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A conserved glycine harboring disease-associated mutations is required for slow deactivation and high Ca^{2+} permeability in NMDA receptors

Johansen B. Amin^{1,2,3}, Xiaoling Leng⁴, Aaron Gochman⁵, Huan-Xiang Zhou^{4,6}, and Lonnie P. Wollmuth^{3,5,7*}

¹Graduate Program in Cellular and Molecular Pharmacology, ²Medical Scientist Training Program (MSTP), ³Center for Nervous System Disorders, ⁵Department of Neurobiology & Behavior, ⁷Department of Biochemistry & Cell Biology, Stony Brook University, Stony Brook, NY 11794-5230

⁴Department of Physics and Institute of Molecular Biophysics, Florida State University, Tallahassee, FL 32306

⁶Departments of Chemistry and Physics, University of Illinois at Chicago, Chicago, IL 60607

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Address for correspondence: Dr. Lonnie P. Wollmuth
Depts. of Neurobiology & Behavior and Biochemistry &
Cell Biology
Center for Nervous System Disorders
Stony Brook University
Stony Brook, New York 11794-5230
Tel: (631) 632-4186
Fax: (631) 632-6661
ORCID: 0002-8179-1259
E-mail: lonnie.wollmuth@stonybrook.edu

ABSTRACT

A variety of *de novo* and inherited missense mutations associated with neurological disorders are found in the NMDA receptor M4 transmembrane helices, which are peripheral to the pore domain in eukaryotic ionotropic glutamate receptors. Subsets of these mutations affect receptor gating with dramatic effects, including in one instance halting it, occurring at a conserved glycine near the extracellular end of M4. Functional experiments and molecular dynamic simulations of constructs with and without substitutions at this glycine indicate that it acts as a hinge, permitting the intracellular portion of the ion channel to laterally expand. This expansion stabilizes long-lived open states leading to slow deactivation and high Ca^{2+} permeability. Our studies provide a functional and structural framework for the effect of missense mutations on NMDARs at central synapses and highlight how the M4 segment may represent a pathway for intracellular modulation of NMDA receptor function.

Word count: 144

INTRODUCTION

Aberrant ion channel functions, or channelopathies, are a major cause of neurological disorders¹. Ionotropic glutamate receptors (iGluRs), including AMPA (AMPA) and NMDA (NMDAR) receptor subtypes, are glutamate-gated ion channels found throughout the brain and participate in almost all brain functions. In terms of channelopathies, iGluR autoantibodies are associated with paraneoplastic encephalopathies and the neurological dysfunction in neuropsychiatric lupus^{2,3}. Further, numerous *de novo* and inherited missense mutations have been identified in iGluR subunits that are associated with a wide spectrum of disorders, including epilepsy, intellectual disability, movement disorders, and schizophrenia⁴⁻⁷.

iGluRs are highly modular proteins composed of four domains (Figure 1a) and function as tetrameric complexes⁸. Interestingly, missense mutations in the ligand-binding domain (LBD) and transmembrane domain (TMD) forming the ion channel are typically associated with more severe clinical phenotypes^{7,9}, reflecting their central role in agonist binding and ion channel opening. The ion channel core is formed by two transmembrane helices, M1 and M3, and an intracellular reentrant pore helix, M2^{10,11}. In eukaryotic iGluRs, there is an additional transmembrane helix, M4, that packs against the M1 and M3 helices of a neighboring subunit. The M4 helix plays an important role in receptor biogenesis and ion channel function¹²⁻¹⁴.

In the NMDAR M4 segments, there are numerous missense mutations associated with clinical pathologies (Figures 1b & 1c; Supplementary Table 1). These mutations are primarily located in the extreme extracellular third of the M4 segments (Figure 1c), which participates strongly in ion channel gating^{15,16}, implicating gating deficits as a potential cause for these disease phenotypes.

Here we address the contribution of these M4 missense mutations to receptor function.

Although many of these mutations impact receptor gating, the most notable and robust effects occur at a highly conserved glycine ([Supplementary Figure 1](#)) positioned near the extracellular end of the M4 segments. Missense mutations at this position in the GluN1 and GluN2B subunits have been identified in multiple patients, and the resulting changes in side chain properties can be either great (glycine-to-arginine) or subtle (glycine-to-alanine). For both NMDAR subunits, but most notably the obligatory GluN1 subunit, mutations at this conserved glycine drastically impair the stability of long-lived open states, greatly enhancing the rate of receptor deactivation as well as attenuating Ca^{2+} permeability. Molecular dynamics simulations show that this glycine acts as a hinge, allowing for the C-terminal portion of the GluN1 M4 segment and the ion channel core to laterally expand. These results have strong implications for how neurological disorders are caused at the ion channel level and implicate the M4 as a major hub for modulation of ion channel function.

Word Count: 425

RESULTS

Disease-associated missense mutations in the NMDAR M4 segments are prominent at glycines

At present, twenty-three patients with neurological disorders have been identified with *de novo* missense mutations in the M4 segments of NMDAR subunits ([Figure 1](#)). The clinical phenotype for these patients is typically severe, encompassing epileptic encephalopathies and intellectual disabilities ([Supplementary Table 1](#)). For the majority of patients, the mutations occur at glycines (15 out of 23 patients). In transmembrane regions, the most common missense mutation associated with disease is glycine-to-arginine^{17,18}. Glycines often participate in critical structural roles in transmembrane segments, including acting as a notch for transmembrane segment interactions¹⁹ and to provide flexibility to transmembrane segments²⁰. Substitution of a charged side chain for a glycine can strongly disrupt these functions.

Of the 15 patients with missense mutations at glycines, 8 carry mutations to charged side chains: 6 are arginines (R) [GluN1(G815R), GluN1(G827R)] and 2 are glutamate (E) [GluN2B(G820E), GluN2B(G826E)]. However, for a glycine at the external end of M4, a number of patients have mutations that have less dramatic changes in the nature of the side chain, from glycine to alanine (A) or valine (V). This glycine, G815 in GluN1, G819 in GluN2A, and G820 in GluN2B, is one of only three positions completely conserved across all mammalian iGluR M4 segments ([Supplementary Figure 1a](#)) and also shows considerable conservation across many different species ([Supplementary Figure 1b](#)). That less dramatic missense mutations (e.g., alanine) can have a clinical phenotype and its conservation suggest that this glycine plays an important role in iGluR function.

Missense mutations in the NMDAR M4 segments dramatically alter receptor gating

The association of NMDAR missense mutations with neurological diseases could reflect alterations in the rules of NMDAR subunit assembly, trafficking²¹, interaction with other intracellular or extracellular proteins, and/or receptor functional properties such as gating, ion permeation or channel block^{22,23}. The majority of missense mutations in the M4 segment are positioned at the external ends, which participate strongly in gating and make no apparent contribution to a cell biological function (e.g., assembly)¹⁵. We therefore characterized the effect of many of these missense mutations on receptor gating (Figure 2 & Supplementary Figure 2).

A critical determinant of synaptic NMDARs is their slow deactivation time course, which is GluN2-subunit specific²⁴⁻²⁶. Currents in human GluN1/GluN2A (hN1/hN2A) (Figure 2a, *left*) and GluN1/GluN2B (hN1/hN2B) (Figure 2b, *top*), induced by a brief glutamate application as occurs at synapses, decay slowly with the time courses best fit by double exponentials (weighted τ s, 81 ± 2 ms, $n = 11$ and 660 ± 30 ms, $n = 8$, respectively) (mean $\pm$ SEM, n = number of recordings) (Figures 2c & 2d; Supplementary Table 2). As expected, the decay was much slower for GluN2B-containing receptors²⁵.

Several of the missense mutations had no significant effect on deactivation times (Figures 2c & 2d, *open bars*), whereas two, hGluN2A(M817V)¹⁶ and hGluN2(G826E), significantly slowed deactivation. However, the most prominent effects of the M4 missense mutations was a significant speeding of deactivation, which was notable for mutations at the conserved glycine in hGluN1, G815V (35 ± 4 ms, $n = 5$) and G815R (39 ± 2 ms, $n = 6$) (Figure 2c, *filled bars*) and in hGluN2B, G820A (205.2 ± 12 ms, $n = 7$) (Figure 2d, *filled bars*). hGluN1(G815R) reduced the deactivation times by about half whether in the hGluN2A or hGluN2B background. We could not detect glutamate-activated whole-cell current for hGluN1(G827R)/hGluN2A or

hGluN1/hGluN2B(G820E), either with brief or more sustained (Supplementary Figure 2) applications.

Although the missense mutations could have additional effects on receptor function (e.g., permeation) or cell biology, these strong effects on receptor deactivation (Figures 2c & 2d) as well as those on desensitization (Supplementary Figure 2), presumably contribute to their associated clinical phenotype. Here we will focus on how these mutations impact receptor function.

Subsets of missense mutations strongly alter single channel activity

To begin to understand how the missense mutations impact receptor gating, we recorded single channel activity in the on-cell mode (Figures 2e-2h; Supplementary Figure 3 & Table 3). Wild type hGluN1/hGluN2A showed a mean equilibrium open probability (eq. P_{open}) of 0.69 ± 0.04 , $n = 6$ (mean $\pm$ SEM, n = number of single channel patches) (Figures 2e & 2f) whereas hGluN1/hGluN2B showed a reduced eq. P_{open} (0.34 ± 0.07 , $n = 5$) (Figures 2g & 2h)(Supplementary Table 3), as found previously^{27,28}. Certain missense mutations had no effect on activity (Figures 2f & 2h, open bars), whereas one, GluN2A(M817V), showed a significant potentiation¹⁶.

On the other hand, most tested missense mutations reduced single channel activity with the strongest effects occurring at glycines. At the conserved glycine, G815 in GluN1 and G820 in GluN2B, eq. P_{open} was less than 0.02 (G815V, 0.01 ± 0.005 , $n = 4$; G815R, 0.02 ± 0.01 , $n = 5$; and G820A, 0.008 ± 0.0005 , $n = 4$). For GluN1/GluN2B(G820E), which showed no whole-cell current amplitudes, no single channel activity could be detected (Figure 2H; Supplementary Table 3). We detected single channel activity for a missense mutation at a glycine positioned more intracellularly, GluN1(G827R), for which we could not detect whole-cell current. Again,

like other missense mutations at glycines, gating in GluN1(G827R) was strongly disrupted (0.02 ± 0.01 , $n = 4$) (Figure 2H). The lack of detectable whole-cell currents for GluN1(G827R) probably reflects that C-terminal positions in M4, especially this glycine, impact receptor surface expression¹⁵.

A missense mutation at the conserved glycine in GluN2B is pore dead.

The lack of glutamate-activated current for GluN2B(G820E), either in the whole-cell or on-cell configuration (Figure 2), could reflect disrupted cell biology, either assembly and/or trafficking, or a complete lack of gating. To test these alternatives, we measured surface expression of GluN2B(G820E) using pHmystik, a pH-sensitive GFP, and TIRF microscopy¹⁵. In contrast to wild-type GluN1/GluN2A (Figure 3a), wild-type GluN1/GluN2B showed poor surface expression (Figures 3b & 3d) with the strong background fluorescence presumably due to receptors trapped in the endoplasmic reticulum. In contrast, GluN1/GluN2B(G820E) showed robust surface expression (Figures 3c & 3d). Thus, this missense mutation in GluN2B, G820E, at least in a diheteromeric form, is pore dead: it can get to the membrane surface but cannot gate, an effect found for other positions at the extracellular end of GluN2 M4¹⁵.

To further test the importance of the conserved glycine in GluN2B, we performed a rescue experiment in which we recorded single channel activity from triheteromeric receptors, where the receptor contains a single copy of G820E (Figures 3e-3h). To generate triheteromeric receptors, we used NMDAR constructs having coiled-coiled domains²⁹ that permit only specific subunit combinations to reach the plasma membrane. The triheteromeric form, GluN1/GluN2B/GluN2B(G820E), is functional (Figure 3f), but like other missense mutations at this conserved glycine, gating is severely restricted (eq. P_{open} , 0.007 ± 0.001 , $n = 3$) (Figure 3h).

In summary, many of the missense mutations in the M4 segment strongly restrict NMDAR gating (Figures 2 & 3; Supplementary Figure 3). These effects are notable at glycines, but are most severe at a conserved glycine, leading to in the diheteromeric form a receptor that transits to the membrane but is unable to gate (Figures 3a-3d).

The conserved glycine is a key point in the M4 segments for receptor gating

The M4 segments must be displaced for efficient pore opening to occur³⁰. One possible explanation for the effect of missense mutations at glycines in the various M4 segments is that these glycines provide local flexibility for the gating process to proceed. To test this possibility, we substituted each individual glycine in the M4 segments of GluN1, GluN2A, or GluN2B (Figure 4a), including those not associated with missense mutations, with alanine, which would constrain local flexibility³¹ while not greatly altering local interactions as might occur with charged side chains. We measured single channel activity for these constructs (Figures 4b-4e).

Many of the glycine-to-alanine substitutions in GluN1, GluN2A or GluN2B M4s either had no effect (open bars) or weakly altered eq. P_{open} (G822 in GluN1). Notably, a charged missense mutation in a GluN1 glycine (G827R) had a dramatic effect on gating (Figure 2F), but the subtler alanine had no significant effect (Figure 4c; Supplementary Table 4). In contrast, alanine substitutions at the conserved glycine in all three subunits, GluN1(G815A), GluN2A(G819A), and hGluN2B(G820A), dramatically altered gating, reducing eq. P_{open} to less than 0.01. Thus, the conserved glycine plays a unique role irreplaceable by any other side chain.

Alanine substitutions at the conserved glycines dramatically reduced receptor gating by significantly altering mean closed time (MCT) and mean open time (MOT) (Figure 4d & 4e; Supplementary Table 4). In addition, the alanine substitution in GluN1 reduced single channel

activity to a similar extent as the charged (G815R) missense mutation (Figure 2f) or when tryptophan was substituted¹⁵.

The conserved glycine permits entry into long-lived open states allowing slow deactivation characteristic of synaptic NMDARs

To further address the impact of the conserved glycines to gating, we characterized closed and open time distributions of the glycine-to-alanine substitutions in GluN1 and GluN2A (Figures 5a-5c). We focused functional experiments on GluN2A rather than GluN2B because of its more robust eq. P_{open} .

Our wild-type recordings were best described by 5 closed states (Figure 5a, left; Supplementary Table 5)³², as were the recordings for alanine substitutions at the conserved glycine position in GluN1 (Figure 5b, left) and GluN2A (Figure 5c, left). Their time course and specific duration, however, showed significant variation (Supplementary Table 5).

Wild-type GluN1/GluN2A shows modal gating consisting of a brief (O_1) and three longer lived (O_2 , O_3 , and O_4) open states²⁷. For our recordings, open time distributions of wild type were also generally best described by 4 open states (Figure 5a, right; Supplementary Table 6). In contrast, the open time distributions for glycine-to-alanine substitutions were at best only fit by 3 components, with a long-lived open state completely absent (Figures 5b & 5c, right). Further, while GluN1(G815A) and GluN2A(G819A) visited a third open state, the durations were shorter (3.5 ms, $n = 2$ and 4.9 ± 1.0 ms, $n = 3$, respectively, compared to 8.7 ± 0.6 ms, $n = 10$ for wild type). Finally, GluN1(G815A) visited a third open state extremely rarely (2 out of 5 recordings), around 1% of the time compared to $39 \pm 10\%$ for wild type, highlighting the more prominent role of the GluN1 conserved glycine.

The kinetics of the NMDAR single channel activity mediate the slow macroscopic deactivation time courses characteristic of synaptic NMDARs^{24,33}. We therefore directly compared deactivation rates for GluN1/GluN2A, GluN1(G815A)/GluN2A, and GluN1/GluN2A(G819A) under conditions comparable to those done for single channel recordings (extracellular 0.05 mM EDTA) (Figure 5d). Consistent with the reduced long open times, the deactivation times for GluN1(G815A) (11.3 ± 0.8 ms, $n = 5$) and GluN2A(G819A) (23.7 ± 0.7 ms, $n = 5$) were significantly faster than that for wild type (44.2 ± 1.5 ms, $n = 5$). Paralleling the results for MOTs, deactivation for GluN1(G815A) was significantly faster than that for GluN2A(G819A) (Figure 5e).

One potential explanation for the reduced MOTs and speeding of deactivation rates is that alanine substitutions of the conserved glycines disrupt the stability of the clam-shell closed, agonist-bound LBD conformation. To test this possibility, we used cysteine cross-links in the LBD that lock the clam-shell closed (Supplementary Figure 4). The eq. P_{open} (Supplementary Figure 4b) and MOT (Supplementary Figure 4d) for glycine-to-alanine substitutions in either GluN1 or GluN2A was indistinguishable with or without the clam-shell locked close, arguing against any strong effect of the conserved glycine on LBD conformation. We also tested glycine deactivation (Supplementary Figure 5) and it too was significantly faster for both GluN1(G815A) and GluN2A(G819A) relative to wild type (Supplementary Figure 5c). Finally, desensitization properties for the glycine-to-alanine receptors do not appear to contribute strongly to differences in deactivation rates (Figure 5f).

In summary, alanine substitution of the conserved glycine in either GluN1 and GluN2A attenuates long-lived open states and speeds receptor deactivation, with these effects

significantly more prominent in GluN1. These effects are primarily due to this conserved glycine regulating the ion channel conformation as opposed to the LBD conformation.

The conserved glycine maintains open postures of the GluN1 and GluN2 M4 segments

At present there are no open state structures available for NMDARs. To understand how alanine substitutions of the conserved glycines might impact receptor function, we used homology modeling and molecular dynamic (MD) simulations. Based on our previous study³⁴, we built a homology model for the GluN1/GluN2B open state using the AMPAR open structure 5WEO³⁵ as template, introduced glycine-to-alanines, and ran simulations for 500 nanoseconds each for wild type, GluN1(G815A)/GluN2B, and GluN1/GluN2B(G820A) ([Supplementary Figure 7 & Figure 6](#)). Partly due to uncertainty of the wild-type open model, we focused our analysis on the relative difference between glycine-to-alanine mutants and wild type.

During the MD simulations, the C α RMSDs of the entire TMD from the homology model stabilized after about 200 ns for all constructs ([Supplementary Figure 7a](#)). We therefore used the 250-500 ns portion of the MD trajectories to calculate average properties. For wild type the TMD C α RMSD stabilized at 2.5 ± 0.2 Å (mean value $\pm$ SD) ([Supplementary Figure 7b](#)). For both alanine substitutions, the TMD C α RMSDs increased and, paralleling the impact on MOT, the value was higher for GluN1(G815A) (3.1 ± 0.1 Å) compared to GluN2B(G820A) (2.7 ± 0.2 Å). The C α RMSDs for the TMD core (M1-M3) and the M4 segments showed similar trends ([Figure 6a](#)) though with greater changes over time for the M4 segments ([Figure 6b](#)). Thus, relative to wild type, introducing alanine at the conserved glycine in either GluN1 or GluN2B alters not only M4 but the entire TMD structure, with these effects stronger for GluN1(G815A).

For wild type, a notable feature was the splaying of the C-termini of the M4 segments, with the hinge or pivot point occurring around the conserved glycine ([Figures 6c & 6d](#)). In contrast,

this splaying was considerably more limited for the GluN1 M4s in GluN1(G815A) (Figures 6e & 6f), while a less drastic change was observed for the GluN2 M4s in GluN2B(G820A) (Figures 6h & 6i). To quantify this effect, we measured the M4 C α displacements of GluN1(G815A) (Figure 6g) and GluN2B(G820A) (Figure 6j) from wild type. The M4 N-terminal portions of the mutants and wild type remained closely aligned. However, the displacements gradually increased toward the C-termini, where the values were much greater for GluN1 subunits in GluN1(G815A) (Figure 6g; A subunit, 5.9 Å; C, 8.9 Å) than for GluN2 subunits in GluN2B(G820A) (Figure 6j; B subunit, 0.9 Å; D, 5.3 Å). Again, these changes in the positioning of the M4 segments parallel the effect of the alanine substitutions on the stability of the open channel (Figures 2e-2h, 5a-5c).

Glycine-to-alanine mutation in GluN1 but not GluN2 disrupts the selectivity filter and reduces Ca²⁺ permeability

Given the differences in C-terminal splaying between the constructs (Figure 6), we characterized how the glycine-to-alanine mutations might impact the C-terminal portion of the permeation pathway as defined by the M2 pore helix (Figures 7a & 7b). For wild type, the average pore radius over the M2 helix is 2.0 Å (minimum at 1.6 Å) (Figures 7a & 7b, gold). In comparison, for GluN1(G815A), the pore is narrower, averaging around 1.6 Å (minimum, 1.2 Å) (Figure 7a, *right*), whereas for GluN2B(G820A), it is unchanged 2.1 Å (minimum at 1.8 Å) (Figure 7b, *right*).

The M2 helix regulates Ca²⁺ permeability in NMDARs⁸. We therefore measured relative Ca²⁺ permeability using changes in reversal potential (Figures 7c-7d)³⁶. Wild-type GluN1/GluN2A, on going from 0 to 10 mM Ca²⁺, showed a change of reversal potential (ΔE_{rev}) of about 10 mV (9.5 ± 0.9 mV, $n = 7$), yielding a $P_{\text{Ca}}/P_{\text{Na}}$ of 4 (4.1 ± 0.6). For GluN1(G815A), where the M2 pore size was reduced in the MD trajectory, the reversal potential change was

significantly reduced (5.7 ± 0.5 mV, $n = 8$), corresponding to a decrease in P_{Ca}/P_{Na} by about half (2.0 ± 0.2 , $n = 8$). In contrast, GluN1/GluN2A(G819A), where pore size was not altered, P_{Ca}/P_{Na} was not significantly changed (3.7 ± 0.6 , $n = 7$).

We also measured Ca^{2+} permeability for a missense mutation, G815R, at the conserved glycine in GluN1. For this construct, Ca^{2+} permeability was altered to the same extent as the alanine (Figure 7d) indicating that electrostatics do not contribute to the role of the GluN1 conserved glycine in modifying Ca^{2+} permeability.

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DISCUSSION

A key function of NMDARs at synapses is to mediate a time-dependent Ca^{2+} influx that defines synaptic strength and dynamics of integration^{8,26}. These features are dependent on the slow deactivation time course of NMDARs in response to transient glutamate released presynaptically and a high Ca^{2+} permeability. Here, using a combination of functional experiments and MD simulations, our data on clinically relevant mutations show that the C-terminal splaying of the M4 segments, mainly in the GluN1 subunit, regulates both deactivation and high Ca^{2+} permeability (Figure 8). These observations have multiple implications for iGluR gating and permeation, including the role of NMDARs in disease and modulation via the C-terminal domain.

The conserved glycine mainly in GluN1 permits C-terminal expansion of the ion channel

The NMDAR open state model was built using homology modeling of an AMPAR open structure³⁵ (see Online Methods). Although this model (Figures 6c & 6d) may capture the open state of wild-type NMDARs, we did not test this rigorously in the present context. Rather, we focused on the relative difference between wild type and constructs containing glycine-to-alanine substitutions either in GluN1 (Figures 6e-6g) or GluN2B (Figures 6h-6j). Both of these constructs, relative to wild type, showed greater deviations for all transmembrane segments with these effects consistently stronger for GluN1(G815A) (Figure 6b). The most notable difference between wild type and the glycine-to-alanine constructs was in the M4 segments, as clearly revealed by GluN1(G815A), where the conserved glycine maintains the bottom two-thirds of the helix in an open posture, leaving the upper one-third in close proximity to other TMD elements (Figure 6g). We assume that the major effect of the glycine-to-alanine in GluN1 is to limit this splaying of M4, reducing the stability of long-open states (Figure 8). The glycine-to-alanine

substitution in GluN2B also alters the positioning of M4 relative to wild type (Figure 6j), but this effect is small and may not reflect the major pathway by which the conserved glycine in GluN2 impacts receptor function.

One prediction arising from the MD simulations is that the glycine-to-alanine in GluN1 but not GluN2 restricts the pore diameter of the M2 pore loop (Figures 7a & 7b). Supporting this observation is that Ca^{2+} permeation in GluN1(G815A) but not GluN2A(G819A) was significantly reduced (Figures 7c & 7d). This result supports the relative differences in the MD simulations and also indicates that pore expansion impact the high Ca^{2+} influx mediated by NMDARs.

That glycine-to-alanine substitutions at neighboring positions in the M4 segments had no significant effects on receptor gating (Figure 4) suggest that, rather than merely providing local flexibility to the M4 segments, the conserved glycines play unique roles irreplaceable by any other side chain. In part it may reflect that the relative positioning of the glycine in the M4 helix is critical, with structural elements positioned external to the glycine being involved in critical interactions²² and those internal to the glycine requiring flexibility¹⁵. In addition, the open state model suggests a Ca hydrogen bond that connects the S1-M1 directly to the conserved glycine in the M4³⁴, which could also alter receptor gating.

We also envision that the M4 is passively, not actively, splaying in response to pore opening. Consistent with this idea, specific side chain interactions of the C-terminal two-thirds of the M4 helix appear unimportant to gating¹⁵ as would be expected if it acted permissively.

Stability of NMDAR open state in the pathogenesis of neurological disorders

Given the high number of patients identified with mutations in the conserved glycine relative to other M4 transmembrane segment mutations, it is unlikely that mutations at this site are merely

epiphenomena. Indeed, such drastic alterations in NMDAR structure and subsequent dysfunction imply a causal relationship. One therefore asks what aspect of this dysfunction plays the dominant role in the pathogenesis of these disorders. Notably, most M4 missense mutations that we measured had reduced channel activity characterized by a destabilized open state (Figure 2). Though a reduction in excitatory glutamatergic activity leading to epilepsy may seem counterintuitive, it most likely changes the balance of excitation and inhibition in the nervous system.

The most identifiable pathology amongst these mutations is epilepsy. However, a dysfunction common to nearly all M4 missense mutations is intellectual disability ([Supplementary Table 1](#)), suggesting that the problem may lie in development. The GluN2B subunit, which carries numerous mutations at the conserved glycine, is most highly expressed before birth²⁶. GluN1, being an obligate subunit, is expressed at all stages of development. Interestingly, the absence of any conserved G mutants in GluN2A, a subunit that is primarily expressed in the adult brain, suggest that pathogenesis of these disorders strongly involves the NMDARs role in the developing brain.

The M4 segments as a hub of NMDAR modulation

The M4 transmembrane helix in iGluRs is the most peripheral transmembrane segment in the iGluR ion channel, interacts with lipids, and is attached to the regulatory C-terminal domain. Lipids³⁷ and post-translational modifications of the C-terminal domain³⁸ can modulate ion channel gating and Ca²⁺ permeation, but how these modifications arise is unknown. We hypothesize that a major pathway by which they act is by regulating the permissiveness of the M4. Future experiments will be needed to directly test this idea.

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Author Checklist

J.A., H-X.Z., and L.P.W. designed research; J.A., A.G. and L.P.W. carried out and/or analyzed the functional experiments; X.L. and H-X.Z., performed the computational studies including modeling and molecular dynamic simulations; J.A., X.L, H-X. Z., and L.P.W. wrote the paper.

Competing Interests statements.

“The authors declare no competing financial interest’.

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FIGURE LEGENDS

Figure 1. Topology of NMDA receptors and distribution of missense mutations in and around the M4 transmembrane segments

- (a)** Model NMDAR structure (structure based on 4TLM of GluN1/GluN2B)¹⁵ lacking the intracellular C-terminal domain (CTD). Subunits are colored light orange (GluN1) and gray 60% (GluN2). For iGluRs, the tetrameric complex is composed of four highly modular domains: the extracellularly located amino-terminal (ATD) and ligand-binding (LBD) domains; the membrane-spanning transmembrane domain (TMD) forming the ion channel; and the CTD. Positions highlighted in magenta are disease-associated missense mutations in the GluN1, GluN2A, or GluN2B M4 transmembrane segment or the S2-M4 linker (L812M and I814T in GluN2A).
- (b)** An enlarged view of the extracellular end of the TMD highlighting the distribution of missense mutations in and around the M4 segments.
- (c)** The GluN1, GluN2A, and GluN2B M4 segments indicating the specific location of missense mutations. Each dot represents a patient in which the mutation has been identified ([Supplementary Table 1](#)). The dashed line separates M3 (*leftside*) versus lipid (*rightside*) facing portions of the M4 transmembrane segment.

Figure 2. Missense mutations in M4 segments alter NMDAR gating.

- (a & c)** Whole-cell currents from HEK 293 cells expressing human NMDAR subunits, either hGluN1/hGluN2A (**a**) or hGluN1/GluN2B (**c**) or the same receptor with a missense mutation at the conserved glycine. Currents were elicited by a 2 ms application of glutamate (1 mM) in the continuous presence of glycine (0.1 mM), as approximately occurs at synapses. Gray traces are wild type. Holding potential, -70 mV.

(b & d) Bar graphs (mean $\pm$ SEM) ($n \geq 5$) showing deactivation rates for hGluN1/hGluN2A **(b)** or hGluN1/hGluN2B **(d)** ([Supplementary Table 2](#)). No whole-cell current could be detected for hGluN1(G827R)/hGluN2A or hGluN1/hGluN2B(G820E), which were tested on at least three different transfection cycles ([Supplementary Figure 2](#)). Solid bars indicate values significantly different from wild type ($p < 0.05$, *t*-test).

(e & g) Example single channel recordings of hGluN1/hGluN2A **(e)** or hGluN1/hGluN2B **(g)** or the same receptor containing missense mutations at the conserved glycine. Recordings were performed in the on-cell configuration (holding potential, +100 mV). Downward deflections are inward currents. For each construct, the top half shows a low-resolution (filtered at 1 kHz) and the bottom half a higher resolution portion of same record (3 kHz).

(f & h) Equilibrium open probability (*eq. P_{open}*) (mean $\pm$ SEM, $n \geq 4$ patches) for hGluN1/hGluN2A **(f)** or hGluN1/hGluN2B **(h)** ([Supplementary Table 3](#)). Solid bars indicate values significantly different from wild type ($p < 0.05$, *t*-test). For GluN1/GluN2B(G820E), we could not detect glutamate-activated current either in the on-cell patch or whole-cell mode.

Figure 3. The diheteromeric missense mutation of a conserved glycine, GluN2B(G820E), is pore dead.

(a-c) Assaying surface expression using a pH-sensitive GFP¹⁵ indicates that GluN1/GluN2B(G820E) is expressed on the membrane. GFP intensity as the extracellular solution pH was changed from 7.4 to 5.5 (gray bar, 30 seconds) and back again. The representative cell images are from the time-points labeled in panel A: image (i) was taken around baseline fluorescence (F_o), whereas image (ii) around the test fluorescence (F_{test}). The change in fluorescence ($\Delta F = F_o - F_{test}$) was used as an index of surface expression.

Sampling rate, 5 seconds. The strong fluorescent background for GluN2B constructs presumably reflects subunits trapped in endoplasmic reticulum.

- (d)** Changes in cell fluorescence at low pH (ΔF). Significant differences from pHmystick-GluN1/GluN2A and pHmystick-GluN1 alone are indicated by # and **, respectively ($p < 0.05$, t -test). The numbers (far right) indicate the number of cells that showed detectable changes in fluorescence relative to the total number of cells tested.
- (e-g)** Single channel recordings of NMDAR constructs containing the coiled-coiled domain in C-terminus²⁹, either wild type **(e)**, the triheteromeric containing a single copy of G820E **(f)** or the diheteromeric containing 2 copies of G820E **(g)**, which does not show detectable glutamate-activated current (Figure 2), though it expresses on the membrane. The triheteromeric receptor shows detectable single channel activity.
- (h)** Mean ($\pm$ SEM) showing *eq. P_{open}* for wild type ($n = 4$) and triheteromeric GluN1/GluN2B/GluN2B(G820E) ($n = 3$).

Figure 4. Alanine substitutions of glycine in the M4 segments reveal that the conserved glycine has a unique functional role in gating.

- (a)** Sequence of M4 segments and adjacent regions highlighting glycines.
- (b)** Example single channel recordings in the on-cell configuration of wild type rat GluN1/GluN2A and various receptors containing glycine-to-alanine substitutions. Recordings displayed as in Figure 2E.
- (c-e)** Mean ($\pm$ SEM) ($n \geq 4$ patches) showing equilibrium open probability (*eq. P_{open}*) **(c)**, mean closed time (MCT) **(d)**, and mean open time (MOT) **(e)** for alanine substitutions of glycines in the M4 segments of rat GluN1 and GluN2A and human GluN1 (hGluN1) and GluN2B

(hGluN2B) ([Supplementary Table 4](#)). Solid bars indicate values significantly different from their respective wild types ($p < 0.01$, *t-test*).

Figure 5. Constraining the conserved glycine prevents entry into long-lived open states and speeds deactivation.

(a-c) Closed (*left*) and open (*right*) time histograms for wild type **(a)** or for receptors containing an alanine substitution at the conserved glycine in GluN1 **(b)** or GluN2A **(c)**. All closed time histograms were best fit with 5 exponentials ([Supplementary Table 5](#)), whereas open time histograms were best fit typically with 4 (wild type), 2 (GluN1(G815A)), or 3 (GluN2A(G18A)) exponentials ([Supplementary Table 6](#)) (see Online Methods). Smooth lines are associated exponential fits.

Insets, mean closed and open state durations (τ , ms), occupancies (α , %), and for open time distributions how many recordings contained that open time ([Supplementary Tables 5 & 6](#)). For GluN1(G815A), the longest-lived open state (3.5 ms) was identified in only 2 out of 5 recordings; # indicates that we did not do statistics on this state.

(d) Whole-cell currents in response to a 2 ms application of glutamate (1 mM, gray bars) applied in the continuous presence of glycine (light gray bars). Recordings were made and analyzed as in Figure 2A but included extracellular 0.05 mM EDTA to remove effects of Zn^{2+} as done for single channel recordings.

(e & f) Mean ($\pm$ SEM) ($n \geq 5$) showing deactivation rates **(e)** or desensitization properties **(f)** for constructs shown in D. Solid bars indicate values significantly different from wild type, whereas asterisks indicate values different from each other ($p < 0.05$, *ANOVA, Tukey*).

Figure 6. MD simulations show that constraining the conserved glycine in GluN1 prevents C-terminal expansion of the M4 as observed in wild type.

- (a) C α RMSDs of the ion channel cores (M1-M3, thick lines) and M4s (thin lines) as a function of simulation time for open state models: wild type GluN1/GluN2B, light orange; GluN1(G815A)/GluN2B, light blue; GluN1/GluN2B(G820A), maroon (see Online Methods).
- (b) Average RMSD values (250-500 ns). Error bars are standard deviations.
- (c & d) Open state of wild type GluN1/GluN2B TMD shown either top down (c) or side views (d) of the GluN1 (*left*) or GluN2B (*right*) M4s. All displayed structures are snapshots close to the average structures calculated over 250-500 ns.
- (e & f) GluN1(G815A) overlaid on wild type either from top down (e) or with a side view (f).
- (g) Displacements between GluN1(G815A) and wild type for M4 residues in the A and C subunits.
- (h & i) GluN2B(G820A) overlaid on wild type either from top down (h) or with a side view (i).
- (j) Displacements between GluN2B(G820A) and wild type for M4 residues in the B and D subunits.

Figure 7. Constraining the conserved G in GluN1 reduces the dimensions of the selectivity filter and Ca²⁺ permeability.

- (a) *Left*, Cytoplasmic view of model open state structures of wild type and GluN1(G815A). The constrained M4 collapses the adjacent GluN2B ion channel core (M1-M3) including the pore size as defined by the M2 loop. *Right*, Average pore radius along the channel axis for the M2 pore loop for wild type (gold) and GluN1(G815A) (light blue). '0' references the serine (S) in SYTANLAAF in M3. Thick lines are mean values and thinner lines the error bars, which were calculated using block averages based on a time block of 20 ns.
- (b) Same as (A) except construct is GluN2B(G820A) (maroon).

- (c) Current-voltage (IV) relationships in an external solution containing high Na^+ (140 mM) and 0 added Ca^{2+} (open circles) or 10 mM Ca^{2+} (solid circles). The 0 Ca^{2+} IV is the average of that recorded before and after the 10 mM Ca^{2+} recording. We used changes in reversal potentials (ΔE_{rev}) to calculate $P_{\text{Ca}}/P_{\text{Na}}$ ³⁶.
- (d) Mean ($\pm$ SEM) showing relative calcium permeability ($P_{\text{Ca}}/P_{\text{Na}}$) calculated from ΔE_{rev} s for rat GluN1/GluN2A ($n = 7$), GluN1(G815A) ($n = 8$), or GluN2A(G819A) ($n = 7$) as well as human GluN1/GluN2A ($n = 11$) and the missense mutation GluN1(G815R) ($n = 7$). Values either are not (open bars) or are (solids bars) significantly different from each other ($p < 0.05$, ANOVA, Tukey).

Figure 8. Expansion of the inner pore permitted mainly by the GluN1 M4 conserved glycine facilitates NMDAR gating and Ca^{2+} permeation.

Cartoon of NMDAR pore structure including the influence of the GluN1 M4 on open state stability and selectivity filter integrity. Subunits are colored light orange (GluN1) and gray (GluN2).

ONLINE METHODS

Molecular biology and cell culture

All manipulations were made in rat GluN1 (GluN1a) (NCBI Protein database accession no. P35439), GluN2A (Q00959), or GluA2 (P19491) subunits or human GluN1 (hGluN1; Q05586), GluN2A (hGluN2A; Q12879), or GluN2B (hGluN2B; Q13224) subunits. Unless otherwise noted constructs tested were rat. In all instances, numbering included the signal peptide (GluN1, 18 residues; GluN2A, 19 residues; GluN2B, 26 residues; GluA2, 21 residues). Mutations were generated via QuickChange site-directed mutagenesis (Agilent) with XL1-Blue super-competent cells.

Cell Culture and Transfection. For details on cell culture and transfection see Amin et al., 2017. Briefly, human embryonic kidney 293 (HEK 293) or HEK 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), for 24 hours prior to transfection. Non-tagged cDNA constructs were co-transfected into HEK 293 cells along with a separate pEGFP-Cl vector at a ratio of 4.5:4.5:1 (N1/N2/EGFP for NMDARs) using X-tremeGene HP (Roche). GFP-tagged constructs were transfected at a 1:1 ratio. To improve cell survivability, HEK 293 cells were bathed in a media containing the NMDAR competitive antagonist APV (100 μ M) and Mg^{2+} (100 μ M) (single channel experiments) or the transfection mixture was replaced four hours after transfection with fresh 5% FBS-DMEM culture media containing APV (100 μ M) and Mg^{2+} (1 mM) (whole-cell and imaging experiments). All experiments were performed 18-48 hours post-transfection.

Triheteromeric expression system. NMDARs are obligate heterotetramers typically composed of 2 GluN1 and 2 GluN2 subunits. Co-expression of wild-type GluN1 and GluN2A and a subunit with a mutation[e.g., GluN2A(mutation)] would result in three populations of cell-surface

expressed receptors: GluN1/GluN2A (di-heteromeric); GluN1/GluN2A/GluN2A(mutation) (tri-heteromeric); and GluN1/GluN2A(mutation) (di-heteromeric). To restrict surface expression to defined tri-heteromeric receptors, we used the ‘triheteromeric’ system developed by the Traynelis lab²⁹, which is based on leucine zipper motifs from GABA_B receptors and ER retention/retrieval motifs introduced into the C-terminal domain of NMDAR subunits. All constructs were kindly provided by Dr. Kasper Hansen (University of Montana).

Molecular modeling and simulations

Previously, we constructed an NMDAR open-state model by repacking the transmembrane helices³⁴. The model was very similar to the GluA2 open-state structure (Protein Data Bank (PDB) entry 5WEO)³⁵, indicating that 5WEO could be a useful template for modeling the open state of the NMDAR TMD.

Homology modeling. We used homology modeling to build an initial model for the GluN1/GluN2B receptor in the open state (without the disordered C-terminal domain)³⁹. We then introduced glycine-to-alanine mutations at the conserved position, producing the N1(G815A)/N2B and N1/N2B(G820A) mutants. For the wild-type open model, we chose PDB entry 5FXG⁴⁰ as the template for the extracellular amino-terminal domain (ATD) and LBD³⁴, and 5WEO³⁵ as the template for the TMD. The templates were both aligned to the closed NMDAR structure 4TLM⁴¹. We then used Modeller 9.18⁴² for homology modeling, including generation of missing residues.

Our previous study³⁴ suggested that interactions between the pre-M1 helix and the extracellular end of the M4 helix are important for stabilizing the open state. Among these interactions are special hydrogen bonds formed with the C α atoms of the conserved glycines as donors. Here we introduced such hydrogen bonds into our initial open model, in a 14-ps fine-

tuning simulation in vacuum using NAMD 2.11. In this simulation, all C α atoms were restrained with a force constant of 5 kcal mol⁻¹Å⁻² except for residues K544-Q559 in GluN1 and R540-S555 in GluN2B containing the pre-M1 helices. In addition, C α atoms of G815 in GluN1 and G820 in GluN2B were restrained to a hydrogen-bonding distance of 3.2 Å from backbone carbonyl oxygen atoms of L551 in GluN1 and P547 on GluN2B, respectively, with a force constant of 0.1 kcal mol⁻¹Å⁻². The G815A mutation in GluN1 and G820A mutation in GluN2B were made using VMD⁴³ to produce open models for the two mutants.

Molecular dynamics (MD) simulations. Following our previous study³⁴, MD simulations were carried out for the TMD construct, consisting of residues L541-P670 and R794-A841 in GluN1 and M539-K669 and G799-F845 in GluN2B, truncated from our full-length models. The four LBD-proximal residues of the LBD-TMD linkers (L541-K544, I667-P670, R794-E797 in GluN1 and M537-R540, L666-K669, G799-H802 in GluN2B) were restraint to mimic the agonist-bound form of the LBD that maintains channel opening. The TMD construct was embedded in a membrane bilayer composed of 222 POPC lipids, and solvated by 25,000 water molecules and 0.15 M NaCl, using CHARMM-GUI⁴⁴. CHARMM-GUI's standard 6-step equilibration protocol was followed to equilibrate the system. An additional equilibration step of 12 ns was used to ramp down the restraints on the C α atoms except for the LBD-proximal linker residues. The final production run was 500 ns with restraints on the LBD-proximal linker C α remain at 5 kcal mol⁻¹Å⁻².

Starting from the CHARMM-GUI's equilibration step, simulations were performed using AMBER 14 on GPUs⁴⁵ with CHARMM36 force field⁴⁶. The time step was 2 fs. Nonbonded interactions were cut off at 8.0 Å, and the particle mesh Ewald method was used for long-range electrostatic interactions⁴⁵. The NPT ensemble was used in simulation, with temperature

controlled at 300 K by Langevin dynamics and pressure controlled at 1 bar by the Berendsen method⁴⁷.

Analysis. MD trajectories were saved once every 100 ps (5,000 frames for each construct). Root-mean-square-deviations (RMSDs) were calculated using C α atoms of either all the helical segments (M1-M4) or only M1-M3 in the ion channel core. In the latter case, the RMSD for M4 was also calculated using the structural alignment on M1-M3. The reference for all the RMSD calculations was the homology model. The aligned structures were used to calculate an average structure, which was then used to select a representative snapshot from each MD trajectory. The pore radii along the pore axis were calculated using the HOLE program⁴⁸ on every 10th of the saved frames.

Macroscopic current recordings

Macroscopic currents in the whole-cell mode or outside-out patches, isolated from HEK 293 cells, were recorded at room temperature (20-23°C) using an EPC-9 or EPC-10 amplifier with Patchmaster software (HEKA Elektronik, Lambrecht, Germany), digitized at 10 kHz and low-pass filtered at 2.8 kHz (-3 dB) using an 8 pole low pass Bessel filter. Patch microelectrodes were filled with our standard intracellular solution (in mM): 140 KCl, 10 HEPES, 1 BAPTA, pH 7.2 (KOH). Our standard extracellular solution consisted of (in mM): 140 NaCl, 1 CaCl₂, 10 HEPES, pH 7.2 (NaOH). Pipettes had resistances of 2-6 M Ω when filled with the pipette solution and measured in the standard Na⁺ external solution. We did not use series resistance compensation nor did we correct for junction potentials. Currents were measured within 15 minutes of going whole-cell.

External solutions were applied using a piezo-driven double barrel application system. The open tip response (10-90% rise time) of the application system was between 400-600 μ s. For display, NMDAR currents were digitally refiltered at 500 Hz and resampled at 1 kHz.

Rates of deactivation and desensitization. To determine the rates of activation and deactivation, we applied glutamate for 2 ms at -70 mV in the whole-cell mode. For activation times, we used the 10-90% rise time. Deactivation times (weighted τ s) were derived by fitting the decay phase of currents with a double-exponential function. To determine the extent and rate of desensitization, we applied glutamate at -70 mV for 2.5 secs in the whole-cell mode. Percent desensitization (% des) was calculated from the ratio of peak (I_{peak}) and steady-state (I_{ss}) current amplitudes: %des = 100 x (1 - $I_{\text{ss}}/I_{\text{peak}}$). Time constants of desensitization were determined by fitting the decaying phase of currents a double-exponential function. In some instances when current amplitudes were small, we averaged 3-12 records.

Ca²⁺ permeability. To quantify Ca²⁺ permeability, we measured changes in reversal potentials, ΔE_{rev} , for glutamate-activated currents on replacing a reference solution (140 mM NaCl, 10 mM HEPES, 0 added Ca²⁺, pH 7.2, NaOH) with a test solution (the same solution but with added 10 mM CaCl₂). Ca²⁺ permeability ratios ($P_{\text{Ca}}/P_{\text{Na}}$) were calculated using the Lewis equation³⁶. We did not correct for activity coefficients.

Single-channel recordings and analysis

All single-channel recordings were performed in the on-cell configuration at steady state. The pipette solution, which mimicked extracellular agonist conditions, contained (in mM): 150 NaCl, 10 HEPES, 0.05 EDTA, 1 glutamate, and 0.1 glycine, pH 8.0 (NaOH). The high pH and EDTA were used to minimize proton and divalent mediated inhibitory effects, respectively²⁷. Recording pipettes were pulled from thick-wall borosilicate capillary glass (Sutter Instruments) and fire-

polished to final pipette resistances ranging from 5-30 M Ω when measured in the bath solution (with an applied positive pipette pressure of approximately 200 mbar). Cells were identified by GFP fluorescence and patched to resistances exceeding 1.5 G Ω . To elicit inward current amplitudes, we held the electrode voltage at +100 mV. Currents were recorded using a patch clamp amplifier (Axopatch 200B; Molecular Devices), filtered at 10 kHz (four-pole Bessel filter), and digitized at 40 kHz (ITC-16 interfaced with PatchMaster). Experiments ran for approximately 3-20 min to ensure a significant number of events for analysis.

NMDAR single channel analysis. Analysis of single-channel records was comparable to Talukder and Wollmuth⁴⁹. Data was exported from PatchMaster to QuB. Processed data was idealized using the segmental k-means (SKM) algorithm in QuB with a dead time of 20 μ s. Closed and open state fits were performed using the maximum interval likelihood (MIL) algorithm in QuB. For most recordings, we measured only single-channel amplitudes, mean closed time (MCT), and mean open time (MOT) as well as equilibrium open probability (eq. P_o).

For certain constructs, we explored in more detail the occupancy of different closed and open states. Kinetic models of NMDA receptor gating activation have been proposed to contain approximately 5 closed states and 3-4 open states^{27,28}. For each individual record, state models with increasing closed (3 to 6) and open (2 to 4) states were constructed and fitted to the recordings until log-likelihood (LL) values improved by less than 10 LL units/added state or if the next added state showed 0% occupancy⁴⁹. To verify single channels in patches, especially for those constructs with a low equilibrium P_o , we used an approach as described previously³⁰.

Assaying surface expression using pH sensitive GFP

Surface expression was assayed as described previously¹⁵. Briefly, HEK 293T cells were imaged 24-48 hours post-transfection using a Nikon Ti Eclipse microscope equipped with the Nikon

TIRF slider. Excitation used the 488 nm line from a fiber-coupled argon ion laser (Lasos, Jena, Germany). The fluorescence emission was collected with a Nikon 60x 1.45 NA oil-immersion TIRF objective and relayed to an iXon DU897 emCCD camera (Andor Technologies, South Windsor, CT). The fluorescence emission was transmitted using a dual band 488/561 TIRF filter cube with a GFP band pass of 525/50 (Chroma, Bellows Falls, VT). Cells were observed at 5 second intervals with 30-50 exposures collected for each field of view, which contained 1-4 fluorescent cells. Cells were imaged in either a bath solution at pH 7.4, consisting of (in mM): 140 NaCl, 10 HEPES, pH 7.4 (NaOH); or a bath solution at pH 5.5, consisting of (in mM): 140 NaCl, 30 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.5 (HCl). These bathing solutions were exchanged using a continuous flow perfusion system.

Fluorescence intensity was quantified in ImageJ⁵⁰. Cells were selected for analysis that were at least 50% isolated from other cells. The fluorescence intensity (F) of a cell of interest was calculated for each frame: $F = F_{\text{cell}} - F_{\text{backgnd}}$, where F_{cell} is the mean fluorescence of the cell, defined by a polygon circumscribing it, and F_{backgnd} is the mean fluorescence of an acellular region directly adjacent to the cell of interest. For display and analysis, we normalized fluorescence intensity to a baseline fluorescence (F_0), which was the mean F just before the solution was changed from pH 7.4 to 5.5. The change in fluorescence (ΔF) was defined as $\Delta F = F_0 - F_{\text{test}}$, where F_{test} is the fluorescence intensity taken 15-30 seconds after the solution was switched from pH 7.4 to 5.5. We defined ‘detectable’ surface expression as occurring when the fluorescence intensity decreased rapidly upon switching to pH 5.5 and returned to baseline with a return to pH 7.4. For some constructs, we detected both positive and negative (no detectable ΔF); in these instances, we averaged surface expression only for those cells that were positive.

Statistics

Data analysis was performed using IgorPro, QuB, Excel and MiniTab 18. All average values are presented as mean $\pm$ SEM. The number of replicates is indicated in the figure legend or in a table associated with the figure. Often, we were only interested in whether outcomes were statistically different from that for wild type. In these instances, we used an unpaired two-tailed Student's t-test to test for significant differences. An analysis of variance (ANOVA), followed by the Tukey or Dunnet's test, was used for multiple comparisons. Unless otherwise noted, statistical significance was set at $p < 0.05$.

Data availability: Igor and/or Excel files of all raw data is available upon request.

Figure 1. Topology of NMDA receptors and distribution of missense mutations in and around the M4 transmembrane segments

- (a)** Model NMDAR structure (structure based on 4TLM of GluN1/GluN2B)[Amin, 2017 #2943] lacking the intracellular C-terminal domain (CTD). Subunits are colored light orange (GluN1) and gray 60% (GluN2). For iGluRs, the tetrameric complex is composed of four highly modular domains: the extracellularly located amino-terminal (ATD) and ligand-binding (LBD) domains; the membrane-spanning transmembrane domain (TMD) forming the ion channel; and the CTD. Positions highlighted in magenta are disease-associated missense mutations in the GluN1, GluN2A, or GluN2B M4 transmembrane segment or the S2-M4 linker (L812M and I814T in GluN2A).
- (b)** An enlarged view of the extracellular end of the TMD highlighting the distribution of missense mutations in and around the M4 segments.
- (c)** The GluN1, GluN2A, and GluN2B M4 segments indicating the specific location of missense mutations. Each dot represents a patient in which the mutation has been identified (Table S1). The dashed line separates M3 (*leftside*) versus lipid (*rightside*) facing portions of the M4 transmembrane segment.

Figure 2. Missense mutations in M4 segments alter NMDAR gating.

(a & c) Whole-cell currents from HEK 293 cells expressing human NMDAR subunits, either hGluN1/hGluN2A **(a)** or hGluN1/hGluN2B **(c)** or the same receptor with a missense mutation at the conserved glycine. Currents were elicited by a 2 ms application of glutamate (1 mM) in the continuous presence of glycine (0.1 mM), as approximately occurs at synapses. Gray traces are wild type. Holding potential, -70 mV.

(b & d) Bar graphs (mean $\pm$ SEM) ($n \geq 5$) showing deactivation rates for hGluN1/hGluN2A **(b)** or hGluN1/hGluN2B **(d)** ([Table S2](#)). No whole-cell current could be detected for

hGluN1(G827R)/hGluN2A or hGluN1/hGluN2B(G820E), which were tested on at least three different transfection cycles (Figure S2). Solid bars indicate values significantly different from wild type ($p < 0.05$, *t-test*).

- (e & g)** Example single channel recordings of hGluN1/hGluN2A (**e**) or hGluN1/hGluN2B (**g**) or the same receptor containing missense mutations at the conserved glycine. Recordings were performed in the on-cell configuration (holding potential, +100 mV). Downward deflections are inward currents. For each construct, the top half shows a low-resolution (filtered at 1 kHz) and the bottom half a higher resolution portion of same record (3 kHz).
- (f & h)** Equilibrium open probability (*eq. P_{open}*) (mean $\pm$ SEM, $n \geq 4$ patches) for hGluN1/hGluN2A (**f**) or hGluN1/hGluN2B (**h**) (Table S3). Solid bars indicate values significantly different from wild type ($p < 0.05$, *t-test*). For GluN1/GluN2B(G820E), we could not detect glutamate-activated current either in the on-cell patch or whole-cell mode.

Figure 3. The diheteromeric missense mutation of a conserved glycine, GluN2B(G820E), is pore dead.

- (a-c)** Assaying surface expression using a pH-sensitive GFP [Amin, 2017 #2943] indicates that GluN1/GluN2B(G820E) is expressed on the membrane. GFP intensity as the extracellular solution pH was changed from 7.4 to 5.5 (gray bar, 30 seconds) and back again. The representative cell images are from the time-points labeled in panel A: image (i) was taken around baseline fluorescence (F_o), whereas image (ii) around the test fluorescence (F_{test}). The change in fluorescence ($\Delta F = F_o - F_{test}$) was used as an index of surface expression. Sampling rate, 5 seconds. The strong fluorescent background for GluN2B constructs presumably reflects subunits trapped in endoplasmic reticulum.
- (d)** Changes in cell fluorescence at low pH (ΔF). Significant differences from pHmystik-GluN1/GluN2A and pHmystik-GluN1 alone are indicated by # and **, respectively ($p < 0.05$, t -test). The numbers (far right) indicate the number of cells that showed detectable changes in fluorescence relative to the total number of cells tested.
- (e-g)** Single channel recordings of NMDAR constructs containing the coiled-coiled domain in C-terminus [Hansen, 2014 #2888], either wild type (**e**), the triheteromeric containing a single copy of G820E (**f**) or the diheteromeric containing 2 copies of G820E (**g**), which does not show detectable

glutamate-activated current (Figure 2), though it expresses on the membrane. The triheteromeric receptor shows detectable single channel activity.

- (h)** Mean ($\pm$ SEM) showing *eq. P_{open}* for wild type (n = 4) and triheteromeric GluN1/GluN2B/GluN2B(G820E) (n = 3).

Figure 4. Alanine substitutions of glycine in the M4 segments reveal that the conserved glycine has a unique functional role in gating.

(a) Sequence of M4 segments and adjacent regions highlighting glycines.

(b) Example single channel recordings in the on-cell configuration of wild type rat GluN1/GluN2A and various receptors containing glycine-to-alanine substitutions. Recordings displayed as in Figure 2E.

(c-e) Mean ($\pm$ SEM) ($n \geq 4$ patches) showing equilibrium open probability (eq. P_{open}) (c), mean closed time (MCT) (d), and mean open time (MOT) (e) for alanine substitutions of glycines in the M4 segments of rat GluN1 and GluN2A and human GluN1 (hGluN1) and GluN2B (hGluN2B) (Table S4). Solid bars indicate values significantly different from their respective wild types ($p < 0.01$, t -test).

Figure 5. Constraining the conserved glycine prevents entry into long-lived open states and speeds deactivation.

(a-c) Closed (*left*) and open (*right*) time histograms for wild type (a) or for receptors containing an alanine substitution at the conserved glycine in GluN1 (b) or GluN2A (c). All closed time histograms were best fit with 5 exponentials (Table S5), whereas open time histograms were best fit typically with 4 (wild type), 2 (GluN1(G815A)), or 3 (GluN2A(G18A)) exponentials (Table S6) (see Online Methods). Smooth lines are associated exponential fits.

Insets, mean closed and open state durations (τ , ms), occupancies (α , %), and for open time distributions how many recordings contained that open time (Tables S5 & S6). For GluN1(G815A), the longest-lived open state (3.5 ms) was identified in only 2 out of 5 recordings; # indicates that we did not do statistics on this state.

(d) Whole-cell currents in response to a 2 ms application of glutamate (1 mM, gray bars) applied in the continuous presence of glycine (light gray bars). Recordings were made and analyzed as in Figure 2A but included extracellular 0.05 mM EDTA to remove effects of Zn^{2+} as done for single channel recordings.

(e & f) Mean ($\pm$ SEM) ($n \geq 5$) showing deactivation rates (e) or desensitization properties (f) for constructs shown in D. Solid bars indicate values significantly different from wild type, whereas asterisks indicate values different from each other ($p < 0.05$, ANOVA, Tukey).

Figure 6. MD simulations show that constraining the conserved glycine in GluN1 prevents C-terminal expansion of the M4 as observed in wild type.

- (a) C α RMSDs of the ion channel cores (M1-M3, thick lines) and M4s (thin lines) as a function of simulation time for open state models: wild type GluN1/GluN2B, light orange; GluN1(G815A)/GluN2B, light blue; GluN1/GluN2B(G820A), maroon (see Online Methods).
- (b) Average RMSD values (250-500 ns). Error bars are standard deviations.
- (c & d) Open state of wild type GluN1/GluN2B TMD shown either top down (c) or side views (d) of the GluN1 (left) or GluN2B (right) M4s. All displayed structures are snapshots close to the average structures calculated over 250-500 ns.

- (e & f)** GluN1(G815A) overlaid on wild type either from top down **(e)** or with a side view **(f)**.
- (g)** Displacements between GluN1(G815A) and wild type for M4 residues in the A and C subunits.
- (h & i)** GluN2B(G820A) overlaid on wild type either from top down **(h)** or with a side view **(i)**.
- (j)** Displacements between GluN2B(G820A) and wild type for M4 residues in the B and D subunits.

Figure 7. Constraining the conserved G in GluN1 reduces the dimensions of the selectivity filter and Ca^{2+} permeability.

- (a) *Left*, Cytoplasmic view of model open state structures of wild type and GluN1(G815A). The constrained M4 collapses the adjacent GluN2B ion channel core (M1-M3) including the pore size as defined by the M2 loop. *Right*, Average pore radius along the channel axis for the M2 pore loop for wild type (gold) and GluN1(G815A) (light blue). '0' references the serine (S) in SYTANLAAF in M3. Thick lines are mean values and thinner lines the error bars, which were calculated using block averages based on a time block of 20 ns.
- (b) Same as (A) except construct is GluN2B(G820A) (maroon).
- (c) Current-voltage (IV) relationships in an external solution containing high Na^+ (140 mM) and 0 added Ca^{2+} (open circles) or 10 mM Ca^{2+} (solid circles). The 0 Ca^{2+} IV is the average of that recorded before and after the 10 mM Ca^{2+} recording. We used changes in reversal potentials (ΔE_{rev}) to calculate $P_{\text{Ca}}/P_{\text{Na}}$ [Jatzke, 2002 #1569].
- (d) Mean ($\pm$ SEM) showing relative calcium permeability ($P_{\text{Ca}}/P_{\text{Na}}$) calculated from ΔE_{rev} for rat GluN1/GluN2A ($n = 7$), GluN1(G815A) ($n = 8$), or GluN2A(G819A) ($n = 7$) as well as human GluN1/GluN2A ($n = 11$) and the missense mutation GluN1(G815R) ($n = 7$). Values either are not (open bars) or are (solids bars) significantly different from each other ($p < 0.05$, ANOVA, Tukey).

Figure 8. Expansion of the inner pore permitted mainly by the GluN1 M4 conserved glycine facilitates NMDAR gating and Ca²⁺ permeation.

Cartoon of NMDAR pore structure including the influence of the GluN1 M4 on open state stability and selectivity filter integrity. Subunits are colored light orange (GluN1) and gray (GluN2).