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

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ABSTRACT

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.

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 over-activation, 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 on 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 in 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 helixes form a physical constriction in the ion channel pore that prevents ion conduction when the channels are in closed or desensitized states (Karakas 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 (A8T) 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 iGluR gating. Importantly, study of the structural determinants of GluN2C/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. ^{1}H-NMR spectra were measured on Bruker Avance III 300, 400, or 500 MHz spectrometers. Chemical shifts (in ppm) were referenced to internal standard tetramethylsilane ($\delta = 0$ 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-4*H*-1,2,4-triazol-3-amine (3.0 g, 24%) as a white solid. LCMS, $m/z = 127.0$ $[M + H]^+$. ^{1}HNMR (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 (30/1) 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, $m/z = 193.0$ $[M+H]^+$. ^{1}HNMR (400 MHz, $CDCl_3$) 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, $m/z = 238.1$ $[M+H]^+$. ^{1}HNMR (400 MHz, $CDCl_3$) 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 Pd/C (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, $m/z = 208.1$ $[M+H]^+$. ^{1}HNMR (300 MHz, $CDCl_3$) 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-9278):

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 (1.17 g, 54%) as a white solid. LCMS, $m/z = 430.2$ $[M+H]^+$. ^{1}HNMR (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),