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Allosteric modulation of GluN1/GluN3 NMDA receptors by GluN1-selective competitive antagonists

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NMDA-type ionotropic glutamate receptors are critical for normal brain function and are implicated in central nervous system disorders. Structure and function of NMDA receptors composed of GluN1 and GluN3 subunits are less understood compared to those composed of GluN1 and GluN2 subunits. GluN1/3 receptors display unusual activation properties in which binding of glycine to GluN1 elicits strong desensitization, while glycine binding to GluN3 alone is sufficient for activation. Here, we explore mechanisms by which GluN1-selective competitive antagonists, CGP-78608 and L-689,560, potentiate GluN1/3A and GluN1/3B receptors by preventing glycine binding to GluN1. We show that both CGP-78608 and L-689,560 prevent desensitization of GluN1/3 receptors, but CGP-78608-bound receptors display higher glycine potency and efficacy at GluN3 subunits compared to L-689,560-bound receptors. Furthermore, we demonstrate that L-689,560 is a potent antagonist of GluN1^{FA+TL}/3A receptors, which are mutated to abolish glycine binding to GluN1, and that this inhibition is mediated by a non-competitive mechanism involving binding to the mutated GluN1 agonist binding domain (ABD) to negatively modulate glycine potency at GluN3A. Molecular dynamics simulations reveal that CGP-78608 and L-689,560 binding or mutations in the GluN1 glycine binding site promote distinct conformations of the GluN1 ABD, suggesting that the GluN1 ABD conformation influences agonist potency and efficacy at GluN3 subunits. These results uncover the mechanism that enables activation of native GluN1/3A receptors by application of glycine in the presence of CGP-78608, but not L-689,560, and demonstrate strong intra-subunit allosteric interactions in GluN1/3 receptors that may be relevant to neuronal signaling in brain function and disease.

Introduction

NMDA-type glutamate receptors mediate excitatory neurotransmission in the central nervous system (CNS) and are involved in many neuronal functions, including synaptic plasticity and neuronal development, but have also been implicated in several neurological and psychiatric disorders (Hansen et al., 2021). Seven NMDA receptor subunits have been cloned; the glycine-binding GluN1, four glutamate-binding GluN2 (GluN2A–D), and two glycine-binding GluN3 subunits (GluN3A–B; Pérez-Otaño et al., 2016; Hansen et al., 2018). Most NMDA receptors in the CNS are assembled from two GluN1 and two GluN2 subunits (i.e., GluN1/2 receptors; Benveniste and Mayer, 1991; Clements and Westbrook, 1991; Karakas and Furukawa, 2014; Lee et al., 2014), and decades of studies have provided ample insights to

the structure, function, and pharmacology of GluN1/2 receptors (Dingledine et al., 1999; Traynelis et al., 2010; Paoletti et al., 2013; Sanz-Clemente et al., 2013; Glasgow et al., 2015; Iacobucci and Popescu, 2017; Hansen et al., 2018; Hansen et al., 2021). By contrast, the structural and functional properties of GluN1/3 receptors remain elusive, although recent studies implicate GluN3-containing receptors in synapse maturation and synaptic plasticity, as well as several behaviors and CNS disorders (Larsen et al., 2011; Henson et al., 2012; Huang et al., 2013; Marco et al., 2013; Yuan et al., 2013; Wesseling and Pérez-Otaño, 2015; Creed et al., 2016; Mahfooz et al., 2016; Huang et al., 2017; Lee et al., 2018; Otsu et al., 2019; Christian et al., 2021; Bossi et al., 2022).

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Many properties are strikingly different between GluN1/2 and GluN1/3 receptors. GluN1/2 receptors require simultaneous binding of glycine (or D-serine) to GluN1 and glutamate to GluN2 for activation (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988; Benveniste and Mayer, 1991; Clements and Westbrook, 1991), while GluN1/3 receptors only require glycine (or D-serine) for activation (Chatterton et al., 2002). GluN1/2 receptors weakly desensitize (Monyer et al., 1992), whereas GluN1/3 receptors strongly desensitize following glycine exposure (Awobuluyi et al., 2007; Madry et al., 2007; Kvist et al., 2013a; Cummings and Popescu, 2016; Grand et al., 2018). GluN1/2 receptors have high Ca^{2+} -permeability (Wollmuth, 2018) and are blocked by extracellular Mg^{2+} at resting membrane potentials (Mayer et al., 1984; Nowak et al., 1984), while GluN1/3 receptors have low permeability to Ca^{2+} and are almost insensitive to Mg^{2+} block (Chatterton et al., 2002; Piña-Crespo et al., 2010; Otsu et al., 2019). Furthermore, GluN1/3 receptors have unusual activation properties, since agonist binding to GluN1 triggers strong desensitization, while agonist binding to GluN3 alone is sufficient for receptor activation (Awobuluyi et al., 2007; Madry et al., 2007; Kvist et al., 2013a; Grand et al., 2018). GluN1/3 receptor desensitization can therefore be prevented by introducing mutations in the GluN1 agonist binding pocket (e.g., F484A+T518L; hereafter FA+TL) to abolish agonist binding to GluN1 (Awobuluyi et al., 2007; Kvist et al., 2013a; Skrenkova et al., 2019). Alternatively, GluN1-selective competitive antagonists, such as CGP-78608, can prevent glycine binding to GluN1 and thereby enhance activation of GluN1/3 receptors (Madry et al., 2007; Madry et al., 2008; Grand et al., 2018). These unusual activation properties have impeded studies to define the pharmacology of GluN3-containing NMDA receptors and the development of GluN3-selective ligands to investigate their roles in neuronal signaling (Kvist et al., 2013a; Kvist et al., 2013b; Zhu et al., 2020; Zeng et al., 2022).

Radioligand binding assays using soluble, isolated agonist binding domains (ABDs) have determined dissociation binding constants (K_d) for glycine of 40 nM at the GluN3A ABD and 26 μM at the GluN1 ABD (Furukawa and Gouaux, 2003; Yao and Mayer, 2006). Thus, glycine binds the isolated GluN3A ABD with 650-fold higher affinity compared to the isolated GluN1 ABD. These results are in stark contrast to the glycine potency at GluN1 ($\text{EC}_{50} \sim 0.1\text{--}1 \mu\text{M}$) measured in GluN1/2A-D receptors (Hansen et al., 2021), and the relatively low glycine potency at GluN3A measured in GluN1^{FA+TL}/3A ($\text{EC}_{50} = 60 \mu\text{M}$) and CGP-78608-bound GluN1/3A receptors ($\text{EC}_{50} = 39 \mu\text{M}$; Kvist et al., 2013a; Grand et al., 2018). This apparent discrepancy may result from differences in experimental preparations (i.e., binding on isolated ABDs versus function of full receptors), since EC_{50} values are expected to be similar or lower than K_d values, depending on agonist efficacy (Colquhoun, 1998; Kenakin, 2016). Binding studies also revealed subunit-selective competitive antagonists, including CGP-78608 and L-689,560, which bind the GluN1 ABD with 860- and 10,400-fold higher affinity, respectively, compared to the isolated GluN3A ABD (Furukawa and Gouaux, 2003; Yao and Mayer, 2006). It was later demonstrated that the GluN1-selectivity of CGP-78608 enabled remarkable enhancement of current responses from GluN1/3A

receptors by preventing glycine binding to GluN1 (Grand et al., 2018). Paradoxically, the more GluN1-selective antagonist L-689,560 displayed surprisingly poor enhancement of glycine responses compared to CGP-78608 (Grand et al., 2018). In this study, we investigate the mechanisms that enable modulation of GluN1/3A and GluN1/3B receptor responses by GluN1-selective antagonists. These results highlight a complex structure-function relationship that have important implications for our understanding of GluN1/3 receptor pharmacology.

Materials and methods

Animals

All procedures and experiments involving mice were approved by the Institutional Animal Care and Use Committee at the University of Montana. Mice were housed in breeding pairs on a 12 h light/dark cycle and allowed ad libitum access to food and water. Sex of the mice was not considered as a biological variable. Wild-type (WT) mice (C57BL/6J; strain 000664) and *Grin3a*^{-/-} knock-out mice (B6;129x1-*Grin3a*^{tm1Nnk}/J, strain 029974; Das et al., 1998) were obtained from The Jackson Laboratory. *Grin3a*^{-/-} mice were backcrossed with WT C57BL/6J mice to minimize genetic variation. Heterozygous *Grin3a*^{+/-} mice were crossed with WT mice for seven generations, after which heterozygous *Grin3a*^{+/-} mice were crossed together to yield homozygous *Grin3a*^{-/-} mice on the C57BL/6J background (hereafter 3A-KO mice).

Genotyping

During backcrossing, the mice were ear punched after postnatal day 14 (P14) and the ear tissue was used for genotyping. Ear punches were placed in individual 1.5 ml tubes and the Hot Sodium Hydroxide and Tris (HotSHOT) method was used to extract the genomic DNA for genotyping (Truett et al., 2000). Briefly, 50 μl of alkaline solution (25 mM NaOH and 0.2 mM EDTA, pH ~ 12 with no adjusting needed) was added to each tube containing ear tissue, and the samples were incubated for 20–30 min at 95°C. The tubes were then briefly vortexed to manually break up the tissue before the samples were placed on ice and allowed to cool. Next, 50 μl of acidic solution (40 mM Tris-HCl, pH ~ 5 with no adjusting needed) was added to each tube and briefly vortexed. The tubes were centrifuged for 1 min to pellet the remaining tissue, and the supernatant containing the extracted genomic DNA was transferred to a new tube and stored at -20°C until needed for PCR genotyping.

The genomic DNA samples were genotyped using a multiplex PCR protocol with the following primers: WT_3A, 5'-TTGGGG AGCGCCCTGCATGG-3'; KO_3A, 5'-GCCTGAAGAACGAGATCA GC-3'; common_rev, 5'-CACTCCACGCACCAGACTCC-3'. Amplification was performed with 1 μl of genomic DNA sample in 25 μl reactions from the KAPA2G Robust HotStart kit (Roche Sequencing Solutions) using an initial 3 min denaturation at 95°C, followed by 25 cycles with 15 s at 95°C, 15 s at 60°C, and 15 s at 72°C, and a final extension of 1 min at 72°C. Using this protocol, the WT *Grin3a* allele generates a PCR product of 434 bp and the 3A-KO allele generates a PCR product of 457 bp, which can be resolved on a 2% agarose gel.

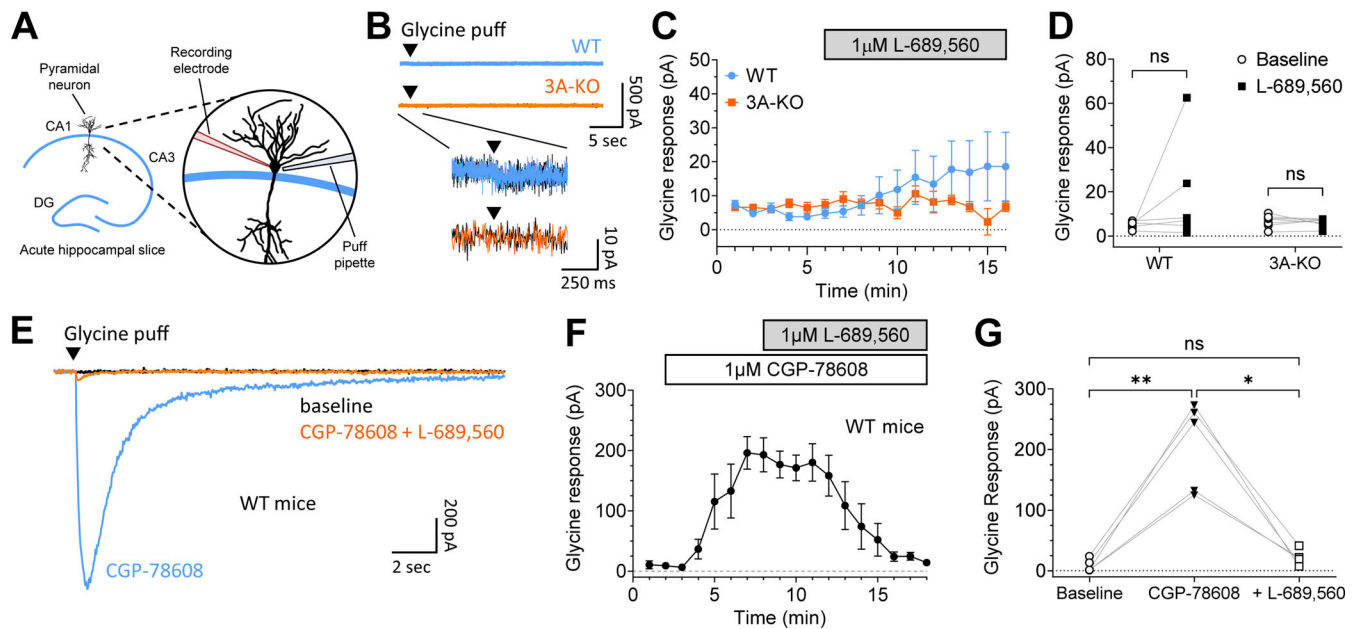


Figure 1. L-689,560 inhibits GluN1/3A-mediated responses in acute hippocampal slices. (A) Schematic depiction of whole-cell patch-clamp recordings from CA1 pyramidal neurons in acute hippocampal slices from P8–12 mice. Responses to pressure-applied application (puffs) of 10 mM glycine were recorded in the absence and presence of bath-applied GluN1-selective competitive antagonists. DG, dentate gyrus. (B) Representative recordings of responses to glycine puffs (10 mM) in the absence (baseline; black trace) and presence of 1 μ M L-689,560 from CA1 pyramidal neurons in acute hippocampal slices from WT (blue) or GluN3A knock-out (3A-KO; orange) mice. (C) Average time course of responses to glycine puffs from WT ($n = 6$) and 3A-KO ($n = 7$) neurons. L-689,560 was bath-applied during the time indicated by the bar above graph. (D) Summary of current responses to glycine puffs in the absence (baseline) and presence of 1 μ M L-689,560. Application of L-689,560 did not significantly potentiate current responses in WT ($n = 6$; $P = 0.227$; paired t test) or 3A-KO ($n = 7$) neurons ($P = 0.522$; paired t test). (E) Representative recordings of responses to glycine puffs from WT neurons in the absence (baseline; black) and presence of 1 μ M CGP-78608 (blue), as well as in the presence of 1 μ M CGP-78608 + 1 μ M L-689,560 (orange). (F) Average time course of responses to glycine puffs from WT hippocampal CA1 neurons ($n = 5$). (G) Summary of glycine responses from WT neurons in the absence (baseline), in the presence of 1 μ M CGP-78608, and in the presence of 1 μ M CGP-78608 + 1 μ M L-689,560. Application of CGP-78608 significantly potentiated current responses compared to baseline ($P = 0.006$), and the CGP-78608-mediated potentiation was inhibited by the addition of L-689,560 ($P = 0.011$), resulting in glycine responses that were not significantly different from baseline ($P = 0.068$; paired one-way ANOVA with Tukey's posttest). Data are mean $\pm$ SEM; ns indicates not significant.

Patch-clamp recordings from acute hippocampal brain slices

At P8–P12, WT and 3A-KO mice of both sexes were induced into a deep anesthetic plane with isoflurane followed by cardiac perfusion with ice-cold, oxygenated sucrose solution containing (in mM) 3 KCl, 24 NaHCO₃, 1.25 NaH₂PO₄, 10 glucose, 230 sucrose, 0.5 CaCl₂, and 10 MgSO₄, saturated with 95% O₂/5% CO₂. A craniotomy was performed, and brains were transferred to a beaker with the same solution. After removing the cerebellum, the brains were hemisected, mounted onto a cutting stage, and transferred into a chamber containing an ice-cold, oxygenated cutting solution containing (in mM) 130 NaCl, 3 KCl, 24 NaHCO₃, 1.25 NaH₂PO₄, 10 glucose, 1 CaCl₂, and 3 MgSO₄ following an established protocol (Bischofberger et al., 2006). Finally, transverse slices (300 μ m) containing ventral hippocampus were cut on a vibrating microtome (VT1200S; Leica Microsystems) and incubated in cutting solution for ≥ 1 h at room temperature.

Hippocampal slices were transferred to a recording chamber (RC-26GLP; Warner Instruments) mounted on a SliceScope Pro 200 (Scientifica). Slices were continuously perfused with an oxygenated artificial cerebral spinal fluid containing (in mM) 130 NaCl, 3 KCl, 24 NaHCO₃, 1.25 NaH₂PO₄, 10 glucose, 2 CaCl₂, and 1 MgSO₄ at a flow rate of 2–3 ml/min, maintained at 32°C with a dual in-line and heated platform temperature control (TC-344A; Warner Instruments). Recording electrodes

with tip resistance of 1.5–3 M Ω were made from thin-walled borosilicate glass micropipettes (TW150F-4; World Precision Instruments) prepared using a micropipette puller (P-1000; Sutter Instruments) and filled with an internal solution containing (in mM) 120 Cs-methanesulfonate, 4.6 MgCl₂, 10 HEPES, 15 BAPTA, 4 Na₂-ATP, 0.4 Na-GTP, 1 QX-314, and 10 K₂-creatine phosphate, pH 7.25, 280–290 mOsm. Whole-cell voltage-clamp recordings of hippocampal CA1 pyramidal cells were made at a holding potential of -70 mV using a Multiclamp 700B amplifier (Molecular Devices) with filtering at 4 kHz (Bessel) and digitized at 20 kHz using Digidata 1440A with pClamp 10 software (Molecular Devices). Series resistance was monitored throughout the recording with a 200 ms, 5 mV hyperpolarizing voltage step. Recordings were excluded from analysis if the series resistance exceeded 20 M Ω or changed by 20% over the course of the experiment. To elicit glycine-induced current responses in hippocampal CA1 pyramidal cells, glycine (10 mM) “puffs” were pressure-applied (100 ms duration, 5–7.5 psi) to the soma using a custom-made picospritzer (Forman et al., 2017) every 30 or 60 s through a borosilicate capillary tube. To isolate the glycine responses, 10 μ M gabazine, 2 μ M NBQX, 100 μ M APV, and 50 μ M strychnine were bath applied to block GABA_A, AMPA, GluN1/GluN2 NMDA, and glycine receptors, respectively.

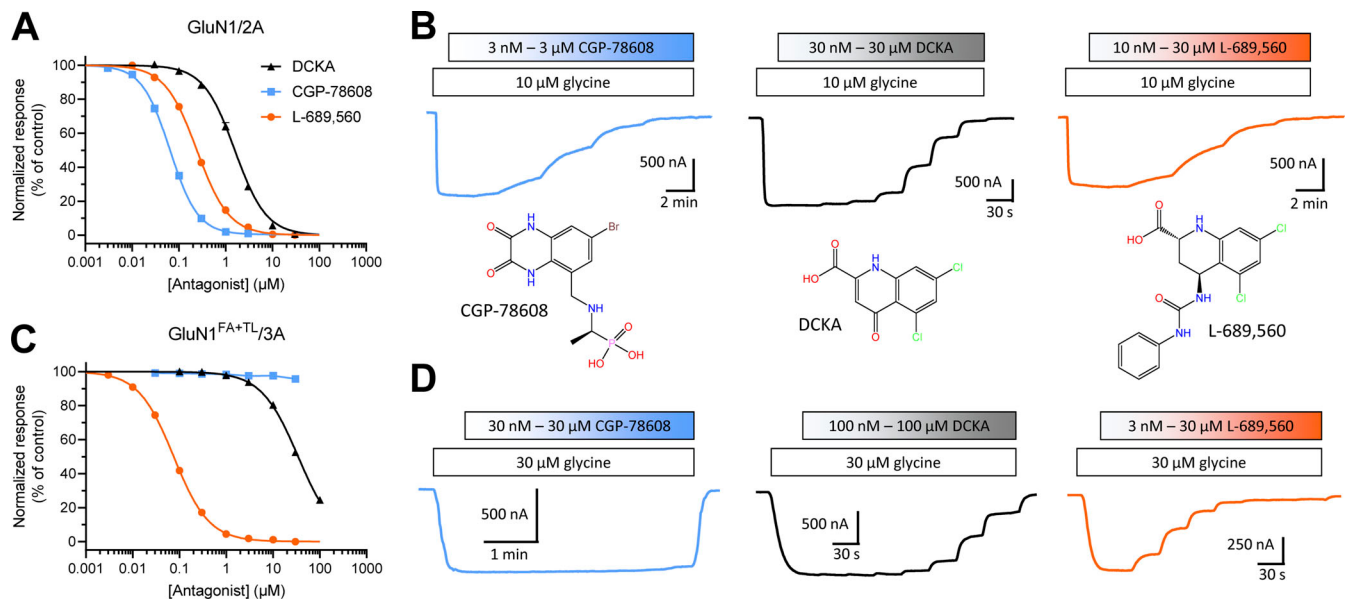


Figure 2. L-689,560 is a potent inhibitor of GluN1^{FA+TL}/3A receptors. (A) Concentration–response data for inhibition of recombinant GluN1/2A receptors by competitive glycine site antagonists. Responses were activated by 10 μM glycine in the continuous presence of 300 μM glutamate and measured using two-electrode voltage-clamp recordings. IC₅₀ values for CGP-78608, L-689,560, and DCKA were 0.065 ± 0.003 μM ($100 \pm 1\%$ inhibition; $n_H = 1.5$; $n = 12$), 0.25 ± 0.01 μM ($100 \pm 1\%$ inhibition; $n_H = 1.3$; $n = 14$), and 1.5 ± 0.1 μM ($102 \pm 1\%$ inhibition; $n_H = 1.3$; $n = 12$), respectively. Data are mean $\pm$ SEM. (B) Representative responses from GluN1/2A receptors inhibited by increasing concentrations of competitive glycine site antagonists. (C) Concentration–response data for inhibition of recombinant GluN1^{FA+TL}/3A receptors. Responses were activated by 30 μM glycine and measured using two-electrode voltage-clamp recordings. CGP-78608 produced no inhibition at 30 μM ($n = 16$). IC₅₀ values for L-689,560 and DCKA were 0.077 ± 0.01 μM ($95 \pm 1\%$ inhibition; $n_H = 1.2$; $n = 16$) and 29 ± 1 μM ($93 \pm 1\%$ inhibition; $n_H = 1.2$; $n = 12$), respectively. Data are mean $\pm$ SEM. (D) Representative responses from GluN1^{FA+TL}/3A receptors inhibited by increasing concentrations of competitive glycine site antagonists. See Table 1 for L-689,560 IC₅₀ values.

Fast-application whole-cell voltage-clamp recordings

HEK293T cells were plated onto glass coverslips coated with poly-D-lysine (0.1 mg/ml) ~48 h before recordings and maintained in Dulbecco's modified Eagle's medium (DMEM) with GlutaMax-I and sodium pyruvate (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 10 U/ml penicillin, and 10 mg/ml streptomycin (Thermo Fisher Scientific). Cells were transfected the following day using the calcium phosphate precipitation method (Chen and Okayama, 1987) with a 1:1:1 ratio of plasmid cDNAs encoding green fluorescent protein (EGFP), rat GluN1-4b (GenBank accession no. U08268), and rat GluN3A (U29873).

Whole-cell voltage-clamp recordings were performed ~24 h following transfection at a holding potential of -60 mV and at room temperature (20°C) using an Axopatch 200B amplifier (Molecular Devices). Data was filtered at 8 kHz (8-pole Bessel, Frequency Devices) and digitized at 1 kHz using Digidata 1322B with pClamp 10 software (Molecular Devices). Recording electrodes with tip resistance of 2–3 MΩ were made from thin-walled borosilicate glass micropipettes (TW150F-4; World Precision Instruments) prepared using a micropipette puller (P-1000; Sutter Instruments) and filled with internal solution containing (in mM) 110 D-gluconate, 110 CsOH, 30 CsCl, 5 HEPES, 4 NaCl, 0.5 CaCl₂, 2 MgCl₂, 5 BAPTA, 2 NaATP, and 0.3 NaGTP (pH 7.35 with CsOH). The extracellular recording solution was composed of (in mM) 150 NaCl, 10 HEPES, 3 KCl, and 0.5 CaCl₂ (pH 7.4 with NaOH). Rapid solution exchange (10–90%

open-tip solution exchange times were 0.6–0.8 ms) on lifted cells was performed with gravity-driven perfusion through a theta-glass pipette controlled by a piezoelectric translator (MXPZT-300; Siskiyou Corporation).

Two-electrode voltage-clamp recordings

DNA constructs encoding rat GluN1-1a (GenBank accession no. U11418), rat GluN1-4a (U08267), rat GluN2A (D13211), rat GluN3A (U29873), and rat GluN3B (NM_133308) were linearized using restriction enzymes and used as templates to synthesize cRNA using the mMessage mMachine kit (Ambion, Life Technologies). For some experiments, GluN1 subunits included F484A + T518L or F484H + R523K mutations in the GluN1 agonist binding site to prevent desensitization of GluN1/3 receptors. *Xenopus laevis* oocytes were obtained from Xenopus 1 Corporation, injected with cRNAs encoding GluN1-1a + GluN2A, GluN1-1a + GluN3A, or GluN1-4a + GluN3B at a 1:2 ratio. Incubation of *Xenopus* oocytes and two-electrode voltage-clamp recordings were performed 2–4 d following cRNA injection at a holding potential of -40 mV and at room temperature (21 – 23°C) as previously described (Hansen et al., 2013). During recordings, the oocytes were perfused with recording solution containing (in mM) 90 NaCl, 1 KCl, 10 HEPES, 0.5 BaCl₂, and 0.01 EDTA (pH 7.4 with NaOH). Ligand applications were prepared in recording solution from 20 to 100 mM ligand stock solutions prepared in DMSO. The concentration of DMSO was kept constant in all applications and never exceeded 0.6%.

Table 1. Concentration–inhibition data for L-689,560 at NMDA receptor subtypes

	[Glycine] (μM)	IC ₅₀ (μM)	% Inhibition	Hill slope	n
GluN1/2A	10 ^a	0.25 ± 0.01	100 ± 1	1.3	14
GluN1/3A	30 ^b	0.53 ± 0.08	96 ± 1	1.3	8
	30 ^c	3.8 ± 0.3	92 ± 1	0.94	6
GluN1/3B	100 ^b	1.9 ± 0.1	87 ± 2	1.2	12
	100 ^c	8.9 ± 0.6	75 ± 3	1.7	8
GluN1 ^{FA+TL} /3A	30	0.077 ± 0.046	95 ± 1	1.2	16
	100 ^d	0.15 ± 0.01	75 ± 4	1.4	5
	1,000 ^d	0.30 ± 0.01	44 ± 1	1.2	5
	3,000 ^d	0.38 ± 0.03	41 ± 2	1.0	4
GluN1 ^{FH+RK} /3A	100	5.6 ± 0.2	85 ± 1	1.3	5
GluN1 ^{FA+TL} /3B	100 ^d	0.14 ± 0.01	29 ± 1	1.8	8
	1,000 ^d	0.20 ± 0.01	30 ± 1	2.1	6
	3,000 ^d	0.20 ± 0.01	40 ± 2	1.6	6

Concentration–inhibition data for L-689,560 were recorded using two-electrode voltage-clamp electrophysiology at recombinant NMDA receptors expressed in *Xenopus* oocytes. Responses were activated by glycine at the indicated concentrations. The fitted maximal inhibition is shown as percentage inhibition of the response in the absence of antagonist. Data are presented as mean ± SEM of values from individual oocytes, and *n* is the number of oocytes used to generate the data.

^aMeasured in the continuous presence of 300 μM glutamate.

^bMeasured in the continuous presence of 1 μM CGP-78608.

^cMeasured in the continuous presence of 10 μM CGP-78608.

^dBiphasic concentration–response relationships were observed, but only data for high-potency components are listed.

Data analysis

Concentration–response data from two-electrode voltage-clamp recordings were analyzed using GraphPad Prism (GraphPad Software). Agonist data for individual oocytes were fitted to the following Hill equation: $I = I_{\min} + (I_{\max} - I_{\min}) / (1 + 10^{(\log EC_{50} - \log[A]) \times n_H})$, where $I_{\max}$ and $I_{\min}$ are the fitted maximum and minimum current response to the agonist ($I_{\min}$ for agonists was typically negligible), respectively, EC_{50} is the agonist concentration that produces half-maximum response, $[A]$ is the agonist concentration, and n_H is the Hill slope. Antagonist data for individual oocytes were fitted to the above Hill equation, except using IC_{50} and antagonist concentrations. Biphasic concentration–inhibition data were analyzed by simultaneously fitting to the following three equations: $I_{\text{high}} = F_{\text{high}} \times (I_{\max} - I_{\min}) / (1 + 10^{(\log IC_{50, \text{high}} - \log[A]) \times n_{H, \text{high}}})$, $I = (1 - F_{\text{high}})(I_{\max} - I_{\min}) / (1 + 10^{(\log IC_{50, \text{low}} - \log[A]) \times n_{H, \text{low}}})$, and $I = I_{\min} + I_{\text{high}} + I_{\text{low}}$, where F_{high} is the fraction of inhibition produced by the high-potency component, and $IC_{50, \text{high}}$ and $IC_{50, \text{low}}$ are for the high- and low-potency components, respectively. $I_{\max}$ for antagonists was normalized to the current response to agonist alone without antagonist in the same recording and the unsigned value of n_H is reported.

Inhibition was also evaluated using agonist concentration–response data in the absence and presence of increasing concentrations of L-689,560 (i.e., Schild analysis). This data was then used to determine the allosteric constant α and dissociation binding constant K_b for L-689,560 inhibition using a global nonlinear least-squares fitting method, in which the concentration–response data were simultaneously fit to the Hill equation and the following equation: $EC_{50}' = EC_{50} \times (1 + [B]/K_b) / (1 + \alpha[B]K_b)$,

where $[B]$ is the L-689,560 concentration, EC_{50} is for the agonist in the absence of L-689,560, and EC_{50}' is the for agonist in the presence of increasing concentrations of L-689,560. This method will perform a global fit to the data and provide the agonist EC_{50} in the absence of L-689,560, the allosteric constant α , and the dissociation binding constant K_b that best describes all the data as previously described (Christopoulos and Kenakin, 2002; Hansen et al., 2012; Yi et al., 2016). For graphical representation, data points from individual oocytes were normalized and the averaged data points were plotted with the fitted concentration–response curve.

Molecular dynamics simulations

All starting structures for molecular dynamics simulations were modeled using the GluN1 ABD with bound L-689,560 from the GluN1/2A ABD heterodimer structure (PDB ID 6USU; Chou et al., 2020). Briefly, the GluN2A ABD, including bound glutamate and associated water molecules, was deleted and the DockPrep feature in UCSF Chimera software (version 1.15; Pettersen et al., 2004) was used to complete missing GluN1 side chains and remove alternate side chain conformation. Missing loops and terminal residues were built using Yasara 18.11.10 software (YASARA Biosciences). Finally, the GluN1 ABD model composed of residues 396–544 and 663–798 joined by a Gly-Thr dipeptide linker was capped at the N-terminus by the addition of an acetyl group (ACE) and the C-terminus by the addition of an N-methyl amide group (NME). This GluN1 ABD model with bound L-689,560 was then used to produce the glycine-bound

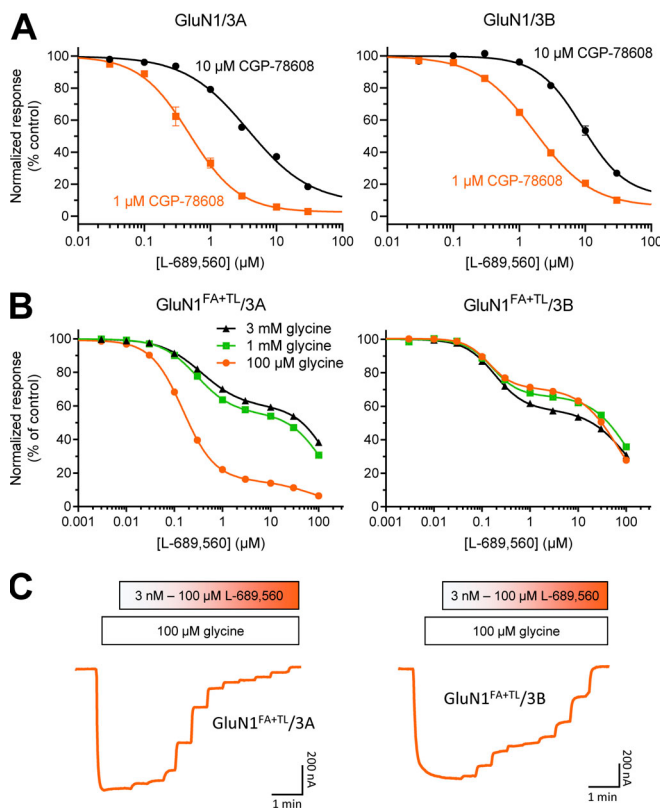


Figure 3. L-689,560 is a negative allosteric modulator of GluN1^{FA+TL}/3A and GluN1^{FA+TL}/3B receptors. (A) Concentration–response data for L-689,560 inhibition of GluN1/3A activated by 30 μM glycine and GluN1/3B receptors activated by 100 μM glycine. Responses were activated in the continuous presence of either 1 or 10 μM CGP-78608. Data are mean ± SEM from 6 to 12 oocytes. (B) Inhibition of GluN1^{FA+TL}/3A and GluN1^{FA+TL}/3B receptors by L-689,560. Responses were activated by either 100 μM, 1 mM, or 3 mM glycine. Increased glycine concentrations resulted in a biphasic concentration–response relationship (see Materials and methods for details on data analysis). Data are mean ± SEM from 4 to 8 oocytes. (C) Representative two-electrode voltage-clamp recordings of responses from GluN1^{FA+TL}/3A and GluN1^{FA+TL}/3B receptors that are inhibited by increasing concentrations of L-689,560. See Table 1 for IC₅₀ values.

GluN1 ABD by deleting all atoms of L-689,560, except the amino acid moiety. CGP-78608-bound GluN1 ABD was generated by docking CGP-78608 into the agonist binding site using AutoDock (Morris et al., 2009; Trott and Olson, 2010) from the SeamDock web interface (Murail et al., 2021). These GluN1 ABD models with either bound glycine, CGP-78608, or L-689,560 were then mutated to F484A + T518L or F484H + R523K using UCSF Chimera. The resulting models, which included crystallographic water molecules bound to the GluN1 ABD, were further solvated by adding ~15,200 water molecules (TIP3P; Jorgensen and Tirado-Rives, 2005) using the tleap tool of AMBER 20 (Case et al., 2005) and a minimum of 12 Å between the solute and the edge of a truncated octahedron box. The system was neutralized and ionized to 150 mM NaCl by adding Na⁺ and Cl⁻ ions (Machado and Pantano, 2020). Ligand parameterization was performed by adding hydrogen atoms to ligands in UCSF Chimera and then obtaining partial charges by calculating molecular electrostatic potentials using quantum

chemistry software (Gaussian 16) through the R.E.D. server (Vanqualef et al., 2011). Net charges of glycine, CGP-78608, and L-689,560 were constrained to 0, −1, and −1, respectively. Other ligand force field parameters were taken from the improved general AMBER force field (GAFF2; He et al., 2020).

Simulations were performed in AMBER 18 or AMBER 20 (Case et al., 2005) with the AMBER ff14SB force field for protein and the TIP3P water model (Maier et al., 2015). The solvated systems were minimized and heated before they were equilibrated under constant pressure and temperature (NPT) conditions. The Monte Carlo barostat method was used to achieve a constant pressure of 1 atmosphere, and the Langevin dynamics method was used to achieve a constant temperature at 310 K (Feller et al., 1995). Periodic boundary conditions were applied to all simulations with the particle mesh Ewald method (Essmann et al., 1995) to calculate long-range electrostatic interactions with a 10 Å cutoff. To allow for integration time steps of 4 fs, the SHAKE algorithm was used to constrain hydrogen bonds (Ryckaert et al., 1997), and the mass of solute hydrogen atoms was repartitioned to 3.024 D with adjustment of the mass to which each hydrogen is bonded to the amount necessary to leave total mass unchanged (i.e., hydrogen mass repartitioning; Hopkins et al., 2015). All production simulations were preceded by a 10 ns equilibrium simulation with random initial velocities and an asymmetric harmonic potential with a force constant of 4 kcal/mol/Å² to prevent ligand dissociation from the open ABD conformation. The asymmetric harmonic potential, which was only active when the distance exceeded 3.0 Å, was applied to the center of mass (COM) distance between guanidinium nitrogen atoms in Arg523 (or the side chain nitrogen of Lys523 in the GluN1 FH+RK mutant) and α-carboxylate oxygen atoms in glycine and L-689,560 or the 2,3-dioxy oxygen atoms in CGP-78608.

Unrestrained production simulations to evaluate ligand binding (500 ns each) were analyzed to determine ligand binding states for each frame using the following three ligand–receptor distances: dist1) distance between COM of guanidinium nitrogens in Arg523 (or nitrogen in Lys523) and COM of α-carboxylate oxygens in glycine/L-689,560 or oxygens in CGP-78608, dist2) distance between backbone carbonyl oxygen in Pro518 and α-amino acid nitrogen in glycine/L-689,560 or nitrogen N1 in CGP-78608, and dist3) distance between Ca in glycine, chlorine in L-689,560, or bromine CGP-78608 and the COM of indole atoms in Trp731. Ligands were categorized as fully bound if dist1 < 3.5 Å, dist2 < 3.5 Å, and dist3 < 6 Å for L-689,560, and dist3 < 7 Å for glycine/CGP-78608. Ligands were defined as unbound if dist1 > 3.5 Å, dist2 > 3.5 Å, and dist3 > 6 Å for L-689,560, and dist3 > 7 Å for glycine/CGP-78608. Ligands were partially bound if they were not categorized as fully bound or unbound.

Hinged movements between the two lobes (D1 and D2) of the GluN1 ABD that form the agonist binding cleft, were described by measuring distances between the COM for atoms N, CA, CB, C, and O of residues 484–485 in GluN1 lobe D1 and residues 688–689 in GluN1 lobe D2 (distance ξ₁), and between residues 405–407 in GluN1 lobe D1 and residues 714–715 in GluN1 lobe D2 (distance ξ₂) as previously described (Yao et al., 2013). Production simulations to evaluate conformational selection (2,500 ns

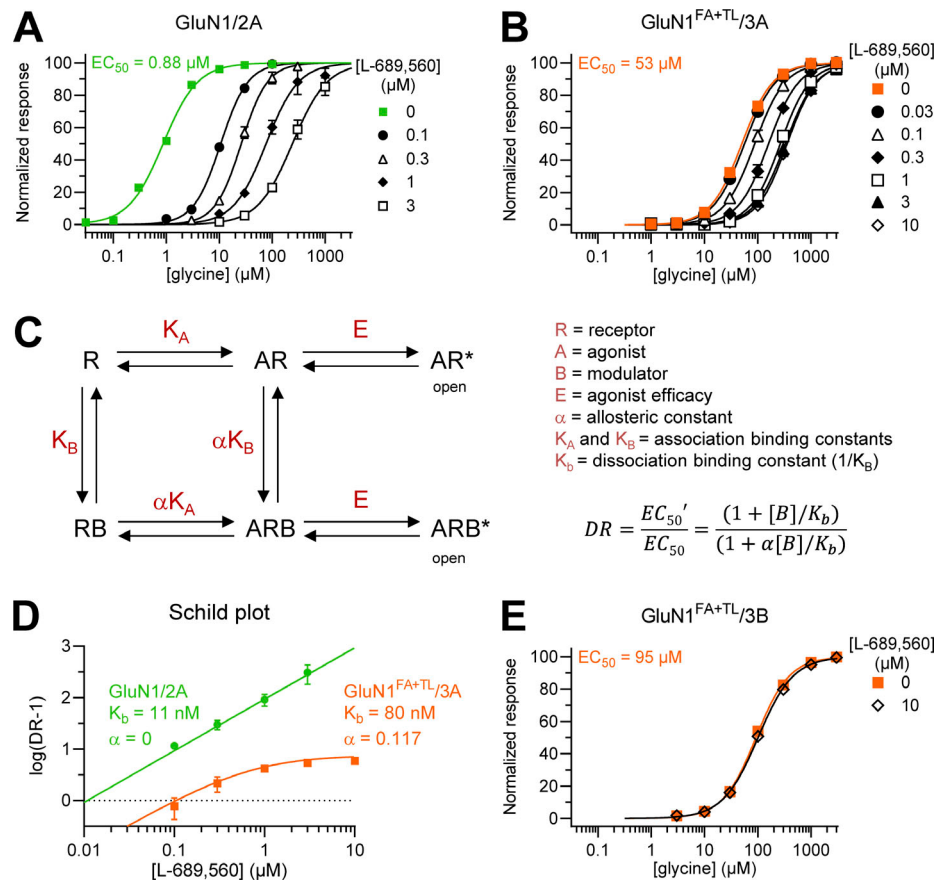


Figure 4. L-689,560 reduces glycine binding to GluN1^{FA+TL}/3A, but not GluN1^{FA+TL}/3B receptors. (A) Glycine concentration–response data in continuous presence of 300 μM glutamate at GluN1/2A receptors in the absence and presence of increasing concentrations of by L-689,560. The glycine EC_{50} value in the absence L-689,560 is shown. Data are mean ± SEM from 3 to 6 oocytes. (B) Glycine concentration–response data at GluN1^{FA+TL}/3A receptors in the absence and presence of increasing concentrations of by L-689,560. The glycine EC_{50} value in the absence L-689,560 is shown. Data are mean ± SEM from 6 to 12 oocytes. (C) Overview of an equilibrium model for L-689,560 inhibition. A and B represent the agonist (glycine) and modulator (L-689,560), respectively. Agonist K_A is changed by the allosteric constant α upon binding of modulator and vice versa. E is the agonist efficacy that, in this model, remains unchanged upon modulator binding. The dose ratio (DR) is the ratio of agonist EC_{50} values in the presence (EC_{50}') and absence (EC_{50}) of modulator as a function of modulator concentration [B], modulator binding dissociation constant K_b , and the allosteric constant α. (D) Schild plot of data shown in A and B demonstrating competitive antagonism of L-689,560 on GluN1/2A receptors ($K_b = 11 \text{ nM}$), and allosteric antagonism of L-689,560 on GluN1^{FA+TL}/3A receptors ($K_b = 80 \text{ nM}$). (E) Concentration–response data for glycine at GluN1^{FA+TL}/3B receptors in the absence and presence of L-689,560. The glycine EC_{50} value in the absence L-689,560 is shown. Data are mean ± SEM from 13 to 21 oocytes. See Table 2 for EC_{50} values and Materials and methods for details on data analysis.

each) included the restraint to prevent ligand dissociation by applying an asymmetric harmonic potential, only active when the distance exceeded 3.0 Å, with a force constant of 4 kcal/mol/Å² to the COM distance between guanidinium nitrogen atoms in Arg523 and α-carboxylate oxygen atoms in glycine and L-689,560 or the 2,3-dioxy oxygen atoms in CGP-78608. Furthermore, the production simulations included asymmetric harmonic potentials with force constants of 20 kcal/mol/Å² on both ξ_1 and ξ_2 , which were only active when these distances exceeded 16 Å, to prevent the GluN1 ABD from visiting hyper-extended conformations considered unrealistic in the context of published crystal and cryo-EM structures (Karakas and Furukawa, 2014; Lee et al., 2014; Tajima et al., 2016; Zhu et al., 2016; Zhang et al., 2018; Chou et al., 2020; Wang et al., 2021). To determine 2D protein conformational free energy landscapes, or potentials of mean force (PMFs), values of (ξ_1 , ξ_2) from each frame of 8 × 2,500 ns simulation trajectories were pooled to calculate 2D kernel probability densities using Origin software (version

2022b, OriginLab Corporation). To determine 1D PMFs, values of (ξ_1 , ξ_2) were projected onto a single coordinate using $\xi_{12} = (\xi_1 + \xi_2)/2$ as previously described (Yao et al., 2013). Probability densities were converted to energies according to the Boltzmann distribution to provide $W(\xi_1, \xi_2)$ for 2D PMFs or $W(\xi_{12})$ for 1D PMFs.

Block-averaging analyses of ξ_1 and ξ_2 coordinates were used to assess convergence and sampling quality in the molecular dynamics simulations (Grossfield and Zuckerman, 2009). Briefly, all trajectories for each condition were combined to produce $N = M \times n$ frames that were divided into M segments with block length n , and averages of ξ_1 and ξ_2 coordinates were calculated for each segment. This was performed with an initial short block length and with gradually increasing block lengths. For each block length n , the blocked standard error (BSE) was calculated as $BSE = \sigma_n/\sqrt{M}$, where σ_n is the standard deviation of either ξ_1 or ξ_2 averages among the blocks and M is the number of segments with block length n . Thus, BSE values estimate the

Table 2. Concentration–response data for glycine at NMDA receptor subtypes

	[L-689,560] (μM)	EC ₅₀ (μM)	Hill slope	n
GluN1/2A	0 ^a	0.87 $\pm$ 0.01	1.3	6
	0.1 ^a	11 $\pm$ 1	1.7	3
	0.3 ^a	27 $\pm$ 2	1.8	6
	1 ^a	80 $\pm$ 7	1.6	6
	3 ^a	267 $\pm$ 42	1.4	6
GluN1/3A	0 ^b	14 $\pm$ 1	1.9	13
	0 ^c	19 $\pm$ 1	1.9	18
	1	714 $\pm$ 71	1.8	8
	10	802 $\pm$ 60	1.8	14
GluN1/3B	0 ^b	19 $\pm$ 1	1.8	20
	0 ^c	26 $\pm$ 1	1.6	17
	1	351 $\pm$ 11	1.6	8
	10	410 $\pm$ 9	1.6	14
GluN1 ^{FA+TL} /3A	0	53 $\pm$ 3	1.5	12
	0.03	58 $\pm$ 5	1.5	10
	0.1	92 $\pm$ 8	1.6	10
	0.3	164 $\pm$ 14	1.6	6
	1	269 $\pm$ 12	1.6	8
	3	331 $\pm$ 15	1.5	6
	10	356 $\pm$ 7	1.6	6
GluN1 ^{FH+RK} /3A	0	162 $\pm$ 8	1.4	12
GluN1 ^{FA+TL} /3B	0	95 $\pm$ 7	1.4	13
	10	102 $\pm$ 3	1.3	21
GluN1 ^{FH+RK} /3B	0	270 $\pm$ 3	1.4	16

Glycine concentration–response data were recorded using two-electrode voltage-clamp electrophysiology at recombinant NMDA receptors expressed in *Xenopus* oocytes. Responses were measured in the continuous presence of L-689,560 at the indicated concentrations. Data are presented as mean $\pm$ SEM of values from individual oocytes, and *n* is the number of oocytes used to generate the data.

^aMeasured in the continuous presence of 300 μM glutamate.

^bMeasured in the continuous presence of 1 μM CGP-78608.

^cMeasured in the continuous presence of 10 μM CGP-78608.

standard error of ξ_1 or ξ_2 based on trajectory segments of length *n*. BSE is predicted to underestimate the true standard error for short block lengths, which are highly correlated, but will increase with larger block lengths as the blocks become statistically independent. Simulations can be considered to have reasonable sampling quality when BSE values no longer change (i.e., asymptotes) with increasing block lengths. These block-averaging analyses estimated that BSE values for ξ_1 and ξ_2 from $8 \times 2,500$ ns trajectories used for PMF calculations reached 95% of the asymptote after 333–2,286 ns, depending on GluN1 mutations or the ligand bound. Statistical uncertainties in 2D and 1D PMFs were evaluated as standard deviations among individual PMFs calculated for each of the $8 \times 2,500$ ns trajectories.

Statistical analysis and data presentation

Averaged data are presented as mean $\pm$ SEM and *P* < 0.05 was considered statistically significant. All sample sizes (*n* values) represent independent biological replicates. Statistical comparisons were performed as described in figure legends using GraphPad Prism (GraphPad Software). Normality tests were not performed, but statistical comparisons of decay time constants (τ_{decay}) were performed on normally distributed rate constants ($1/\tau_{\text{decay}}$; Christopoulos, 1998) using unpaired two-way ANOVA with Tukey's correction for multiple comparisons. Mice and cells used for experiments were selected randomly among mice with the desired genotype or cells with the indicated NMDA receptor subtype expression. Experimenters were not blinded to genotype or NMDA receptor subtype expression used for the experiments. Experimental data were repeated on at least two different days as independent experiments (i.e., using new recording solutions) with multiple mice for recordings from brain slices, multiple transfections for recordings from HEK cells, and multiple batches of oocytes for two-electrode voltage-clamp recordings.

Online supplemental material

Fig. S1 shows block-averaging analyses for ξ_1 and ξ_2 coordinates to assess convergence and sampling quality in trajectories used for PMF calculations. Fig. S2 shows statistical uncertainties in 2D PMFs for WT and mutated GluN1 ABDs in the apo state or with bound ligands.

Results

L-689,560 inhibits GluN1/3A-mediated glycine responses in acute hippocampal slices

Binding studies show that the competitive antagonist L-689,560 displays 10,400-fold higher affinity for the orthosteric binding site in GluN1 over GluN3A (Yao and Mayer, 2006) suggesting that this antagonist may enable activation of native GluN1/3A receptors by pressure-applied application of glycine (i.e., glycine puffs). To test this prediction, we measured current responses to glycine puffs onto hippocampal CA1 pyramidal neurons in acute brain slices from WT and 3A-KO mice in the absence and presence of 1 μM L-689,560, but in the presence of bath-applied antagonists of GABA_A, AMPA, glycine, and GluN1/2 receptors to isolate responses from native GluN1/3A receptors (Fig. 1). However, L-689,560 did not significantly enhance glycine responses in neurons from either WT or 3A-KO mice (Fig. 1, B–D). Using the same experimental conditions, we evaluated potentiation of native GluN1/3A receptors by CGP-78608 in hippocampal CA1 pyramidal neurons from WT mice (Fig. 1, E–G). Importantly, glycine-induced responses in the presence of CGP-78608 are mediated by GluN1/3A receptors and are absent in GluN3A knock-out mice (Grand et al., 2018; Zhu et al., 2020). In stark contrast to L-689,560, the application of 1 μM CGP-78608 produced ~25-fold potentiation of current responses to glycine puffs. However, the potentiation by CGP-78608 was fully reversed by the co-application of 1 μM L-689,560.

These results demonstrate that despite high selectivity for GluN1, which prevents glycine binding at this subunit,

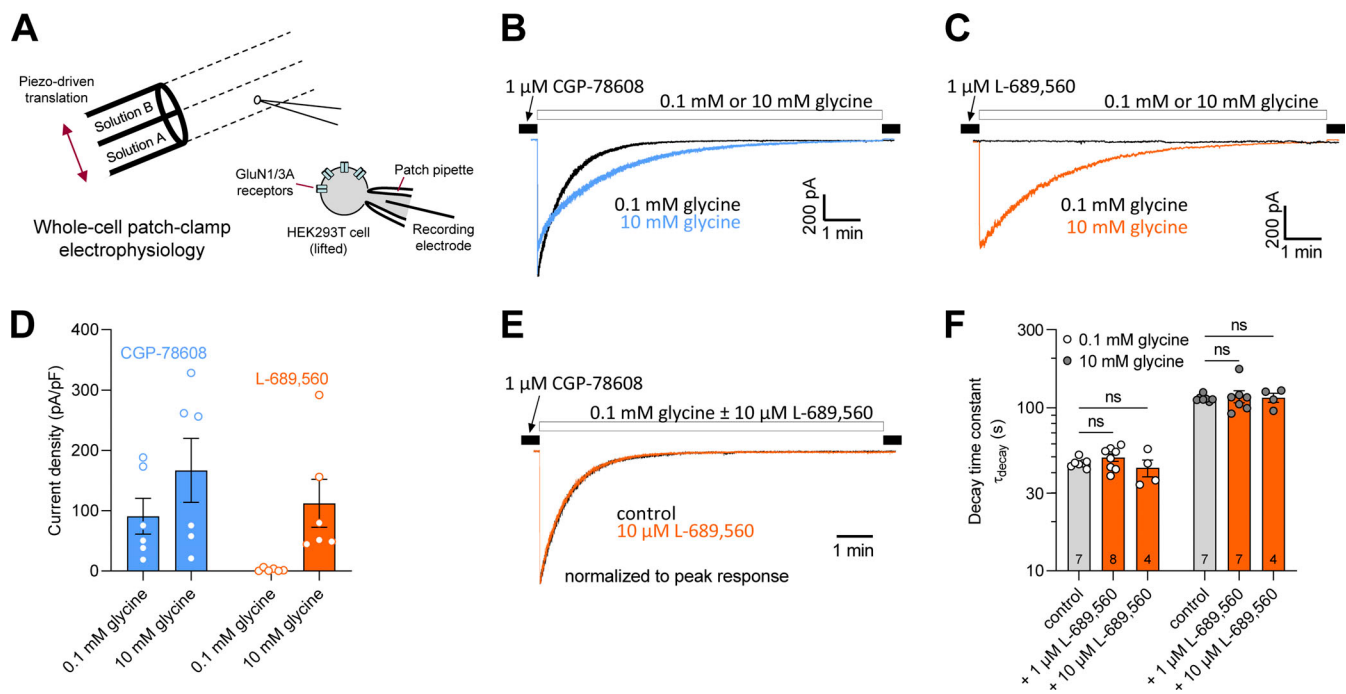


Figure 5. L-689,560 inhibition of GluN1/3A requires unbinding of CGP-78608 from GluN1. (A) Schematic representation of fast-application whole-cell patch-clamp electrophysiology on lifted HEK293T cells expressing recombinant GluN1/3A receptors. Rapid solution exchange is achieved by piezo-driven translation of a theta-tube to move the lifted HEK293T cell between two distinct solutions. (B) Representative whole-cell patch-clamp recordings of responses from recombinant GluN1/3A receptors expressed in HEK293T cells. Responses were activated by long applications (9.5 min) of 0.1 or 10 mM glycine. CGP-78608 (1 μ M) was present before and after, but not during, glycine applications. The decay of the response amplitude reflects desensitization following CGP-78608 unbinding and subsequent glycine binding to the GluN1 subunit. Glycine binding to GluN3A slows CGP-78608 unbinding from GluN1. (C) Representative recordings of responses from GluN1/3A receptors activated with 1 μ M L-689,560 present before and after, but not during, glycine applications. (D) Bar graph summarizing current density of responses activated by 0.1 or 10 mM glycine from GluN1/3A receptors pre-exposed to either 1 μ M CGP-78608 or 1 μ M L-689,560. Pre-exposure to CGP-78608 resulted in robust current responses to both 0.1 and 10 mM glycine, whereas pre-exposure to L-689,560 only resulted in responses to 10 mM glycine. (E) Representative recordings of responses activated with CGP-78608 (1 μ M) present before and after, but not during, glycine applications. Responses were activated by 0.1 mM glycine in the absence (control) or presence of 10 μ M L-689,560. (F) Bar graph summarizing time constants for the decay (τ_{decay}) of current responses from GluN1/3A receptors pre-exposed to 1 μ M CGP-78608 and activated by 0.1 mM or 10 mM glycine. Time constants were measured in the absence (control) and presence of 1 or 10 μ M L-689,560 as shown in B. The presence of high or low L-689,560 concentrations did not change $1/\tau_{\text{decay}}$ values compared to control (control 0.1 mM glycine vs. + 1 μ M L-689,560, $P = 0.9243$; control 0.1 mM glycine vs. + 10 μ M L-689,560, $P = 0.6516$; control 10 mM glycine vs. + 1 μ M L-689,560, $P > 0.9999$; control 10 mM glycine vs. + 10 μ M L-689,560, $P > 0.9999$; unpaired two-way ANOVA with Tukey's posttest; statistical test was performed on normally distributed rate constants ($1/\tau_{\text{decay}}$), suggesting that CGP-78608 unbinding from GluN1 is a rate limiting step for inhibition by L-689,560.

L-689,560 is incapable of robustly potentiating native GluN1/3A receptors. Furthermore, L-689,560 is an inhibitor of glycine-activated current responses mediated by native GluN1/3A receptors and potentiated by CGP-78608.

L-689,560 is a potent inhibitor of GluN1^{FA+TL}/3A receptors

We measured responses from recombinant GluN1/2A and GluN1^{FA+TL}/3A receptors using two-electrode voltage-clamp recordings to evaluate inhibition by L-689,560, CGP-78608, and the competitive glycine site antagonists 5,7-dichlorokynurenic acid (DCKA). GluN1 F484A+T518L mutations abolish glycine binding to GluN1 and thereby enable selective glycine binding to the GluN3A subunit (Kvist et al., 2013a). Thus, antagonist activity at GluN1/2A receptors is mediated by binding to the glycine site in GluN1, whereas inhibition of GluN1^{FA+TL}/3A receptors should reflect binding to the glycine site in GluN3A, assuming the GluN1 F484A+T518L mutations also prevent antagonist binding.

In the continuous presence of 300 μ M glutamate, L-689,560 ($\text{IC}_{50} = 250$ nM), CGP-78608 ($\text{IC}_{50} = 65$ nM), and DCKA ($\text{IC}_{50} = 1.5$ μ M) completely inhibited responses to 10 μ M glycine from GluN1/2A receptors (Fig. 2, A and B; and Table 1). Calculated using the Cheng-Prusoff relationship (Cheng and Prusoff, 1973) and a previously published glycine EC_{50} of 1.0 μ M (Zhao et al., 2022), the dissociation binding constant (K_b) values for L-689,560, CGP-78608, and DCKA at GluN1 in GluN1/2A receptors were estimated to be 23, 5.9, and 140 nM, respectively. These K_b values are similar to previously reported values determined in binding studies using the isolated GluN1 ABD (Furukawa and Gouaux, 2003; Yao and Mayer, 2006).

Surprisingly, L-689,560 potently inhibited responses to 30 μ M glycine from GluN1^{FA+TL}/3A receptors ($\text{IC}_{50} = 77$ nM; Fig. 2, C and D; and Table 1). This result is seemingly inconsistent with the previously reported 10,400-fold higher affinity of L-689,560 for GluN1 over GluN3A ABDs (Yao and Mayer, 2006), but supports a mechanism by which inhibition of native GluN1/3A

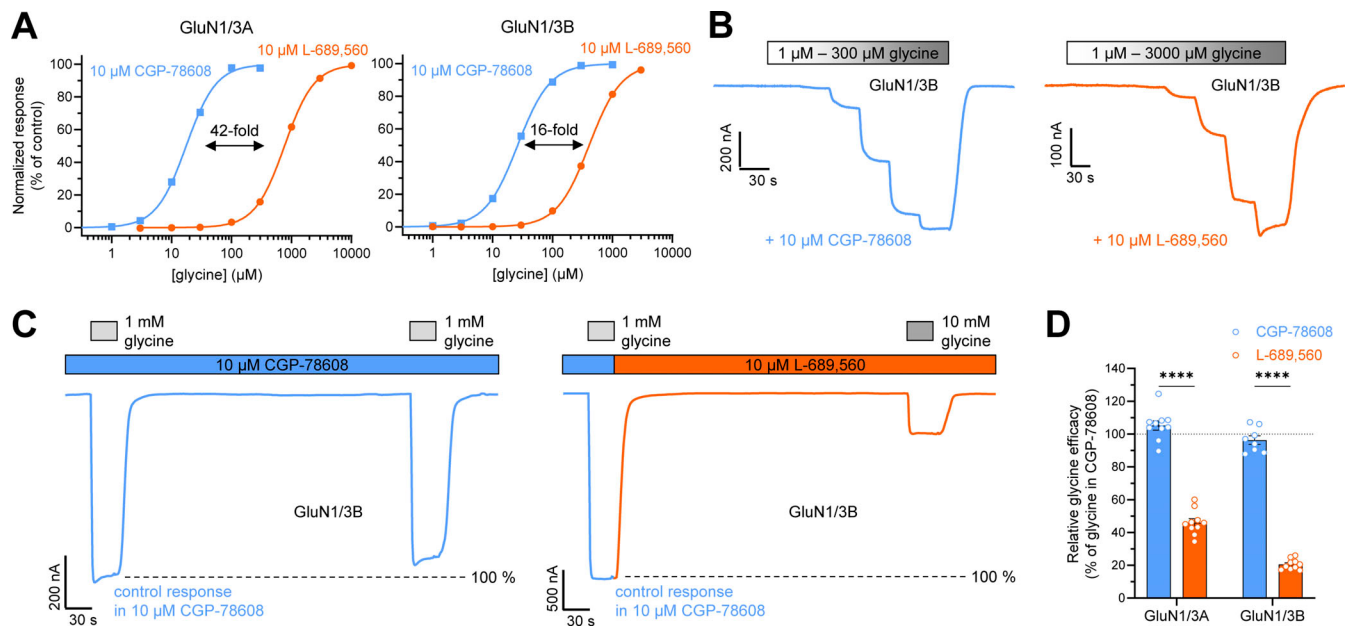


Figure 6. Antagonist binding to GluN1 influences glycine potency and efficacy at the GluN3 subunit. (A) Glycine concentration–response data for recombinant GluN1/3 receptors measured using two-electrode voltage-clamp recordings in the presence of either 10 μ M CGP-78608 or 10 μ M L-689,560. Data are shown as mean $\pm$ SEM from 14 to 18 oocytes. (B) Representative two-electrode voltage-clamp recordings of glycine concentration–response data from GluN1/3B receptors in the presence of either 10 μ M CGP-78608 or 10 μ M L-689,560. (C) Representative recordings to measure the relative agonist efficacy of glycine in the presence of GluN1-selective antagonists. Responses in the presence of CGP-78608 or L-689,560 were normalized to a control response in the presence of CGP-78608 at the beginning of the recording. Responses in CGP-78608 were $105 \pm 3\%$ ($n = 10$) at GluN1/3A and $96 \pm 3\%$ ($n = 8$) at GluN1/3B, and responses in L-689,560 were $46 \pm 2\%$ ($n = 10$) at GluN1/3A and $21 \pm 1\%$ ($n = 10$) at GluN1/3B. (D) Bar graph summarizing data using the recording protocols shown in C. Responses measured in L-689,560 were significantly smaller than responses in CGP-78608 (GluN1/3A, $P < 0.0001$; GluN1/3B, $P < 0.0001$; unpaired two-way ANOVA with Tukey's posttest), demonstrating that binding of L-689,560 reduces glycine agonist efficacy at GluN1/3 receptors compared to binding of CGP-78608.

receptors in hippocampal CA1 pyramidal neurons is mediated by binding of L-689,560 to the GluN1/3A receptor. Concentrations of CGP-78608 up to 30 μ M did not produce inhibition, whereas DCKA inhibited GluN1^{FA+TL}/3A responses ($IC_{50} = 29 \mu$ M), presumably by competing with glycine binding to the GluN3A subunit as previously suggested (Kvist et al., 2013b). These results demonstrate that CGP-78608 is a highly GluN1-selective antagonist and that DCKA is a non-selective GluN1 and GluN3A glycine site antagonist. The activity of L-689,560 at GluN1/3A receptors is puzzling, since this ligand is highly selective for the orthosteric binding site in GluN1 (Yao and Mayer, 2006), yet L-689,560 is a strong antagonist of both native GluN1/3A and recombinant GluN1^{FA+TL}/3A receptors.

L-689,560 is a negative allosteric modulator of GluN1/3A and GluN1/3B receptors

To further evaluate the mechanism of L-689,560 antagonism, we measured inhibition of recombinant GluN1/3A and GluN1/3B receptors potentiated by CGP-78608 (Fig. 3 A and Table 1). L-689,560 produced robust inhibition with IC_{50} values of 0.53 and 1.9 μ M at GluN1/3A and GluN1/3B receptors, respectively, in the presence of 1 μ M CGP-78608. In the presence of 10 μ M CGP-78608, the IC_{50} values for L-689,560 inhibition were reduced 7.2- and 4.7-fold to 3.8 and 8.9 μ M at GluN1/3A and GluN1/3B receptors, respectively. These results indicate that increased CGP-78608 concentrations reduce the potency of L-689,560 inhibition.

We then evaluated L-689,560 inhibition at GluN1^{FA+TL}/3A receptors activated by 100 μ M, 1 mM, or 3 mM glycine in the absence of CGP-78608 (Fig. 3, B and C; and Table 1). L-689,560 inhibition of GluN1^{FA+TL}/3A receptors displayed a biphasic concentration–response relationship containing high-potency and low-potency components with IC_{50} values of 0.15–0.38 and 33–160 μ M, respectively. Moreover, efficacy of the high-potency component was dependent on the glycine concentration with the highest efficacy at 100 μ M glycine (75% inhibition) and similar efficacy at 1 and 3 mM glycine (44 and 41% inhibition, respectively). Concentration–response relationships for L-689,560 at GluN1^{FA+TL}/3B receptors were qualitatively similar to those observed for GluN3A-containing receptors, except L-689,560 inhibition did not display any discernable glycine dependency (Fig. 3 B and Table 1). That is, inhibitory efficacies of the high-potency component were similar at 100 μ M, 1 and 3 mM glycine (29, 30, and 40% inhibition, respectively). These observations are inconsistent with a competitive mechanism of L-689,560 binding to agonist binding sites in GluN3 subunits. Rather, the experiments suggest that L-689,560 is a negative allosteric modulator of both GluN1/3A and GluN1/3B receptors.

L-689,560 reduces glycine binding to GluN1/3A, but not GluN1/3B receptors

We performed Schild analyses by measuring glycine concentration–response data in the absence and presence of

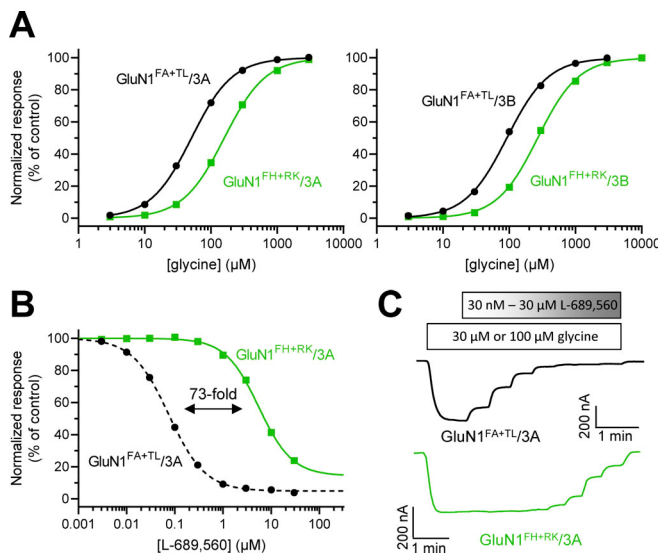


Figure 7. GluN1 mutations influence glycine potency at the GluN3 subunit and modulation by L-689,560. (A) Glycine concentration–response data for GluN1^{FA+TL}/3A and GluN1^{FH+RK}/3A receptors. F484A+T518L and F484H+R523K mutations both abolish glycine binding to GluN1 and prevent receptor desensitization. Data are shown as mean $\pm$ SEM from 12 to 16 oocytes. (B) Concentration–response data for L-689,560 inhibition of GluN1^{FA+TL}/3A activated by 30 μ M glycine and GluN1^{FH+RK}/3A receptors activated by 100 μ M glycine. Data for GluN1^{FA+TL}/3A was reproduced from Fig. 2 C. Data are shown as mean $\pm$ SEM from 5 to 16 oocytes. (C) Representative two-electrode voltage-clamp recordings of responses from GluN1^{FA+TL}/3A and GluN1^{FH+RK}/3A receptors activated by 30 and 100 μ M glycine, respectively, and inhibited by L-689,560. See Tables 1 and 2 for IC₅₀ and EC₅₀ values.

increasing concentrations of L-689,560 (Fig. 4). The concentration–response data were analyzed by fitting to equations derived from an equilibrium model for allosteric modulation of agonist binding to obtain the glycine EC₅₀ in the absence of L-689,560, the allosteric constant α , and the dissociation binding constant K_b for L-689,560 in the absence of glycine (Fig. 4 C; see Materials and methods). According to this model, the association constant for agonist binding (K_A) is changed by the allosteric constant α upon binding of the allosteric modulator (Ehlert, 1988; Christopoulos and Kenakin, 2002; Hansen et al., 2012; Yi et al., 2016). Similarly, the association constant for modulator binding (K_B) is also changed by the allosteric constant α upon binding of agonist. This model assumes that agonist efficacy E is unchanged by the allosteric modulator, although this is likely oversimplified. In this model, competitive antagonists have an allosteric constant $\alpha = 0$, neutral ligands have $\alpha = 1$, positive modulators have $\alpha > 1$, and negative modulators have $0 < \alpha < 1$.

As expected, L-689,560 displayed a competitive mechanism of inhibition at GluN1/2A receptors with allosteric constant $\alpha = 0$ and $K_b = 11$ nM (Fig. 4 D). However, L-689,560 displayed a noncompetitive mechanism on GluN1^{FA+TL}/3A receptors with allosteric constant $\alpha = 0.117$ and $K_b = 80$ nM (Fig. 4 D). By contrast, L-689,560 did not change glycine potency at GluN1^{FA+TL}/3B receptors (Fig. 4 E and Table 2), consistent with data shown in Fig. 3 B. Thus, negative modulation of glycine binding is saturable at GluN1^{FA+TL}/3A with glycine EC₅₀ of ~ 50 μ M in the absence of

L-689,560 and glycine EC₅₀ of ~ 430 μ M (i.e., EC₅₀/ α) when L-689,560 is fully bound to the receptor. The shift in glycine EC₅₀ upon L-689,560 binding to GluN1^{FA+TL}/3A can explain the glycine-dependent reduction in the efficacy of the high-potency component shown in Fig. 3 B. That is, the highest efficacy (75% inhibition) was observed at 100 μ M glycine, which is below the EC₅₀ of ~ 400 μ M when L-689,560 is bound. Efficacy for the high-potency component was saturated at 1 and 3 mM glycine (44 and 41% inhibition, respectively) since these glycine concentrations are above the EC₅₀ of ~ 400 μ M when L-689,560 is bound. Moreover, the $\sim 40\%$ inhibition at 3 mM glycine shown in Fig. 3 B suggests that L-689,560 also reduces agonist efficacy in addition to reducing glycine potency. The results further corroborate that L-689,560 is a negative allosteric modulator of GluN1/3A and GluN1/3B receptors.

L-689,560 inhibition of GluN1/3A requires CGP-78608 unbinding from GluN1

L-689,560 was developed to bind the GluN1 orthosteric site with high affinity (Foster et al., 1992). We therefore hypothesized that allosteric modulation of GluN1/3 receptors by L-689,560 is mediated by binding to the GluN1 glycine site. If allosteric inhibition by L-689,560 requires binding to the GluN1 orthosteric site, then occupancy at this site with another ligand, such as CGP-78608, would prevent inhibition, consistent with data shown in Fig. 3 A. According to this mechanism, the rate of L-689,560 inhibition at GluN1/3 receptors would be limited by the unbinding rate of CGP-78608 bound to the GluN1 orthosteric site.

To test our hypothesis, we examined kinetic relationships between CGP-78608 and L-689,560 binding to recombinant GluN1/3A receptors using fast-application whole-cell voltage-clamp recordings (Fig. 5 A). We first measured CGP-78608 unbinding rate from GluN1. In this experiment, the receptors were pre-exposed to 1 μ M CGP-78608 at baseline and then switched into a solution of either low (0.1 mM) or high (10 mM) glycine without CGP-78608 (Fig. 5 B). Given that glycine binding to GluN1 results in fast and strong desensitization of GluN1/3A receptors, CGP-78608 unbinding from GluN1 would result in a decay of the response back to baseline levels with a time constant (τ_{decay}) that is mainly governed by the time constant for CGP-78608 unbinding. CGP-78608 τ_{decay} values were relatively slow at 45 ± 1 s ($n = 7$) in 0.1 mM glycine, and even slower at 114 ± 2 s ($n = 7$) in 10 mM glycine (Fig. 5, B and F), suggesting positive cooperation between glycine binding to GluN3A and CGP-78608 binding to GluN1. We repeated the same experimental protocol except with pre-exposure to 1 μ M L-689,560 and found that 10 mM glycine, but not 0.1 mM glycine, activated robust responses (Fig. 5, C and D). L-689,560 τ_{decay} was slow at 136 ± 10 s ($n = 6$) in 10 mM glycine (Fig. 5 C). Thus, binding of L-689,560 to GluN1/3A results in the complete inhibition of responses to 0.1 mM glycine but not to 10 mM glycine.

In the next experiment, we pre-exposed GluN1/3A receptors to 1 μ M CGP-78608 and then switched into a solution of 0.1 mM glycine + 1 or 10 μ M L-689,560 (Fig. 5 E). Values for τ_{decay} did not change by co-application of 1 μ M (50 ± 3 s; $n = 8$) or 10 μ M L-689,560 (43 ± 5 s; $n = 4$) compared to τ_{decay} in the absence of L-689,560 (45 ± 1 s; $n = 7$; Fig. 5 F). Similarly, τ_{decay} values for

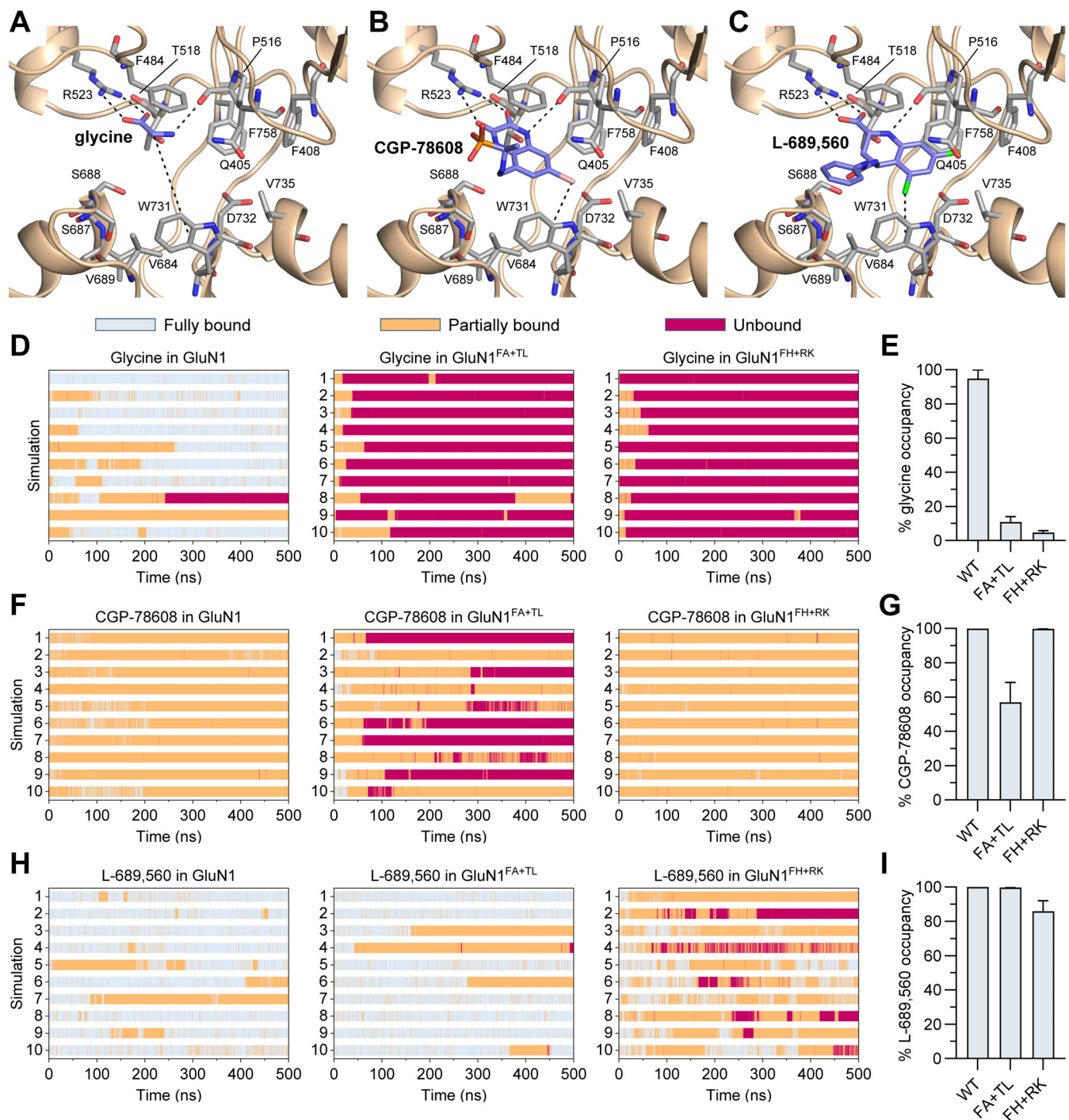


Figure 8. Binding to the GluN1 orthosteric site mediates L-689,560 inhibition of GluN1^{FA+TL}/3A receptors. (A–C) GluN1 agonist binding pockets with bound (A) glycine, (B) CGP-78608, or (C) L-689,560 in starting structures for molecular dynamics simulations modeled from the GluN1 ABD in the GluN1/2A ABD heterodimer structure in complex with L-689,560 (PDB ID: 6USU; Chou et al., 2020). The GluN2A ABD was omitted from simulations. Dashed lines indicate interactions that define the ligands as fully bound, partially bound, or unbound (see Materials and methods for details). Simulations with CGP-78608 and L-689,560 started from the fully bound pose, whereas simulations with glycine started from a partially bound pose. Each trajectory frame was analyzed to determine if the ligand was fully bound (light grey), partially bound (orange), or unbound from the ABD (red). (D–I) Time series from 10 molecular dynamics simulations (500 ns each) with glycine (D), CGP-78608 (F), or L-689,560 (H) bound to the isolated GluN1, GluN1^{FA+TL}, and GluN1^{FH+RK} ABDs. The bar graphs summarize the percentage of time glycine (E), CGP-78608 (G), or L-689,560 (I) spent in the fully and partially bound pose (percent occupancy) for all simulations with GluN1 (WT), GluN1^{FA+TL} (FA+TL), and GluN1^{FH+RK} (FH+RK) ABDs. Data are mean ± SEM from 10 simulations.

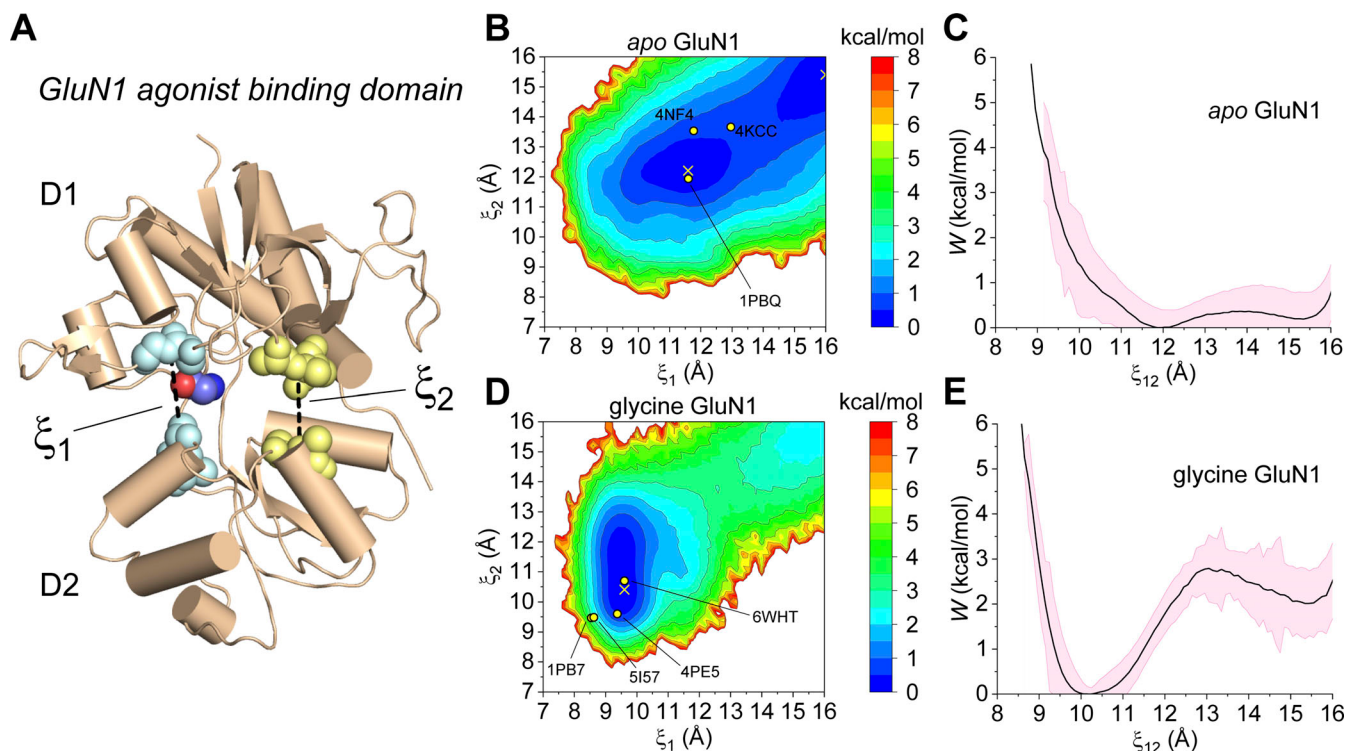


Figure 9. Glycine binding promotes closed GluN1 agonist binding domain conformations. (A) Conformations of the GluN1 ABD are described by measuring ξ_1 and ξ_2 distances between center of mass of atoms (shown as spheres) in the two lobes (D1 and D2) of the GluN1 ABD on each site of the agonist binding cleft. (B) Molecular dynamics trajectories were produced with the isolated GluN1 ABD in the absence of ligand binding (apo), and values of (ξ_1 , ξ_2) from each frame of $8 \times 2,500$ ns simulation trajectories were pooled to calculate the 2D protein conformational free energy landscape (i.e., 2D PMF). X markings indicate local free energy minima. Yellow dots indicate values of (ξ_1 , ξ_2) measured in crystal structures of the isolated DCKA-bound GluN1 ABD (PDB ID: 1PBQ; Furukawa and Gouaux, 2003), the isolated GluN1/2A ABD heterodimer with bound DCKA and glutamate (PDB ID: 4NF4; Jespersen et al., 2014), and the isolated apo GluN1 ABD (PDB ID: 4KCC; Yao et al., 2013). (C) The 1D PMF for the apo GluN1 ABD was calculated by projecting values of (ξ_1 , ξ_2) onto a single coordinate using $\xi_{12} = (\xi_1 + \xi_2)/2$. Red shade indicates standard deviations. (D) Contour plot of the 2D PMF for the glycine-bound GluN1 ABD. The X marking indicates the global free energy minimum. Yellow dots indicate values of (ξ_1 , ξ_2) measured in crystal structures of the isolated glycine-bound GluN1 ABD (PDB ID: 1PB7; Furukawa and Gouaux, 2003), the isolated GluN1/2A ABD heterodimer with bound glycine and glutamate (PDB ID: 5157; Yi et al., 2016), and the glycine- and glutamate-bound GluN1/2B receptor (PDB ID: 4PE5; Karakas and Furukawa, 2014), as well as in the cryo-EM structure of the glycine- and glutamate-bound GluN1/2B receptor (PDB ID: 6WHT; Chou et al., 2020). (E) Graph of the 1D PMF for the glycine-bound GluN1 ABD. Red shade indicates standard deviations.

responses pre-exposed to $1 \mu\text{M}$ CGP-78608 and activated by 10 mM glycine did not change by co-application of $1 \mu\text{M}$ ($117 \pm 10 \text{ s}$; $n = 7$) or $10 \mu\text{M}$ L-689,560 ($115 \pm 7 \text{ s}$; $n = 4$) compared to τ_{decay} in the absence of L-689,560 ($114 \pm 2 \text{ s}$; $n = 7$; Fig. 5 F). These results demonstrate that the rate of L-689,560 inhibition is limited by the unbinding rate of CGP-78608 from the GluN1 orthosteric site. These results support a mechanism in which positive allosteric interactions exist between binding of CGP-78608 to GluN1 and glycine to GluN3A, whereas negative allosteric interactions exist between binding of L-689,560 to GluN1 and glycine to GluN3A.

L-689,560 enabled activation of GluN1/3A receptors by 10 mM glycine, but not 0.1 mM glycine (Fig. 5, C and D), suggesting low glycine potency at GluN1/3 receptors pre-exposed to L-689,560 compared to receptors pre-exposed to CGP-78608. To test this prediction, we measured glycine concentration–response data at GluN1/3A and GluN1/3B receptors in the presence of either CGP-78608 or L-689,560 (Fig. 6, A and B). CGP-78608 binding to GluN1 resulted in a glycine EC_{50} of $14\text{--}19 \mu\text{M}$, whereas L-689,560 binding to GluN1 yielded a glycine EC_{50} of $714\text{--}802 \mu\text{M}$ (Table 2). Thus, glycine potency was 38- to 57-fold higher at GluN1/3A receptors with bound CGP-78608 compared to receptors with bound

L-689,560. The difference was less pronounced for GluN1/3B receptors that displayed 14- to 22-fold higher glycine potency with bound CGP-78608 compared to L-689,560 (Table 2).

To determine if agonist efficacy of glycine at GluN1/3 receptors is affected by binding of GluN1-selective antagonists, we compared responses to saturating concentrations of glycine in the presence of CGP-78608 to responses in the presence of L-689,560. Response amplitudes measured in L-689,560 were $46 \pm 2\%$ ($n = 10$) at GluN1/3A and $21 \pm 1\%$ ($n = 10$) at GluN1/3B relative to responses in CGP-78608 (Fig. 6, C and D). These results demonstrate that glycine agonist efficacy is reduced at GluN1/3 receptors bound to L-689,560 compared to receptors bound to CGP-78608. Desensitization of GluN1/3 receptors can therefore be blocked by binding of L-689,560 to the GluN1 subunit, but this will markedly reduce glycine potency and efficacy at the GluN3 subunit and thereby limit activation of GluN1/3 receptors at lower glycine concentrations.

L-689,560 binds the mutated GluN1 orthosteric site to mediate inhibition of GluN1^{FA+TL}/3A receptors

In order to explain inhibition of GluN1^{FA+TL}/3A receptors by L-689,560, we hypothesized that L-689,560 binds the GluN1

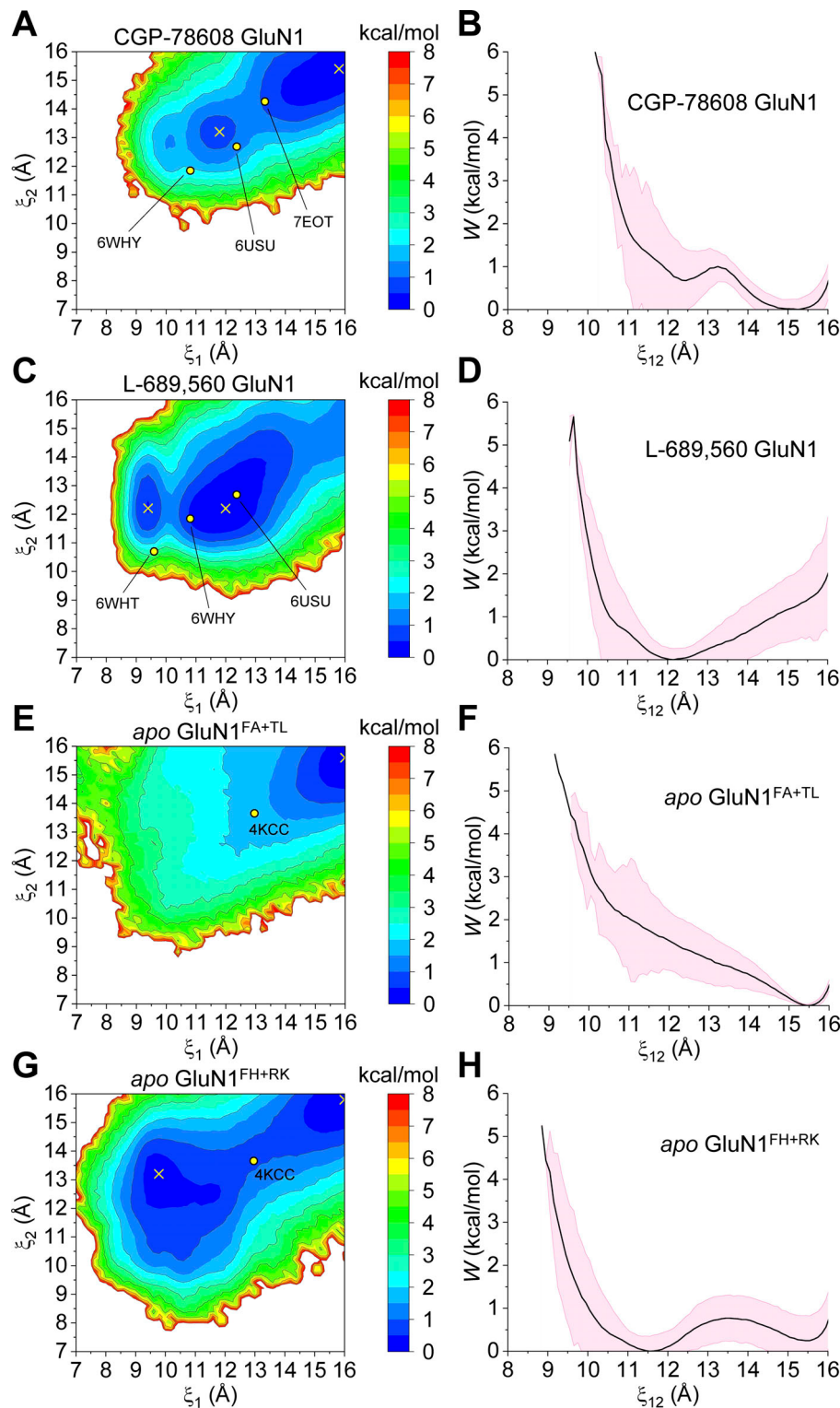


Figure 10. CGP-78608, L-689,560, and mutations select distinct GluN1 agonist binding domain conformations. (A, C, E, and G) Contour plots of 2D PMFs for GluN1 ABDs with bound CGP-78608 (A) or L-689,560 (C), as well as apo GluN1^{FA+TL} (E) and GluN1^{FH+RK} (G) ABDs. X markings indicate global or local free energy minima. Yellow dots indicate values of (ξ_1, ξ_2) measured in crystal structures of the GluN1/2A ABD heterodimer with bound L-689,560 and glutamate (PDB ID: 6USU; Chou et al., 2020), and the isolated apo GluN1 ABD (PDB ID: 4KCC; Yao et al., 2013), as well as in cryo-EM structures of the glycine- and glutamate-bound GluN1/2B receptor (PDB ID: 6WHT; Chou et al., 2020), the L-689,560- and glutamate-bound GluN1/2B receptor (PDB ID: 6WHT; Chou et al., 2020), and the CGP-78608- and glutamate-bound GluN1/2A receptor (PDB ID: 7EOT; Wang et al., 2021). (B, D, F, and H) Graphs show 1D PMFs for GluN1 ABDs with bound CGP-78608 (B) or L-689,560 (D), as well as apo GluN1^{FA+TL} (F) and GluN1^{FH+RK} (H) ABDs. Red shades indicate standard deviations.

orthosteric site despite the modification by F484A+T518L mutations (FA+TL). Consistent with this hypothesis, we predicted that the potency of L-689,560 inhibition must be influenced by mutations in the GluN1 agonist binding site. To evaluate this hypothesis, we introduced F484H+R523K mutations (FH+RK) in the GluN1 orthosteric site in order to both abolish glycine binding and interfere with L-689,560 binding. Glycine displayed

a sigmoidal concentration–response relationship at GluN1^{FH+RK}/3A and GluN1^{FH+RK}/3B receptors, indicating that FH+RK abolish glycine binding to GluN1 and thereby prevent desensitization (Fig. 7 A). Glycine potency was modestly reduced by 3.1-fold for GluN1^{FH+RK}/3A ($EC_{50} = 165 \mu\text{M}$) compared to GluN1^{FA+TL}/3A receptors ($EC_{50} = 53 \mu\text{M}$) and by 2.4-fold at GluN1^{FH+RK}/3B ($EC_{50} = 270 \mu\text{M}$) compared to GluN1^{FA+TL}/3B receptors ($EC_{50} =$

113 μM), indicating that glycine potency at the GluN3 subunit is influenced by GluN1 orthosteric site mutations (Fig. 7 A and Table 2). However, the potency of L-689,560 inhibition was markedly reduced by 73-fold in GluN1^{FH+RK}/3A ($\text{IC}_{50} = 5.6 \mu\text{M}$) activated by 100 μM glycine compared to GluN1^{FA+TL}/3A receptors ($\text{IC}_{50} = 77 \text{ nM}$) activated by 30 μM glycine (Fig. 7, B and C; and Table 1). These results are consistent with our hypothesis and demonstrate that the potency of L-689,560 inhibition is influenced by mutations in the GluN1 agonist binding site.

We performed molecular dynamics simulations to further corroborate that L-689,560 can bind the mutated GluN1^{FA+TL} orthosteric site. We produced 10 simulations of 500 ns for each of three ligands (glycine, CGP-78608, and L-689,560) bound to isolated GluN1^{WT}, GluN1^{FA+TL}, and GluN1^{FH+RK} ABDs (Fig. 8, A–C; see Materials and methods). Each trajectory frame was analyzed to determine if the ligand was fully bound or if the ligand had unbound the orthosteric site. Ligand binding poses that were not fully bound or unbound were defined as partially bound. Glycine remained stably bound to the GluN1^{WT} ABD, but readily unbound from GluN1^{FA+TL} and GluN1^{FH+RK} ABDs (Fig. 8, D and E). Interestingly, CGP-78608 remained stably bound to both GluN1^{WT} and GluN1^{FH+RK} ABDs but was unstably bound in the GluN1^{FA+TL} ABD (Fig. 8, F and G). As predicted, L-689,560 remained stably bound to both GluN1^{WT} and GluN1^{FA+TL} ABDs but was less stably bound in the GluN1^{FH+RK} ABD compared to the GluN1^{FA+TL} ABD (Fig. 8, H and I). These results are consistent with the reduced L-689,560 potency at GluN1^{FH+RK}/3A receptors (Fig. 7) and support a mechanism in which L-689,560 binds the GluN1 orthosteric site to mediate allosteric inhibition of GluN1/3 receptors. Furthermore, L-689,560 binding is preserved in the mutated GluN1^{FA+TL} orthosteric site, thereby enabling inhibition of GluN1^{FA+TL}/3 receptors.

CGP-78608 and L-689,560 promote distinct GluN1 agonist binding domain conformations

CGP-78608 and L-689,560 both bind the GluN1 orthosteric site to prevent desensitization of GluN1/3 receptors. However, L-689,560 occupancy at the GluN1 orthosteric site results in lower glycine potency and efficacy at the GluN3 subunit compared to CGP-78608 occupancy. Furthermore, mutations in the GluN1 orthosteric site to abolish glycine binding influence glycine potency at the GluN3 subunit. These results suggest that GluN1-selective antagonists or mutations promote distinct conformations of the GluN1 ABD that influence intra-subunit interactions between GluN1 and GluN3 subunits. To investigate conformational selection, we used molecular dynamics simulations to observe hinged movements between the two lobes of the GluN1 ABD, D1 and D2, that together form the agonist binding pocket. Specifically, we produced trajectories for each ligand or mutation and measured two center-of-mass distances (ξ_1 , ξ_2) between D1 and D2 as previously described (Yao et al., 2013; Fig. 9 A; see Materials and methods for details). Values of (ξ_1 , ξ_2) from each frame of $8 \times 2,500$ ns simulation trajectories were pooled to calculate 2D protein conformational free energy landscapes $W(\xi_1, \xi_2)$; i.e., PMFs). 1D protein conformational free energy landscapes $W(\xi_{12})$ were calculated by projecting values of (ξ_1 , ξ_2) onto a single coordinate using $\xi_{12} = (\xi_1 + \xi_2)/2$. The simulations included a restraint to prevent

ligand dissociation and asymmetric harmonic potentials to discourage ξ_1 and ξ_2 from exceeding 16 Å and visit hyper-extended ABD conformations during long simulations (see Materials and methods for details).

We initially determined 2D PMFs for the ligand-free (apo) and the glycine-bound GluN1 ABD (Fig. 9; see also Figs. S1 and S2 for estimation of convergences and statistical uncertainties). GluN1 apo 2D and 1D PMFs displayed a relatively broad and flat free energy basin, consistent with conformational flexibility in the absence of bound ligand (Fig. 9, B and C). By contrast, GluN1 glycine PMFs revealed clear selection of a closed ABD conformation, albeit with some flexibility along ξ_2 (Fig. 9, D and E). The apo 2D PMF had local free energy minima at $(\xi_1, \xi_2) = (11.6, 12.2 \text{ Å})$ and $(16.0, 15.4 \text{ Å})$, while the glycine 2D PMF had a global free energy minimum at $(\xi_1, \xi_2) = (9.6, 10.4 \text{ Å})$. Global free energy minima for apo and glycine 1D PMFs were located at $\xi_{12} = 11.9$ and 10.2 Å , respectively. The PMFs and global free energy minima are consistent with previous estimates of conformational free energy landscapes of GluN1 ABDs (Yao et al., 2013; Dai and Zhou, 2015) and crystal and cryo-EM structures of apo and glycine-bound GluN1 ABDs (Furukawa and Gouaux, 2003; Yao et al., 2013; Karakas and Furukawa, 2014; Yi et al., 2016; Chou et al., 2020; Fig. 9).

For the isolated GluN1 ABD with bound antagonists, the 2D PMFs displayed local free energy minima at $(\xi_1, \xi_2) = (11.8, 13.2 \text{ Å})$ and $(15.8, 15.4 \text{ Å})$ for CGP-78608 and $(\xi_1, \xi_2) = (9.4, 12.2 \text{ Å})$ and $(12.0, 12.2 \text{ Å})$ for L-689,560 (Fig. 10, A and C). Global free energy minima for CGP-78608 and L-689,560 1D PMFs were located at $\xi_{12} = 15.2 \text{ Å}$ and 12.1 Å , respectively (Fig. 10, B and D). The mutated GluN1^{FA+TL} ABD without bound ligand (apo) showed a global free energy minimum at $(\xi_1, \xi_2) = (16.0, 15.6 \text{ Å})$ and the mutated apo GluN1^{FH+RK} ABD displayed local free energy minima at $(\xi_1, \xi_2) = (16.0, 15.8 \text{ Å})$ and $(9.8, 13.2 \text{ Å})$; (Fig. 10, E and G). For 1D PMFs, the global free energy minima were located at $\xi_{12} = 15.4 \text{ Å}$ and 11.5 Å for GluN1^{FA+TL} and GluN1^{FH+RK}, respectively (Fig. 10, F and H). The shape of energy basins for these apo GluN1 ABDs indicate that F484A + T518L mutations select more open GluN1 ABD conformations compared to F484H + R523K mutations, which produced a broad and flat free energy basin (Fig. 10, E–H), similar to the WT apo GluN1 ABD (Fig. 9, A and B). In summary, molecular dynamics simulations support a mechanism in which CGP-78608 binding promotes distinct and more open GluN1 ABD conformations compared to L-689,560 binding. Furthermore, GluN1 orthosteric site mutations promote distinct conformations of the GluN1 ABD, which may influence glycine potency at the GluN3 subunit. Together, the functional data and the molecular dynamics simulations suggest that more open conformations of the GluN1 ABD promote glycine binding to GluN3A, whereas more closed GluN1 ABD conformations negatively modulate glycine binding, consistent with glycine EC_{50} values determined in Fig. 6 and shown in Table 2.

Discussion

We demonstrate that the GluN1-selective antagonists, CGP-78608 and L-689,560, both prevent desensitization of GluN1/3 receptors by occluding glycine binding to GluN1, but that

CGP-78608 increases glycine potency and efficacy at the GluN3 subunit, whereas L-689,560 reduces glycine potency and efficacy (Fig. 6 and Table 2). These findings provide a mechanism to explain the observation that pre-exposure to CGP-78608, but not L-689,560, robustly potentiate current responses from native GluN1/3A receptors in hippocampal CA1 pyramidal neurons (Fig. 1). Similarly, we demonstrate that both F484A + T518L and F484H + R523K mutations in the GluN1 glycine binding site prevent desensitization, but that glycine potency is higher at GluN1^{FA+TL}/3A compared to at GluN1^{FH+RK}/3A receptors (Fig. 7 and Table 2). We performed molecular dynamics simulations with the GluN1 ABD in complex with CGP-78608 or L-689,560 (Fig. 10). In these simulations, CGP-78608 selected more open conformations of the GluN1 ABD, whereas L-689,560 selected more closed ABD conformations. Similar results were observed in molecular dynamics simulations with mutated GluN1 ABDs, where F484A + T518L mutations select more open GluN1 ABD conformations compared to F484H + R523K mutations (Fig. 10). In concert, the simulations and functional data indicate that more open conformations of the GluN1 ABD promote glycine binding to GluN3A, whereas more closed GluN1 ABD conformations negatively modulate glycine binding. We also show that L-689,560 is a negative allosteric modulator of glycine potency at the GluN3A subunit in GluN1^{FA+TL}/3A receptors via a mechanism that involves binding to the mutated GluN1 agonist binding pocket (Figs. 3 and 8). This mechanism underscores the complexity of defining GluN1/3 pharmacology and reveals that ligands capable of binding the mutated GluN1 glycine binding site may allosterically modulate GluN1/3 receptors. Previous studies have utilized mutations in the GluN1 glycine binding site to prevent desensitization in pharmacological assays (Awobuluyi et al., 2007; Madry et al., 2007; Kvist et al., 2013a; Kvist et al., 2013b; Kaniakova et al., 2018; Skrenkova et al., 2019; Zeng et al., 2022), but our results suggest that this approach to potentiate GluN1/3 receptors should be deployed with caution to avoid the potential issue of studying modulation specific to a mutated GluN1 agonist site.

GluN1-selective competitive antagonists can enhance activation of GluN1/3 receptors by preventing glycine binding to GluN1 (Awobuluyi et al., 2007; Madry et al., 2007; Madry et al., 2008, 2010; Kvist et al., 2013b; Mesic et al., 2016; Grand et al., 2018; Otsu et al., 2019; Zhu et al., 2020; Bossi et al., 2022; Zeng et al., 2022). However, CGP-78608 appears unique among the GluN1-selective competitive antagonists by producing remarkably strong potentiation of GluN1/3 receptors. In this study, we uncover important features of competitive antagonists that are essential to their efficacy as GluN1/3 receptor potentiators. First, high selectivity for the GluN1 orthosteric site is required to enable potentiation at higher GluN1 antagonist concentrations and/or low glycine concentrations. The relatively non-selective antagonist DCKA is therefore ineffective at potentiating GluN1/3 receptors (Awobuluyi et al., 2007). Second, GluN1 antagonists should preferably have a slow rate of unbinding from the GluN1 orthosteric site. We measured remarkably slow unbinding of CGP-78608 and L-689,560 with time constants at 114 and 136 s, respectively, in the presence of 10 mM glycine (Fig. 5). When CGP-78608 or L-689,560 are bound to GluN1 in GluN1/3A

receptors, the slow unbinding rates minimize desensitization accompanied by glycine binding to GluN1 during activation by brief exposures to a high glycine concentration. Finally, our findings suggest that the robust potentiation of GluN1/3A receptors by CGP-78608 is further facilitated by positive intra-subunit allosteric interactions that increase glycine potency and efficacy at the GluN3A subunit and slows CGP-78608 unbinding from the GluN1 subunit (Fig. 5).

This study provides insights to the apparent discrepancy between the high glycine affinity measured at the isolated GluN3A ABD ($K_d = 40$ nM; Yao and Mayer, 2006) and the relatively low glycine potency at GluN3A in GluN1^{FA+TL}/3A ($EC_{50} = 53$ μ M) and CGP-78608-bound GluN1/3A receptors ($EC_{50} = 19$ μ M; Table 2). Binding assays performed using the soluble GluN3A ABD lack the intra-subunit allosteric interactions by which the GluN1 subunit influence glycine affinity at the GluN3A subunit. Thus, pharmacology at the GluN3A orthosteric site is dependent on the GluN1 conformation, which is influenced by the mechanism used to prevent GluN1/3A desensitization (e.g., GluN1-selective antagonist or GluN1 mutations). In this regard, binding affinities for competitive antagonists may have been under-estimated at the isolated GluN3A ABD, since these values were determined using displacement of [³H]glycine binding (Yao and Mayer, 2006). That is, CGP-78608 and L-689,560 may have higher affinity for the GluN3A subunit in GluN1/3A receptors than previously suggested from binding assays using the isolated GluN3A ABD. We therefore speculate that competitive binding of L-689,560 to GluN3A mediates the low-potency component of inhibition in the biphasic concentration–response relationships shown in Fig. 3 B. This also raises the possibility that binding affinities for the GluN1 orthosteric site may be influenced by GluN3 subunits. For example, binding experiments to rat brain membranes indicated two binding sites for L-689,560 with similar binding affinities, but distinct association and dissociation kinetics (Grimwood et al., 1992). These two binding sites for L-689,560 may reflect distinct populations of GluN1 subunits located in GluN1/2 or GluN1/3 receptors.

Glycine potencies and effects of GluN1-selective antagonist binding are qualitatively similar for WT GluN1/3A and GluN1/3B receptors (Table 2). By contrast, L-689,560 binding produced distinct effects in the context of the mutated GluN1^{FA+TL} subunit; L-689,560 reduced glycine potency up to sevenfold at GluN1^{FA+TL}/3A receptors, but did not affect glycine potency at GluN1^{FA+TL}/3B receptors (Fig. 4 and Table 2). We speculate that the mutated GluN1^{FA+TL} subunit promotes GluN1 ABD conformations that may engage differently with GluN3A and GluN3B subunits and thereby accentuate variation in intra-subunit allosteric interactions among the two GluN1/3 receptor subtypes. Although crystal structures of the isolated GluN3A and GluN3B homomers have been described (Yao et al., 2008; Yao et al., 2013), there is currently a dearth of structural information for full-length GluN3-containing NMDA receptors. Future structural studies on GluN1/3 receptors may point to the specific receptor elements that undergo conformational changes following ligand binding and thereby reveal context to establish the structural mechanisms that mediate intra-subunit allosteric interactions between GluN1 and GluN3 subunits.

In conclusion, we demonstrate that the robust potentiation of GluN1/3 receptors observed for CGP-78608 is facilitated by positive intra-subunit allosteric interactions that increase glycine potency and efficacy at the GluN3 subunit and slows antagonist unbinding from the GluN1 subunit. We suggest that more open conformations of the GluN1 ABD, such as those selected by CGP-78608, promote glycine binding to GluN3A, whereas more closed GluN1 ABD conformations, like those selected by L-689,560, negatively modulate glycine binding. Thus, the pharmacology of GluN1/3 receptors evaluated in functional assays is highly dependent on the GluN1-selective competitive antagonists or GluN1 agonist binding site mutations that are utilized to prevent receptor desensitization. This conclusion may explain, at least in part, the wide range of potencies previously reported for glycine at GluN3 subunits in functional assays (Awobuluyi et al., 2007; Madry et al., 2007; Smothers and Woodward, 2007; Madry et al., 2008; Smothers and Woodward, 2009; Kvist et al., 2013a; Grand et al., 2018; Skrenkova et al., 2019; Zhu et al., 2020) and ligand binding experiments (Nilsson et al., 2007; Yao et al., 2008; Yao et al., 2013). The intra-subunit allosteric interactions described here therefore highlight a complex structure–function relationship that impact the development of potential therapeutic agents and pharmacological tool compounds for native GluN1/3 NMDA receptors.

Data availability

The data underlying figures are available in the published article and its online supplemental material. DNA constructs, molecular dynamics simulation inputs, and analysis scripts are available from the corresponding author upon reasonable request.

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of the study. N. Rouzbeh and K.B. Hansen wrote the manuscript with help from all co-authors.

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Supplemental material

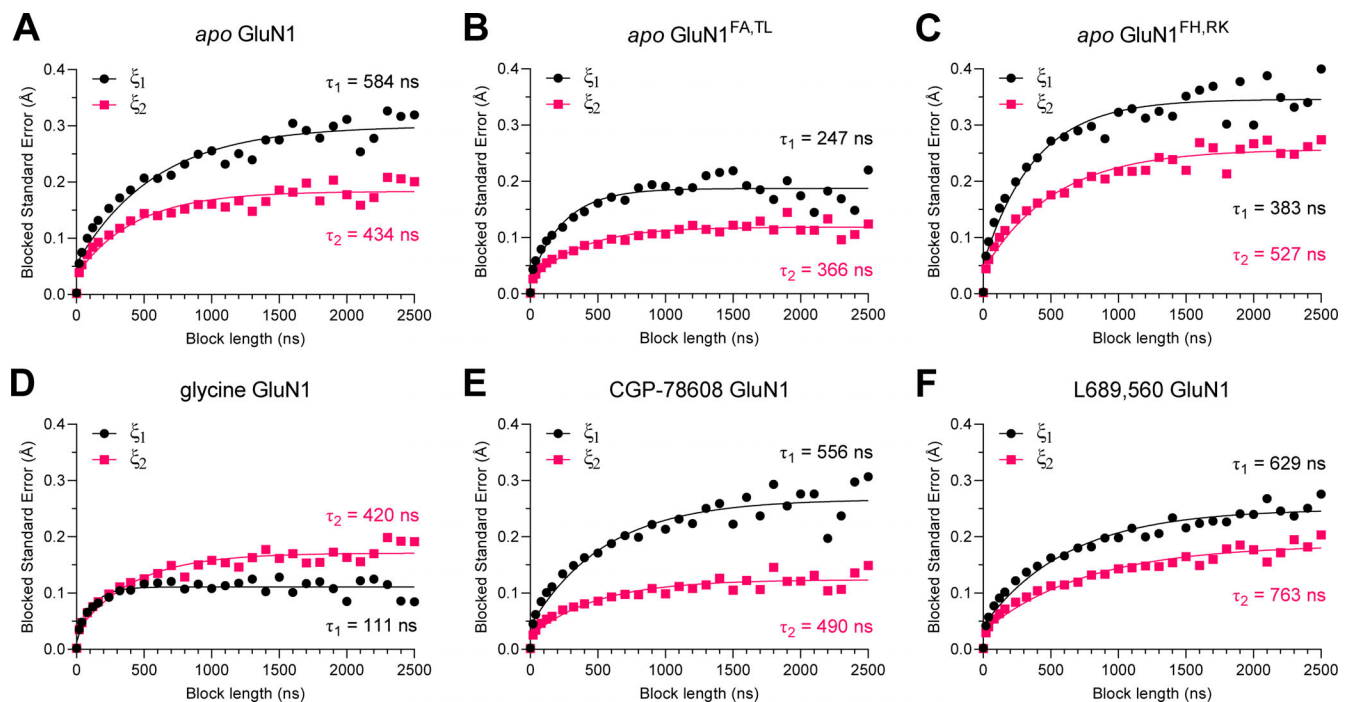


Figure S1. **Block-averaging analyses for ξ_1 and ξ_2 coordinates to assess convergence and sampling quality in molecular dynamics trajectories used for PMF calculations.** All trajectories for each condition were combined to produce $N = M \times n$ frames that were divided into M segments with block length n , and averages of ξ_1 and ξ_2 coordinates were calculated for each segment. This was performed with an initial short block length and with gradually increasing block lengths. For each block length n , the BSE was calculated as $BSE = \sigma_n / \sqrt{M}$, where σ_n is the standard deviation of either ξ_1 or ξ_2 averages among the blocks and M is the number of segments with block length n . Thus, BSE values estimate the standard error of ξ_1 or ξ_2 based on trajectory segments of length n . BSE is predicted to underestimate the true standard error for short block lengths, which are highly correlated, but will increase with larger block lengths as the blocks become statistically independent. Simulations can be considered to have reasonable sampling quality when BSE values no longer change (i.e., asymptotes) with increasing block lengths. BSE values for ξ_1 and ξ_2 from combined $8 \times 2,500$ ns trajectories (i.e., 2 μ s simulation total) were fit to a mono-exponential function to determine the time constant for each coordinate (τ_1 and τ_2). This analysis suggests that BSE values for ξ_1 and ξ_2 reached 95% of the asymptote after 333–2,286 ns, depending on GluN1 mutations or the ligand bound (A–F).

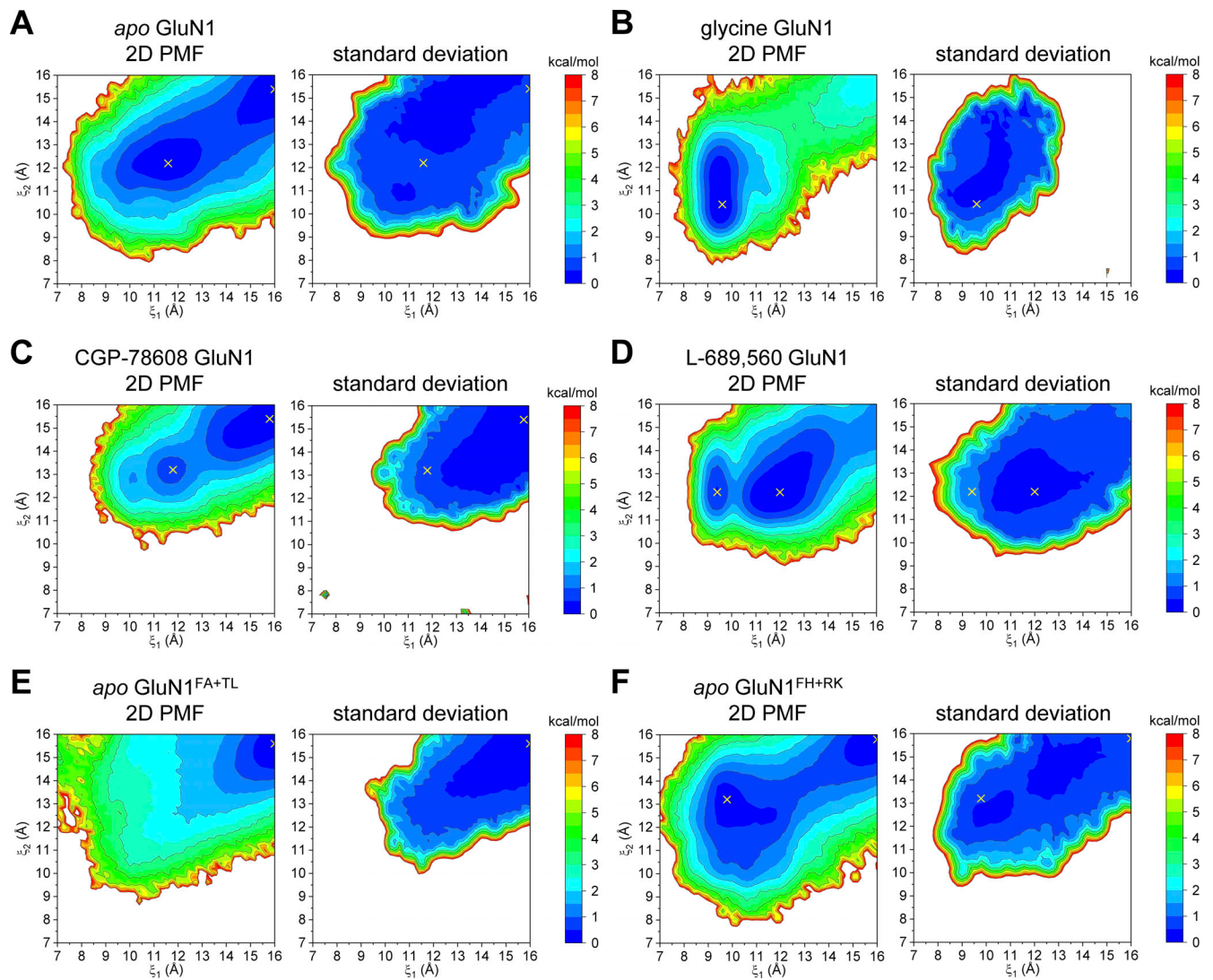


Figure S2. **Statistical uncertainties in 2D PMFs from molecular dynamics simulations with WT and mutated GluN1 ABDs in the apo state or with bound ligands.** (A–F) For each condition, 2D PMFs (A–F, left) were calculated by combining $8 \times 2,500$ ns trajectories (also shown in Figs. 9 and 10). For each condition, the error ranges (A–F, right) were estimated as the standard deviation of 2D PMFs calculated for each of the eight individual molecular dynamics simulations. X markings indicate local free energy minima.