A novel NMDA receptor positive allosteric modulator that acts via the transmembrane domain

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Ionotropic glutamate receptors (iGluRs) mediate fast excitatory neurotransmission and are key nervous system drug targets. While diverse pharmacological tools have yielded insight into iGluR extracellular domain function, less is known about molecular mechanisms underlying the ion conduction gating process within the transmembrane domain (TMD). We have discovered a novel NMDAR positive allosteric modulator (PAM), GNE-9278, with a unique binding site on the extracellular surface of the TMD. Mutation of a single residue near the Lurcher motif on GluN1 M3 can convert GNE-9278 modulation from positive to negative, and replacing three AMPAR pre-M1 residues with corresponding NMDAR residues can confer GNE-9278 sensitivity to AMPARs. Modulation by GNE-9278 is state-dependent and significantly alters extracellular domain pharmacology. The unique properties and structural determinants of GNE-9278 reveal new modulatory potential of the iGluR TMD.

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1. Introduction

Glutamate (Glu) is the main excitatory neurotransmitter in the brain and rapid synaptic responses to Glu release are mediated by ionotropic glutamate receptors (iGluRs). The iGluR family consists of four homologous classes of tetrameric receptors: AMPA, NMDA, kainate, and delta receptors (Dingledine et al., 1999). All iGluRs share a modular structural organization with extracellular amino terminal domains (ATDs), ligand-binding domains (LBDs), intracellular domains, and a pore-forming transmembrane domain (TMD) consisting of three transmembrane helices (M1, M3, and M4) as well as a reentrant loop (M2) between M1 and M3 that serves as part of the selectivity filter. As dysfunction of NMDARs in particular has been implicated in many neurological diseases including schizophrenia, epilepsy, and Alzheimer’s disease, investigation into the potential for pharmacological manipulation of NMDARs has been pursued with great interest (Traynelis et al., 2010; Paoletti et al., 2013; Zhou and Sheng, 2013; Soto et al.,

Abbreviations: ATD, amino terminal domain; iGluR, ionotropic glutamate receptor; LBD, ligand-binding domain; NAM, negative allosteric modulator; PAM, positive allosteric modulator; TMD, transmembrane domain.

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2014). While negative allosteric modulators (NAMs) have been examined for the purpose of normalizing pathological overactivation, positive allosteric modulators (PAMs) could be valuable for correcting hypofunction or enhancing normal physiological function of NMDARs (Coyle et al., 2003; Gonzalez-Burgos and Lewis, 2012).

Much of the insight into the mechanisms of PAM action on iGluRs comes from AMPARs, where multiple classes of PAMs have been described that target the inter-subunit interface of the LBDs (Sun et al., 2002; Jin et al., 2005) and constrain the LBD dimer movement so as to favor the agonist-bound conformation and thereby maintain tension on the linkers that connect to the TMD (Chen et al., 2014). Similarly for NMDARs, we have recently described a class of NMDAR PAM that bind the GluN1/GluN2A LBD interface at an analogous site to where AMPAR PAMs bind (Hackos et al., 2016).

In contrast to the LBD, there is relatively less structural information in the TMD, as there are currently no structures of iGluRs with a TMD in the open pore state, and the recent identification of a NAM binding site at the extracellular aspect of the TMD of AMPARs represents the first published TMD modulator co-crystal structure (Yelshanskaya et al., 2016). Additional insight into the role of this region on channel gating comes from the functional impact of mutations in the TMD. One key region is the highly conserved SYTANLAAF “Lurcher motif” of iGluRs located near the extracellular end of M3. This motif is found where the crossing transmembrane helices form a physical constriction in the ion channel pore that prevents ion conduction when the channels are in closed or desensitized states (Karacas and Furukawa, 2014; Lee et al., 2014).

This region was first identified as important to ion channel gating when it was found that neurodegeneration in the Lurcher mouse results from constitutive channel activity due to an A654T mutation within the SYTANLAAF motif (AST) of the delta 2 glutamate receptor (Glud2) (Zuo et al., 1997). Subsequently, engineered mutations in this region have been shown to cause gain-of-function phenotypes in Glud2 (Kohda et al., 2000), AMPA (Klein and Howe, 2004; Schmid et al., 2007), and NMDA receptors (Hu and Zheng, 2005; Blanke and VanDongen, 2008; Chang and Kuo, 2008; Murthy et al., 2012), suggesting the importance of this region in GluN2/C/D subunit-containing NMDAR potentiation by the modulator CIQ has identified the M1 and pre-M1 regions as contributing to allosteric modulation (Ogden and Traynelis, 2013). Previously, CIQ has represented the only NMDAR PAM with well-characterized TMD structural determinants.

Here we describe the discovery of a new NMDAR PAM, GNE-9278, which has unique modulatory properties and a novel binding site in the TMD. Experiments with TMD mutations demonstrate the positive and negative modulatory potential of this binding site and the transferability of GNE-9278 potentiation to AMPARs. We then used GNE-9278 to examine the impacts of TMD modulation on extracellular domain function and pharmacology.

2. Materials and methods

2.1. GNE-9278 synthesis

GNE-9278 was synthesized in 5 steps from commercial reagents. Unless otherwise indicated, all commercial reagents and anhydrous solvents were used without additional purification. 1H-NMR spectra were measured on Bruker Avance III 300, 400, or 500 MHz spectrometers. Chemical shifts (in ppm) were referenced to internal standard tetramethylsilane (δ = 0 ppm). Reactions were monitored by walkup Shimadzu LCMS/UV system with LC-30AD solvent pump, 2020 MS, Sil-30AC autosampler, SPD-M30A UV detector, CTO-20A column oven, using 2–98% acetonitrile/0.1% formic acid (or 0.01% Ammonia) over 2.5 min OR Waters Acquity LCMS system using 2–98% acetonitrile/0.1% formic acid (or 0.1% Ammonia) over 2 min. Flash column chromatography purifications were done on a Teledyne Isco CombiFlash Rf utilizing Silicycle HP columns.

Step 1: 5-propyl-4H-1,2,4-triazol-3-amine: 1-aminoguanidine carbonate (13.6 g, 0.1 mmol) and butyric acid (9.24 g, 0.105 mol) were added to a 250 mL round-bottom flask and then stirred for 12 h at 120 °C. The resulting mixture was concentrated in vacuo and the residue was then purified by chromatography with dichloromethane/methanol (70/30) to afford 5-propyl-4H-1,2,4-triazol-3-amine (3.0 g, 24%) as a white solid. LCMS, m/z = 127.0 [M + H]+ 1H-NMR (400 MHz, CD3OD) δ 2.53 (t, J = 7.6 Hz, 2H), 1.76–1.62 (m, 2H), 0.97 (t, J = 7.6 Hz, 3H).

Step 2: 5-methyl-2-propyl-1,2,4-triazolo[1,5-a]pyrimidin-7(4H)-one: To a solution of 5-propyl-4H-1,2,4-triazol-3-amine (3 g, 23.8 mmol) in acetic acid (50 mL) was added ethyl 3-oxobutanoate (4.64 g, 35.7 mmol). The resulting solution was stirred for 5 h at 130 °C at which point the mixture was concentrated in vacuo. The residue was purified by silica gel chromatography with dichloromethane/methanol (90/10) to afford 5-methyl-2-propyl-1,2,4-triazolo[1,5-a]pyrimidin-7(4H)-one (3 g, 59%) as a white solid. LCMS, m/z = 193.0 [M + H]+ 1H-NMR (400 MHz, CDCl3) δ 2.84 (t, J = 7.6 Hz, 2H), 2.52 (s, 3H), 1.92–1.83 (m, 2H), 1.03 (t, J = 7.6 Hz, 2H).

Step 3: 5-methyl-6-nitro-2-propyl-1,2,4-triazolo[1,5-a]pyrimidin-7(4H)-one: Sulfuric acid (3 mL) and fuming nitric acid (3 mL) were added to a 25 mL round bottom flask at 0 °C and were allowed stirred for 10 min. 5-methyl-2-propyl-1,2,4-triazolo[1,5-a]pyrimidin-7(4H)-one (1 g, 5.20 mmol) was added and then stirred for 3 h at room temperature. The reaction was then quenched by water/ice (20 g) and the pH value was adjusted to 8 with sodium hydroxide (1 mol/l). The solids were filtered off and dried to afford 5-methyl-6-nitro-2-propyl-1,2,4-triazolo[1,5-a]pyrimidin-7(4H)-one (1 g, 69%) as a yellow solid. LCMS, m/z = 238.1 [M + H]+ 1H-NMR (400 MHz, CDCl3) δ 2.74 (t, J = 7.6 Hz, 2H), 2.55 (s, 3H), 1.88–1.78 (m, 2H), 1.01 (t, J = 7.6 Hz, 2H).

Step 4: amino-5-methyl-2-propyl-1,2,4-triazolo[1,5-a]pyrimidin-7(4H)-one: To a solution of 5-methyl-6-nitro-2-propyl-1,2,4-triazolo[1,5-a]pyrimidin-7(4H)-one (1 g, 4.22 mmol) in methanol (20 mL) was added Phl/D (200 mg), and the resulting mixture stirred for 12 h under a hydrogen atmosphere (5 atm). The solids were filtered off and the filter cake was washed with methanol (10 mL). The filtrate was then concentrated in vacuo to afford 6-amino-5-methyl-2-propyl-1,2,4-triazolo[1,5-a]pyrimidin-7(4H)-one (800 mg, 91%) as a light yellow solid. LCMS, m/z = 208.1 [M + H]+ 1H-NMR (300 MHz, CDCl3) δ 2.70 (t, J = 4.5 Hz, 2H), 2.35 (s, 3H), 1.88–1.75 (m, 2H), 0.99 (t, J = 4.5 Hz, 3H).

Step 5: 4-cyclohexyl-N-{5-(methyl-7-oxo-2-propyl-4-7-dihydro-[1,2,4]triazolo[1,5-a]pyrimidin-6-yl)benzenesulfonamide (GNE-9728): To a solution of 6-amino-5-methyl-2-propyl-1,2,4-triazolo[1,5-a]pyrimidin-7(4H)-one (1 g, 4.83 mmol) in dichloromethane (70 mL) was added pyridine (760 mg, 9.61 mol) and 4-cyclohexylbenzene-1-sulfonyl chloride (1.86 g, 7.19 mmol) at 0 °C. The reaction was stirred overnight at room temperature and then concentrated in vacuo. The residue was purified by silica gel chromatography with dichloromethane/methanol (20/1) to afford 4-cyclohexyl-N-{5-(methyl-7-oxo-2-propyl-4-7-dihydro-[1,2,4]triazolo[1,5-a]pyrimidin-6-yl)benzenesulfonamide (11.7 g, 54%) as a white solid. LCMS, m/z = 430.2 [M + H]+ 1H-NMR (300 MHz, DMSO) 13.30 (brs, 1H), 9.24 (s, 1H), 7.69 (d, J = 8.4 Hz, 2H), 7.36 (d, J = 8.4 Hz, 2H), 2.65–2.60 (m, 3H), 2.26 (s, 3H), 1.81–1.67 (m, 7H),
1.43–1.17 (m, 5H), 0.92 (t, J = 7.5 Hz, 3H).

2.2. Cell lines

Doxycycline-inducible HEK293 cell lines that express GluN1/GluN2A, GluN1/GluN2B, GluN1/GluN2C, GluN1/GluN2D, or GluA2 were used for calcium imaging experiments and a CHO cell line expressing inducible GluN1/GluN2A was used for whole-cell electrophysiology, as previously described (Hackos et al., 2016). Receptor expression was validated by electrophysiology and general cell line authentication and mycoplasma testing were not performed.

2.3. Calcium influx assay

Compounds were tested for their ability to potentiate either NMDAR or AMPAR-expressing cell lines in calcium influx assays using a FDSS 7000 in a 384-well format as previously described (Hackos et al., 2016). For NMDAR assays, EC30 Glu (concentration empirically determined each day the assay was run) and saturating Gly were used to activate the channels. For AMPAR assays, saturating (100 μM) Glu was used for channel activation.

2.4. Whole-cell electrophysiology

Doxycycline (5 μg/mL) was added to the CHO cell culture media in the presence of ketamine (1 mM) to induce GluN1/GluN2A NMDAR expression the night before recording. On the day of recording, cells were detached from the culture dish and kept at room temperature with 1 mM ketamine added to the media. Whole-cell patch clamp recordings from cell lines were obtained using a Molecular Devices Axopatch 200B patch clamp amplifier. Extracellular solution for whole-cell recording contained (in mM): 150 NaCl, 3 KCl, 1 CaCl2, 5 glucose, 10 HEPES, pH 7.4. Intracellular solution contains (in mM): 140 CsF, 10 NaCl, 1.5 MgCl2, 5 EGTA, 10 HEPES, pH 7.2. Rapid solution exchange was achieved using the 16-channel Dynaflow Resolve system (Cellectricon).

2.5. Two-electrode voltage clamp

Expression of NMDAR and AMPAR channels in Xenopus oocytes was achieved by subcloning the human cDNAs for these channels into the pTNT vector (Clontech). To produce RNA for injection into oocytes, the constructs were linearized, purified, and used as the substrate for T7 RNA polymerase-mediated RNA synthesis (mMessage mMachine T7, Ambion). Mutagenesis was carried out using the QuikChange Lightning Multi kit (Agilent) as per manufacturer’s instructions. Xenopus oocytes were purchased from Nasco (LM009353MX; Fort Atkinson, WI) and prepared using standard procedures. In brief, oocytes were digested by type 2 collagenase (720 U/ml final) in OR-2 solution (in mM: 82.5 NaCl, 2.4 KCl, 1 MgCl2, 5 HEPES, pH 7.4) for 2.5 h followed by several washes in ND-96 (in mM: 96 NaCl, 2 KCl, 1 MgCl2, 1.8 CaCl2, 5 HEPES, pH 7.4) after digestion. Oocytes injected with NMDAR mRNAs were incubated at 15 °C for 1–3 days before recording. Oocytes injected with AMPAR mRNAs were incubated for about 1 week before recording. Two-electrode voltage clamp was carried out using standard procedure and the data were digitized at 2 kHz. Unless otherwise stated, the perfusion solutions were prepared using CaCl2 and MgCl2-free ND-96 plus 300 μM BaCl2 and 50 μM Gly. The timing of solution exchange was controlled by custom software developed using MATLAB (MathWorks).

2.6. Data analysis

Data analysis was done using Clampfit (Molecular Devices) and/ or custom scripts written in MATLAB. The deactivation time constant of the NMDAR current was determined by fitting the decaying current with a single-exponential function. Outliers were removed by Grubbs’ test and statistical significance was determined in a two-sided Wilcoxon rank sum test except where otherwise noted in the figure legends. Reported values and error bars in this article represent mean ± SEM.

2.7. GluN2A homology model

A GluN1/GluN2A homology model was constructed using the multiple-sequence alignment and homology modeling functions in the Molecular Operating Environment (MOE, 2015.10; Chemical Computing Group Inc., Montreal, Canada). The human GluN1/GluN2A sequences were first aligned to the respective sequences in GluN1/GluN2B and then a structural model was constructed using the GluN1/GluN2B structure (PDB 4TLM) (Lee et al., 2014) as a template. The model was rendered using the molecular visualization program PyMol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

2.8. Systematic names of compounds used in this study

CIQ: (3-chlorophenyl)[6,7-dimethoxy-1-{(4-methoxyphenox)- methyl]-3,4-dihydroisoquinolin-2(1H)-yl)methanone; Ifenprodil: 2-(4-benzylpiperidino)-1-{(4-hydroxyphenyl)-2-methyl-1-ethanol; GNE-9278: 4-cyclohexyl-N-([5-methyl-7-oxo-2-propyl-4,7-dihydro-[1,2,4]triazolo[1,5-a]pyrimdin-6-yl]benzenesulfonamide; GNE-3419: 7-((ethyl[phenyl]amino)methyl)-2-methyl-5H-[1,3,4]-thiadiazolo[3,2-a]pyrimidin-3-one; GNE-8016: N-ethyl-[2-(2-fluoro-3-[(trifluoromethyl)[benzyl]-2-methyl-5-oxo-5H-thiazolo [3,2-a]pyrimidine-3-carboxamide.

3. Results

3.1. GNE-9278 is a novel NMDAR PAM

We previously reported the results of a high-throughput screen that yielded a series of GluN2A-selective NMDAR PAMs with a binding site in the LBD interface (Hackos et al., 2016). During the course of this screening campaign, we also discovered a distinct NMDAR PAM, GNE-9278 (Fig. 1A), which did not co-crystallize with the LBD. GNE-9278 robustly potentiated GluN2A, 2B, 2C and 2D-containing NMDARs using a rapid perfusion system containing NMDARs with EC50 values of 0.74 ± 0.9, 9.1 ± 4.6 and 6.7 ± 0.9, respectively and maximum fold potentiation of 5.5 ± 0.3, 8.4 ± 0.9, 10.2 ± 0.9 and 7.9 ± 0.6, respectively. At the same time, we found GNE-9278 to be highly selective for NMDARs vs. AMPARs, with no appreciable activity in AMPAR calcium influx assays (Fig. 1B). Electrophysiology experiments in oocytes performed at saturating agonist concentrations confirmed the robust potentiation of GluN2A, 2B, 2C and 2D-containing NMDARs as measured by calcium influx assays from HEK cell lines (Fig. 1A) with EC50 values of 0.74 ± 0.06, 3.07 ± 0.28, 0.47 ± 0.7, and 0.32 ± 0.04 μM, respectively, and maximum fold potentiation of 5.5 ± 0.3, 8.4 ± 0.9, 10.2 ± 0.9 and 7.9 ± 0.6, respectively. At the same time, we found GNE-9278 to be highly selective for NMDARs vs. AMPARs, with no appreciable activity in AMPAR calcium influx assays (Fig. 1B). Electrophysiology experiments in oocytes performed at saturating agonist concentrations confirmed the robust potentiation of GluN2A, 2B, 2C and 2D-containing NMDARs with EC50 values of 3.2 ± 0.8, 15.7 ± 2.3, 6.6 ± 0.2 and 6.7 ± 0.9 μM, respectively, and maximum fold potentiation of 4.9 ± 0.6, 12.4 ± 0.9, 9.1 ± 1.0 and 14.9 ± 1.4, respectively (Fig. 1C). Thus, two distinct assays confirmed robust potentiation of NMDARs by GNE-9278. The differences in the estimates of potency and fold potentiation between the assay formats are likely due to known nonlinearities in the calcium influx assay signal.

Whole-cell recordings from a cell line expressing GluN2A-containing NMDARs using a rapid perfusion system confirmed that GNE-9278 acts as a PAM rather than as an agonist or co-agonist...
(Fig. 1D). GNE-9278 increased current in response to pulses of saturating Glu (in the presence of saturating Glycine) both by increasing the peak current amplitude and by slowing the deactivation process during Glu washout (Fig. 1E). At 100 μM Glu and 50 μM Glycine (Gly), the maximal GNE-9278-mediated potentiation of the peak amplitude was 2.43 ± 0.2 fold with an EC50 of 3.06 ± 0.32 μM (Fig. 1E). Notably, the effect of enhanced peak amplitude occurred with higher potency than the effect of slowing of deactivation (Fig. 1E). In addition, outside-out patch recordings with small numbers of channels showed that GNE-9278 potentiated GluN1/GluN2A NMDARs without altering the single channel conductance (data not shown).

3.2. GNE-9278 slows deactivation and enhances the potency of both Glu and Gly

The slowing of deactivation following removal of Glu suggests that GNE-9278 could act in part by slowing the agonist off-rate and increasing agonist affinity. To further explore this, we performed experiments at a high GNE-9278 concentration and compared the agonists, D-Glu, L-Glu and L-CCG-IV, which have fast, medium and slow deactivation kinetics (in the absence of GNE-9278), respectively. With these agonists, GNE-9278 showed minimal, moderate, and strong slowing of deactivation, respectively, (Fig. 2A and B). This is consistent with GNE-9278 enhancing the affinity of each agonist as reflected in a proportional slowing of off-rate (Fig. 2B). In the case of L-CCG-IV, a slow tail current was observed following GNE-9278 washout, which is expected since when GNE-9278 was removed very little L-CCG-IV dissociation had occurred in the presence of the PAM. Consistent with this, the measured exponential time constant of the slow tail (Tau_tail) once GNE-9278 was removed is the same as the deactivation time constant observed in the absence of GNE-9278 (Fig. 2C).

As our previous series of compounds that bind in the LBD selectively shift Glu but not Gly EC50 of the NMDAR (Hackos et al., 2016), we next determined the effects of GNE-9278 on the EC50s of the co-agonists. These experiments showed a significant lowering of the EC50 for both Glu and Gly in the presence of GNE-9278 (Fig. 2D and E). The results are consistent with increased affinity of both agonists in the presence of GNE-9278.

3.3. GNE-9278 association is rapid and NMDAR activation-dependent

Measurements of GNE-9278 effects indicated rapid association/dissociation kinetics when applied in the presence of saturating Glu (Fig. 3A). However, when tested at lower Glu concentrations, the apparent rate of GNE-9278 association slowed, despite rapid dissociation (Fig. 3A). This Glu-dependence of the rate of GNE-9278 potentiation could be consistent with a requirement of channel activation prior to GNE-9278 binding, with the slow apparent association at low Glu concentrations reflecting the accumulation of activated receptors under low open probability conditions. To further explore this possibility, we pre-applied GNE-9278 and then tested different duration pulses of 1 μM Glu +1 μM Gly application (in the continual presence of 50 μM GNE-9278; Fig. 3B). Sub-saturating Glu/Gly concentrations were used to take advantage of slowed GNE-9278 effects, enabling easier measurement of activation-dependent changes in PAM occupancy over time. Because slowing of channel deactivation increases as a greater fraction of NMDARs are bound by GNE-9278 (Fig. 1E), compound binding that is dependent on channel opening should be reflected in greater slowing of deactivation with increasing durations of agonist application. On the other hand, if compound binding is state-independent, there should be little relationship between the duration of agonist application and the degree of slowing of deactivation since pre-applied 50 μM GNE-9278 (near saturation) would have reached equilibrium at its binding site prior to Glu/Gly application. These experiments revealed an exponential time-dependent slowing of deactivation kinetics with longer agonist pulses (Fig. 3B and D), suggesting that GNE-9278 binding is significantly enhanced following channel activation/opening. Control experiments with agonist pulses in the absence of GNE-9278 demonstrate that the observed slowing of deactivation is dependent on GNE-9278 (Fig. 3C and D) and therefore can serve as a measure of GNE-9278 occupancy. The observed enhanced binding of GNE-9278 following channel activation could be due to the binding site becoming exposed in a use/state-dependent manner, and/or the enhanced affinity of the binding site for GNE-9278 in agonist-activated channels.

3.4. GNE-9278 does not act at known extracellular domain modulatory sites

As a first step in identifying the binding site for GNE-9278, we examined the ability of GNE-9278 to potentiate GluN1/GluN2A NMDARs after disruption of known modulatory sites. The TMD region of NMDARs is a major site for allosteric modulation. Zinc, endogenous polyamines, and GluN2B antagonists including ifenprodil, all bind the GluN2T ADT or at the interface between GluN1 and GluN2 ATDs (Zhu and Paoletti, 2015). Therefore we tested for the ability of GNE-9278 to potentiate NMDARs lacking the GluN2 ADT, which should destroy known ADT binding sites. These experiments showed that NMDARs with the GluN2A ADT deletion could be potentiated similarly to wild-type NMDARs (Fig. 4A and B), largely excluding the ADT as a potential GNE-9278 binding location.

We next examined the NMDAR PAM binding site at the LBD dimer interface by testing the effects of the GluN2A mutations T758A and V783F, which have previously been shown to disrupt PAM binding at this site (Hackos et al., 2016). As a side-by-side comparison, we also tested GNE-8016 (compound 29 from (Volgraf et al., 2016)), a GluN2A-selective PAM that acts at the LBD dimer interface. As expected from the LBD co-crystal structure (PDB 5I2N (Volgraf et al., 2016)), showing GNE-8016 binding at this site (Fig. 4C), GNE-8016 potentiation was eliminated by these mutations (Fig. 4D). On the other hand, GNE-9278 potentiation was not reduced (Fig. 4D), arguing against GNE-9278 binding at this LBD site. The absence of evidence for ADT or LBD binding sites suggested the possibility that GNE-9278 could be acting in the TMD region.

3.5. Identification of specific structural determinants of GNE-9278 potentiation in the TMD

We next performed a mutational scan of likely TMD regions that could be involved in GNE-9278 potentiation. Because mutations or chemical modification within the conserved SYTANLAAF motif can enhance gating of iGluRs, we focused on the residues broadly surrounding this region of M3 in GluN1 and GluN2A. In addition, given the proximity of the M1/pre-M1 helix to this region of M3 in NMDAR crystal structures (Karacas and Furukawa, 2014; Lee et al., 2014), we also examined the adjacent region of GluN1 that includes M1/pre-M1. For this set of experiments, we systematically mutated each residue to alanine, (or leucine in the case of residues that were already alanine), and examined the effects on potentiation of the response to 300 nM Glu (Fig. 5A).

Because mutations could alter basal channel gating and confound interpretation of the effects on GNE-9278 potentiation, we also characterized the baseline effects of each mutation by determining the Glu EC50 in the absence of PAM application. In this analysis many mutations substantially lowered the Glu EC50...
Fig. 1. GNE-9278 is a positive allosteric modulator of NMDARs. (A and B) Calcium influx measurements from GluN1/GluN2-expressing HEK cells. (A) GNE-9278 potentiates calcium influx elicited by an EC_{50} Glu concentration (in the presence of 50 μM Gly) in NMDARs containing each of the four GluN2 subunits. Maximum fold potentiation (left) and normalized potentiation (right) as a function of increasing GNE-9278 concentration. (B) Bar graph showing the percentage of maximal potentiation at 10 μM GNE-9278 compared to the EC_{50} concentration of Glu in the presence of 50 μM Gly, for each GluN2 subunit. (C) Concentration-response curves for GNE-9278 potentiating calcium influx in NMDARs containing each of the four GluN2 subunits. (D) Current tracings showing the effect of 1 μM GNE-9278 on calcium influx at 100 μM Glu and 50 μM Gly. (E) Fold potentiation and time constant (τ) as a function of GNE-9278 concentration.
This increased sensitivity to Glu with some mutations could occlude potentiation and thereby confound identification of residues that are specifically important for GNE-9278 potentiation. Hence, as a control, we measured the effect of each mutation on potentiation by both 1 μM GNE-9278 and 10 μM GNE-8016 (which binds at the LBD interface). These PAM concentrations yield equivalent levels of potentiation in WT NMDARs. To identify residues that were uniquely important for GNE-9278 vs. GNE-8016 potentiation, we looked for mutations that resulted in GNE-9278 potentiation that was meaningfully smaller (at least 30% reduced) and significantly different (p < 0.05) from the level of GNE-8016 potentiation. Experiments testing 64 mutations using these screening criteria identified 5 residues in GluN1 pre-M1 (S549, T550, L551, D552, and F554), 4 residues in GluN1 M3 (Y647, N650, F654, and L655), and one residue in GluN2A M3 (F652) as ones which uniquely reduce GNE-9278 potentiation when mutated (Fig. 5A). We also identified a mutation in GluN2A M3 (A647F) that dramatically increased the effects of GNE-9278. Notably, the residues found to uniquely reduce GNE-9278 potentiation are roughly clustered in regions where effects of mutation on Glu-EC50 were also prominent. These regions also include other residues which when mutated diminished GNE-9278 potentiation, but couldn’t be resolved as unique determinants because GNE-8016 potentiation was also similarly diminished (e.g. GluN1 S553). Importantly, some mutations did reduce GNE-9278 potentiation without affecting the Glu EC50 or reducing GNE-8016 potentiation (most notably the GluN1 mutations T550A and D552A).

As a next step to validate structural determinants of GNE-9278, we measured the GNE-9278 dose-response for key mutations that were found to specifically reduce potentiation in the screen described above (Fig. 6A–D). These experiments were performed at saturating Glu concentrations in order to reduce the confounding effects of the left-shifted Glu EC50 that was observed for some of the mutants (Fig. 5B). In these experiments GluN1 pre-M1 mutations T550A and D552A were found to significantly increase the EC50 of GNE-9278, consistent with a direct contribution of these residues to GNE-9278 binding affinity (Fig. 6D). On GluN1 M3, F654A significantly reduced the efficacy of GNE-9278 (Fig. 6C) without reducing potency (Fig. 6D) and the adjacent L655A mutant completely abolished GNE-9278 potentiation with a small inhibitory effect observed at all concentrations under these conditions (Fig. 6B, gray trace). At the same time, some of the residues identified in the screen caused only subtle reductions of GNE-9278 potentiation (GluN1 S549 and L551), or even showed greater potentiation than wild-type NMDARs (e.g. GluN1 F554 and Y647, Fig. 6C). This could reflect, 1) an increased window for observing potentiation with these mutations due to a lower basal open probability at the saturating Glu concentration and/or 2) false positives from the screen. In either case, as with the broader set of residues identified in the screen, the 4 key residues (GluN1 T550, D552, F654, L655) that confirmed as GNE-9278 structural determinants cluster in the vicinity of where GluN1 pre-M1 and GluN1 M3 are apposed to each other near the constriction of the channel pore (Fig. 6E–G).

### 3.6. Conversion of GNE-9278 modulation from positive to negative by a mutation in GluN1 M3

Of the confirmed structural determinants of GNE-9278 potentiation, GluN1 L655 was particularly interesting as there was a complete loss of potentiation with the alanine mutation even with the highest tested concentration of GNE-9278 (100 μM), and because this residue is located near the Lurcher motif. We further examined this residue by exploring different sized side chain substitutions, including L655V, L655Y, and L655A. In contrast to the near complete loss of GNE-9278 potentiation in GluN2A-containing NMDARs with GluN1 L655A, L655Y caused a partial loss of positive modulation and L655V actually converted GNE-9278 effects into strong negative modulation (Fig. 7A–C). Notably, the GNE-9278 EC50 for WT and L655V mutant NMDARs, and the IC50 for the L655Y mutant, were all in the low micromolar range, suggesting this residue is key to the effects of GNE-9278, but not critical to the binding affinity. In contrast to GNE-9278, GNE-8016 still functioned as a positive modulator of GluN2A-containing NMDARs regardless of the mutations at this residue (Fig. 7D). Similarly, when paired with GluN2C, the three different GluN1 L655 mutations disrupted the positive modulation of GluN2C NMDARs by GNE 9278 (Fig. 7E) but not by the GluN2C/D-selective PAM CIQ (Fig. 7F). Together, these results demonstrate the specificity of these residues in controlling the nature of GNE-9278 modulation.

### 3.7. Three Pre-M1 residues are sufficient to confer GNE-9278 potentiation to AMPARs

The change in efficacy without decreasing the potency of GNE-9278 with mutations of GluN1 M3 residues F654 and L655 (Fig. 6) suggest residues on M3 may mediate the effects of GNE-9278 binding without directly contributing to binding affinity. In contrast, alanine mutations of pre-M1 residues T550 and D552 (which do not alter the Glu EC50 or GNE-8016 mediated potentiation; Fig. 5) resulted in large increases in the GNE-9278 EC50 (Fig. 6D), suggestive of a direct contribution to binding affinity. Therefore, we tested if we could confer GNE-9278 sensitivity to AMPARs by inserting the GluN1 pre-M1 residues T550-L551-D552 into the corresponding portion of pre-M1 in GluA2 (GVF → TLD) (Fig. 8A). Because AMPARs rapidly desensitize in response to Glu application, we measured Glu-induced currents from AMPARs in the presence of GNE-3419, a compound that binds the LBD of AMPARs and prevents desensitization (Hackos et al., 2016). In these experiments, as expected, GNE-9278 failed to potentiate Glu-induced currents in WT AMPARs (Fig. 8B upper panel and 8C). However, in the chimeric AMPARs containing the GluN1 TLD sequence, significant potentiation by GNE-9278 was observed (Fig. 8B lower panel and 8C). While these results are consistent with
Fig. 2. GNE-9278 slows deactivation with multiple agonists and enhances the potency of both Glu and Gly (A) Recordings of the effects of three Glu-site agonists (with different affinities and off-rates) on GluN2A currents in the absence and presence of 50 μM GNE-9278 are shown (50 μM Gly present throughout). The colors of the traces (red, blue, and black) indicate which agonist was applied (1 mM D-Glu, 10 μM L-Glu, and 1 μM L-CCG-IV, respectively; concentrations were chosen to be approximately 10-fold higher than the EC_{50} of each agonist). GNE-9278 slows deactivation in the case of all three agonists. After removal of GNE-9278, a slow tail current (indicated by arrow) is observed in the case of L-CCG-IV. Note that during initial pre-application of GNE-9278 there was apparent agonist activity in this experiment, which was observed to variable extents in experiments using high GNE-9278 concentrations. (B) Deactivation time constants observed with the three agonists in the absence (open squares) and presence (filled circles) of GNE-9278 are shown. Since deactivation after L-CCG-IV removal in the presence of GNE-9278 was very slow, a correction for rundown was applied prior to the exponential fit. Arrows indicate the extent of slowing of deactivation following each agonist application. Values of Tau_deact in the absence and presence of GNE-9278 were as follows: D-Glu, from 57.5 ± 9.1 ms to 238 ± 23.8 ms; L-Glu, from 202.6 ± 32.5 ms to 2206 ± 460 ms; L-CCG-IV, from 1478 ± 118 ms to 16200 ± 1050 ms (n = 7). Note the Tau_deact following D-Glu in the absence of GNE-9278 approaches the limit of the perfusion system (indicated by dashed line). (C) The Tau_tail for the L-CCG-IV recordings was calculated by exponential fits to the tail current following GNE-9278 washout (indicated by the arrow in A). No significant difference was observed between Tau_tail and the control Tau_deact for L-CCG-IV in the absence of GNE-9278, consistent with the interpretation that the tail current is due to L-CCG-IV that remains bound to the NMDAR after agonist washout (in the presence of GNE-9278), and then dissociates normally after removal of GNE-9278. (D) Whole-cell recording from cell lines expressing GluN1/GluN2A during pulses of different Glu concentrations in the constant presence of 50 μM Gly were made before and after application of 50 μM GNE-9278. (D) The Glu EC_{50} shifted from 1.32 ± 0.2 μM in control conditions to 0.11 ± 0.02 μM in the presence of GNE-9278 (n = 9 patches, p = 4.11 × 10^{-2}). The Gly EC_{50} shifted from 1.31 ± 0.11 μM, in control conditions, to 0.12 ± 0.01 μM in the presence of GNE-9278 (n = 10 patches, p = 1.24 × 10^{-3}). Representative traces with (red) or without (black) GNE-9278 are shown on top of the dose response curves. Two-sided Wilcoxon rank sum test was used and data represent mean ± SEM. *p < 0.05, **p < 0.01.
these residues being sufficient for conferring binding to AMPARs, it is also possible that GNE-9278 binds wild-type AMPARs without effect, and these residues are necessary for conferring transduction of the allosteric effect of GNE-9278 binding. This striking ability of 3 pre-M1 residues to confer action of GNE-9278 onto AMPARs confirms understanding of the structural determinants of PAM action in the TMD, and suggests that the GNE-9278 binding site is in the vicinity of the GluN1 pre-M1 helix.

Fig. 3. GNE-9278 has rapid dissociation kinetics, rapid agonist-dependent association kinetics, and exhibits use-dependent slowing of deactivation. (A) A whole-cell patch clamp recording of GluN1/GluN2A channel current shows the effects of addition of 50 μM GNE-9278 when the channel is activated to different extents (0.2, 1, or 100 μM Glu) all in the presence of 50 μM Gly. Exponential fits to GNE-9278 association and dissociation time courses from these recordings are shown graphed as time constants vs. Glu concentration (τ_{obs}, black or τ_{off}, red for association and dissociation respectively). Data represent means ± SEM for n = 6 cells. These data indicate that τ_{obs} is [Glu]-dependent while τ_{off} is not. The limit of the Dynaflow perfusion system was established by switching between extracellular buffer solutions containing 150 vs 300 mM NaCl and is shown as a dashed line (τ = 38.5 ± 4.3 ms). Note that the τ_{obs} of GNE-9278 is around the limit of the perfusion system when it is applied at saturating Glu concentration (100 μM). (B) Use-dependence of the slowing of deactivation by GNE-9278 was examined. Overlays of current elicited by different duration Glu + Gly pulses in the presence of GNE-9278 (pre-applied for 10 s prior to agonist pulses) are shown (left panel, 1 μM Glu, 1 μM Gly, 50 μM GNE-9278). The color of each trace indicates the length of the Glu pulse (black = 100 ms, red = 123 ms, blue = 219 ms, magenta = 499 ms, green = 1 s, dark blue = 3 s). Shorter Glu + Gly pulses were not achievable with our perfusion system. The right panel shows the same data re-plotted using peak alignment and normalization to better illustrate the slowing of deactivation kinetics with longer Glu + Gly pulses. (C) Example traces from control experiments showing measurements of deactivation kinetics as a function of Glu + Gly pulse length in the absence of GNE-9278 (using 50 μM Gly). No slowing of deactivation was observed in the absence of GNE-9278 using either 1 μM Glu (left) or 100 μM Glu (right) pulses. (D) Deactivation rates (τ_{deact}) were calculated by fitting each deactivation time course with an exponential function. In the presence of GNE-9278 there was a significant effect of Glu + Gly pulse duration on the deactivation time constant (H = 9.894, d.f. = 6, p < 0.01, one-way ANOVA on ranks, n = 10 cells), corresponding to a significantly slower deactivation at 499 ms, 1 s, and 3 s compared to baseline deactivation (p < 0.01, Tukey’s test), consistent with the model that GNE-9278 action is facilitated by channel activation and/or opening. An exponential fit to the deactivation time constants is shown in black (τ = 254 ± 40.4 ms). Control Glu + Gly pulses of identical length recorded in the absence of GNE-9278 at either 1 or 100 μM Glu showed no slowing of deactivation (red = 1 μM Glu, blue = 100 μM Glu).
3.8. GNE-9278 interacts with modulators that act via extracellular domain sites

While the above experiments clearly indicate a TMD binding site for GNE-9278, we found evidence of a dominant interaction of GNE-9278 with extracellular domain modulators. Interestingly, we noticed that the LBD PAM GNE-8016 seemed to lose its ability to potentiate L655A NMDARs in the presence of GNE-9278 (Fig. 9A). To follow up on this observation we performed dose-response experiments and found that the maximum effect of GNE-8016 was dramatically reduced in the presence of GNE-9278 (Fig. 9B).

Because these experiments were performed on the L655A channels that lack GNE-9278 potentiation, this doesn’t reflect occlusion of GNE-8016 potentiation by GNE-9278 (i.e. via a ceiling effect), but rather reflects silent allosteric modulation by GNE-9278, which prevents potentiation by the LBD-acting PAM.

Given the evidence that GNE-9278 modulation enhances agonist affinity and diminishes action of LBD PAMs, we asked if GNE-9278 could also reduce allosteric modulation via the ATD. To test this we examined inhibition of NMDAR current by ifenprodil, a GluN2B-selective negative allosteric modulator that acts by binding to the ATD. These experiments showed a dramatic reduction in inhibition by ifenprodil in the presence of GNE-9278 (Fig. 9C). This further demonstrates the ability of GNE-9278 modulation to alter the normal action of modulators that act via the extracellular domain.

4. Discussion

GNE-9278 is a novel PAM that potentiates NMDAR peak currents...
Fig. 5. An Alanine scan of TMD helices identifies regions important for channel gating and GNE-9278 action. (A) Potentiation by 1 μM GNE-9278 and 10 μM GNE-8016 is shown for WT and 64 different mutant GluN1/GluN2A NMDARs expressed in Xenopus oocytes. ‘Hits’ from this screen were defined as having potentiation by 1 μM GNE-9278 that was at least 30X reduced and significantly different from potentiation by 10 μM 8016 (t-test p < 0.05, stars above the bars, n = 3–4 oocytes for each mutant, n = 41 oocytes for wild-type). The black dashed line represents no potentiation and the red dashed line indicates the WT level of GNE-9278 potentiation. Representative traces from WT (upper left) and GluN1 T550A (upper right), which affects GNE-9278 but not GNE-8016 potentiation, are shown (inset). (B) Glu EC₅₀ of each mutant is shown. The hits from panel A are indicated with an arrow. Example traces from WT and GluN1 S553A are shown (inset). All data are shown as mean ± SEM.
and slows deactivation kinetics. GNE-9278 shows fast kinetics and state dependency, apparently potently associating with open but not closed channels. While a precise binding site cannot be fully established in the absence of a co-crystal structure, we took a rigorous approach to identify the site of GNE-9278 action. After excluding potential extracellular domain binding sites, a mutational scan identified key structural determinants of GNE-9278 potentiation near the Lurcher motif. Different side chain mutations of the GluN1 L655 residue were able to alter the polarity of modulation and three pre-M1 residues were identified as sufficient to install the binding site into AMPARs, which were normally not potentiated by GNE-9278. Interestingly, GNE-9278 potentiation is associated with significant alterations to NMDAR extracellular domain function and pharmacology.

4.1. Structural determinants of GNE-9278 effects

An important aspect of our mutational scan was our use of GNE-8016, which binds at the LBD, as a “control” PAM. This allowed discrimination of specific structural determinants of GNE-9278 potentiation from mutations that simply altered channel gating and thus occlude potentiation in general. Interestingly, many of the mutations in the TMD did alter basal Glu sensitivity of NMDARs. This observation builds on previous work showing effects of various TMD mutations on channel gating (Kashiwagi et al., 2002; Hu and Zheng, 2005; Sobolevsky et al., 2007; Blanke and VanDongen, 2008; Chang and Kuo, 2008; Murthy et al., 2012; Ogden and Traynelis, 2013), and emphasizes the role of both GluN1 pre-M1/ M1 and M3 in channel gating.

Of the 4 confirmed GluN1 structural determinants, the pre-M1 residues (T550 and D552) were particularly important for GNE-9278 potency and thus likely contribute directly to GNE-9278 binding. This is convincingly supported by conferral of GNE-9278 potentiation to AMPARs by transferring the 3-amino acid sequence containing these GluN1 residues into GluA2 (Fig. 8). In contrast to the pre-M1 residues, the two critical M3 residues (F654 and L655) are key to the efficacy of GNE-9278. This is seen with the reduced maximal potentiation by F654A, despite somewhat increased potency. Similarly, while the L655A mutation eliminated potentiation, L655V reduced potentiation without dramatically affecting potency and L655Y caused GNE-9278 to act as a NAM with a similar potency as the PAM effects on WT channels (Fig. 7). These observations suggest that L655 determines the nature of GNE-9278 modulation without affecting binding. Interestingly, GNE-8016 can potentiate L655A NMDARs, but this potentiation is greatly diminished in the presence of GNE-9278, indicating that the apparent loss of GNE-9278 modulation with L655A actually represents neutral/silent allosteric modulation that dominates over GNE-8016 potentiation (Fig. 9A and B). Overall our results support a model where, upon channel opening, GNE-9278 binds to the TMD with residues on pre-M1 contributing directly to binding affinity. The effects of GNE-9278 binding then propagate through residues located near the extracellular end of the Lurcher motif in M3.

4.2. Comparison to other NMDAR modulators that act via the TMD

Interestingly, structural determinants of endogenous neurosteroid inhibition of GluN2B NMDARs include residues within the SYTANLAFF motif, and a binding site created by channel opening has been proposed (Vyklicky et al., 2015). However, the proposed neurosteroid inhibitory binding site is an interior funnel formed by the M3 helices, while the structural determinants on pre-M1 support GNE-9278 interaction with an exterior facing aspect of M3 and the extracellular end of the SYTANLAFF motif. At the same time, L655 is among the residues important for blocking NMDARs by MK-801 and TB-3-4 (Kashiwagi et al., 2002), emphasizing the key importance of this residue in channel function and pharmacology.

Other NMDAR PAMs with structural determinants in the TMD include the NMDAR modulator pregnenolone sulfate (Jang et al., 2004) and the GluN2C/2D-selective compound CIQ (Ogden and Traynelis, 2013). Interestingly, while the structural determinants of pregnenolone potentiation include pre-M4/M4 of GluN2, the structural determinants of CIQ and GNE-9278 include pre-M1/M1 of GluN2, and GluN1, respectively. This raises the possibility that CIQ and GNE-9278 bind distinct but related sites involving GluN2 for CIQ (which exhibits GluN2 subunit selectivity) and GluN1 for GNE-9278 (which is GluN2 non-selective). The distinct properties of GNE-9278 and CIQ, however, suggest very different modes of action. In contrast to GNE-9278, CIQ does not prolong channel opening after agonist removal and has minimal effects on Glu and Gly EC50s (Mullasseril et al., 2010). These distinct modes of potentiation by GNE-9278 and CIQ are consistent with their distinct structural determinants.

4.3. Mechanism of PAM action

Due to the lack of open pore iGluR structures, attempting to accurately define a binding pose for GNE-9278, which acts on activated NMDARs, is difficult. Nonetheless, based on the similarity of the iGluR pore to an inverted potassium channel pore (Traynelis et al., 2010), we can assume the TMD undergoes a rearrangement and expansion during channel opening, and that the binding site is somehow created or becomes accessible to GNE-9278 on the exterior aspect of the channel pore. It is striking that the equivalent region in AMPARs to the pre-M1/linker region of GluN1, where the critical residues for GNE-9278 potency were found, is very close to the binding site for the non-competitive AMPAR inhibitors GYKI-53655, CP-465022, and perampanel, which has recently been demonstrated by x-ray crystallography (Yelshanskaya et al., 2016). These AMPAR non-competitive inhibitors exhibit higher affinity for agonist-unbound vs. activated receptors (Balannik et al., 2005), and the proposed mechanism of inhibition involves stabilization of a closed state of the AMPAR by compound binding (Balannik et al., 2005; Yelshanskaya et al., 2016). Conversely, GNE-9278 appears to preferentially bind to agonist-bound NMDARs (Fig. 3) and would appear to function by stabilizing an activated state of the NMDAR.

The ability of GNE-9278 to act in the TMD and alter agonist affinity and slow deactivation is reminiscent of the effects of certain NMDAR blockers like 9-amino-acridine (9-AA), which has been shown to trap glutamate and glycine at their binding sites and slow deactivation while inhibiting channels via an open channel block mechanism (Benveniste and Mayer, 1995). GNE-9278 appears to share this property of increasing agonist affinity while stabilizing an open state of NMDARs via action at the TMD, while lacking the blocking effects of 9-AA. This stabilization of a specific state of the NMDAR is reflected in the insensitivity to LBD and ATD allosteric modulators during GNE-9278 modulation (Fig. 9). In addition, we speculate that negative allosteric modulation of GluN1 L655Y NMDARs and silent allosteric modulation of GluN1 L655A NMDARs by GNE-9278 could possibly reflect an open channel block mechanism that is dominant over the PAM effects in the case of L655Y, and offsets the PAM effects in the case of L655A. The GNE-9278 dose-response profile seen with wild-type NMDARs (Fig. 1C) could also reflect a subtle contribution of less potent channel blocking effects superimposed on the predominant PAM effects. In the case of GluN2C and GluN2D receptors in particular, a contribution of a channel blocking effect could be responsible for the diminished PAM effect observed at the highest GNE-9278 concentration.

An interesting aspect of GNE-9278 potentiation is that both type...
I (increased maximum current) and type II (increased agonist potency and slowing of deactivation) PAM effects are observed (Hackos and Hanson, 2017). Interestingly, the effect on maximum current is more potent (i.e. lower EC50) than the effect on slowing of deactivation.
Fig. 7. Conversion of GNE-9278 potentiation to partial, neutral, or negative by point mutations at GluN1 L655. (A) Example traces showing 100 μM GNE-9278 modulation of GluN1/GluN2A WT, and GluN1 L655V, L655A, and L655Y mutations. Gray bars above the traces represent 300 nM Glu application. (B) Dose-response data for GNE-9278 potentiation in oocytes expressing GluN2A paired with GluN1 WT (EC₅₀ = 8.75 ± 1.18 μM), L655V (EC₅₀ = 5.75 ± 0.23 μM), L655A (EC₅₀ not determined) and L655Y (IC₅₀ = 3.73 ± 1.08 μM) at 300 nM Glu and 50 μM Gly are shown (n = 6 oocytes per receptor type). (C) There was a significant effect of GluN1 mutation type on modulation by 100 μM GNE-9278 (H = 21.136, d.f. = 3, p < 0.001), corresponding to significant differences in responses compared to WT for L655V (p = 0.002), L655A (p = 0.002), and L655Y (p = 0.001). (D) In contrast to GNE-9278, there was no significant effect of mutation type on modulation by 100 μM GNE-8016 (H = 5.261, d.f. = 3, p = 0.15). (E) When GluN2C was paired with the GluN1 L655 mutations, there was also a significant effect of mutation type (H = 25.407, d.f. = 3, p < 0.001), corresponding to significant differences in responses compared to WT for L655V (p = 1.1 × 10⁻⁴), L655A (p = 1.1 × 10⁻³), and L655Y (p = 1.1 × 10⁻³). (F) In contrast to GNE-9278, there was no significant effect of mutation type on modulation by CIQ (H = 2.687, d.f. = 3, p = 0.44). Overall effects of treatment type were determined by one-way ANOVA on the ranks, and individual comparisons were made with the two-sided Wilcoxon rank sum test. Data represent mean ± SEM. **p < 0.01, ***p < 0.001. For experiments in panels C and D, n = 8 WT, 5 L655A, 6 L655V and 5 L655Y oocytes, and in panels E and F, n = 11 WT, 7 L655A, 5 L655V and 6 L655Y oocytes.
Fig. 8. Swapping 3 key residues from GluN1 to GluA2 confers GNE-9278 sensitivity to AMPARs. (A) An alignment of GluN1 and GluA2 is shown. The GluA2-TLD chimeric channel was generated by replacing the corresponding amino acids in GluA2 with amino acids T550 to D552 from the pre-M1 region of GluN1. (B) Representative traces show 100 μM Glu-induced currents in WT AMPARs (top) measured in the presence of 30 μM GNE-3419 (which binds in the LBD interface and prevents desensitization). Under these conditions, application of 30 μM GNE-9278 has no appreciable effect on WT GluA2 AMPARs. In contrast, experiments with the GluA2 chimeric channel (bottom) show that GNE-9278 causes robust reversible potentiation under the same conditions. (C) Quantification showed a significantly greater potentiation with GluA2-TLD chimeric vs. WT GluA2 AMPARs (wild-type, 11 ± 0.02 fold, GluA2-TLD 3.1 ± 0.1 fold, n = 7 oocytes each, p = 5.8 × 10⁻⁴, two-sided Wilcoxon rank sum test). Data represent mean ± SEM.

Fig. 9. GNE-9278 renders NMDARs insensitive to GNE-8016 potentiation and ifenprodil inhibition. (A) Recordings of oocytes expressing GluN1 L655A and GluN2A showed that 100 μM 8016 could not potentiate 100 μM Glu-induced currents in the presence of 100 μM GNE-9278, but could potentiate when GNE-9278 was removed. Timing of Glu, GNE-8016 and GNE-9278 applications is indicated above the trace. (B) Quantification of GNE-8016 potentiation (100 μM Glu, 50 μM Gly) in oocytes expressing GluN1 L655A/GluN2A in the absence or presence of GNE-9278 is shown. GNE-9278 resulted in a significant reduction in the efficacy of GNE-8016 potentiation. The maximum fold potentiation decreased from 2.06 ± 0.24 to 1.15 ± 0.05 (p = 4 × 10⁻⁴, n = 9, 6), while the EC⁵₀ wasn't significantly changed (3.58 ± 1.48 vs. 5.31 ± 1.89 μM). (C) Quantification of ifenprodil inhibition (100 μM Glu, 50 μM Gly) in oocytes expressing GluN1/GluN2B receptors in the absence or presence of GNE-9278 is shown. GNE-9278 resulted in a significant reduction in the maximal inhibition by ifenprodil from 80 ± 2% to 23 ± 4% (p = 6.7 × 10⁻⁴, n = 8, 6) and an increase in the IC⁵₀ from 123 ± 9.8 nM to 961 ± 379 nM (p = 1.7 × 10⁻⁴). All data represent mean ± SEM and two-sided Wilcoxon rank sum tests were used to determine the significance.

deactivation (Fig. 1E). One hypothetical cause for this phenomenon could be that binding of one PAM molecule is sufficient to increase peak potentiation while binding to two binding sites is required to slow deactivation. On the other hand, this phenomenon can also be explained simply by the observed fast GNE-9278 dissociation kinetics. The enhanced peak current observed in the presence of saturating agonists is expected to simply be proportional to receptor occupancy by GNE-9278. However, the fact that GNE-9278 is a PAM means that in the absence of agonists, GNE-9278 can't potentiate the channels once they close. Consequently, the effects on deactivation will be greatly diminished at low GNE-9278 concentrations since the channel will close rapidly (in the absence of Glu) once the PAM dissociates unless GNE-9278 is able to re-bind prior to channel closure, the probability of which is dependent on the PAM concentration. Accordingly, a simple kinetic model of this process suggests that the extent of slowing of NMDAR deactivation kinetics in the presence of a PAM with fast dissociation kinetics should be inversely proportional to the fraction of unoccupied receptors (Supplemental Fig. 1), which is sufficient to explain the less potent slowing of deactivation compared to the potentiation of peak current. Regardless of the mechanism, this mixture of different types of PAM effects, with slowing of deactivation only occurring at higher concentrations, could represent an interesting therapeutic profile if drug-like compounds with similar properties were to be developed.

Overall, the discovery of a novel NMDAR PAM with a TMD
binding site that can be recreated in AMPARs or mutated to cause NAM effects broadens our knowledge of the modulatory potential of iGluRs. These results combined with the previous studies of AMPAR NAMs that bind the TMD (Balannik et al., 2005; Yelshanskaya et al., 2016), predict that state-dependent binding in this region should allow discovery and development of both positive and negative modulators of both NMDA and AMPA receptors. In addition to being useful for further studies of iGluR structure and function, compounds such as GNE-9278 that act via the TMD will help unlock the full potential of iGluR modulation as a therapeutic approach.

Disclosure/conflict of interest

All authors are current or former Genentech employees.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2017.04.041.

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