová-Leca, N., Mazoch, V., Krausová, B. H., Kysilov, B., Smejkalová, T., Vyklický, L., Kohout, M., Hájková, K., Svozil, D., Horsley, R. R., Kuchař, M., & Páleníček, T. (2022). Pharmacokinetic, pharmacodynamic, and behavioural studies of deschloroketamine in Wistar rats. *British Journal of Pharmacology*, *179*(1), 65–83. https://doi.org/10.1111/bph.15680. IF = 9.473 (2021).
2) Hubalkova, P., Ladislav, M., Vyklicky, V., Smejkalova, T., Hrcka Krausova, B., Kysilov, B., Krusek, J., Naimová, Z., Korinek, M., Chodounska, H., Kudova, E., Cerny, J., & Vyklicky, L., Jr (2021) . *The Journal of Neuroscience*, Palmitoylation Controls NMDA Receptor Function and Steroid Sensitivity, *41*(10), 2119–2134. https://doi.org/10.1523/JNEUROSCI.2654-20.2021. IF = 6.709.
3) Tumanovska, L. V., Swanson, R. J., Serebrovska, Z. O., Portnichenko, G. V., Goncharov, S. V., Kysilov, B. A., Moibenko, O. O., & Dosenko, V. E. (2019). Cholesterol enriched diet suppresses ATF6 and PERK and upregulates the IRE1 pathways of the unfolded protein response in spontaneously hypertensive rats: Relevance to pathophysiology of atherosclerosis in the setting of hypertension. *Pathophysiology 26*(3-4), 219–226. https://doi.org/10.1016/j.pathophys.2019.05.005. No IF.