Structural determinants and mechanism of action of a GluN2C-selective NMDA receptor positive allosteric modulator

Alpa Khatri, Pieter B. Burger, Sharon A. Swanger, Kasper B. Hansen, Sommer Zimmerman, Erkan Karakas, Dennis C. Liotta, Hiro Furukawa, James. P. Snyder, Stephen F. Traynelis

Emory University, Pharmacology Department: AK, SAS, SFT
Emory University, Chemistry Department: SZ, PB, DCL, JPS
University of Montana, Dept of Biomedical and Pharmaceutical Sciences, and Center for Biomolecular Structure and Dynamics: KBH
Cold Spring Harbor Labs: EK, HF
Running Title: Analysis of a GluN2C NMDAR positive allosteric modulator

Corresponding author
Stephen F. Traynelis
1510 Clifton Road
RRC 5066
Atlanta GA, 30322
strayne@emory.edu
fax #: 404-727-0365

Number of text pages: 29
Number of Figures: 6
Numbers of Tables: 2
Number of words in the Abstract: 243
Number of words in the Introduction: 637
Number of words in the Discussion: 1259

List of non-standard abbreviations:
NMDA: n-methyl-d-aspartate
PYD: methyl 4-((3-acetyl-4-hydroxy-1-(2-(2-methyl-1H-indol-3-yl)ethyl)-5-oxo-2,5-dihydro-1H-pyrrol-2-yl)benzoate
CNS: Central Nervous System
GABA_A: Gamma-Aminobutyric acid
nAChR: Nicotinic Acetylcholine Receptor
P2X: Purinergic Receptor
DCS: D-cycloserine,
CIQ: (3-chlorophenyl)(6,7-dimethoxy-1-((4- methoxyphenoxy)methyl)-3,4-dihydroisoquinolin-2(1H)-yl)methanone)
5-HT_3A: Serotonin receptor
NIMH-PDSP: National institute of Mental Health-psychoactive drug screening study
HEK: Human Embryonic Kidney cell
QNZ-46: (E)-4-(6-methoxy-2-(3-nitrostyryl)-4-oxoquinazolin-3(4H)-yl)-benzoic acid
DQP-1105: 4-(5-(4-bromophenyl)-3-(6-methyl-2-oxo-4-phenyl-1,2-dihydroquinolin-3-yl)-4,5-dihydro-1H-pyrazol-1-yl)- 4-oxobutanoic acid
AMPA: (α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)
SEM: Standard error of the mean
ANOVA: Analysis of Variance
ER: endoplasmic reticulum
Abstract

NMDA receptors are tetrameric complexes of GluN1, GluN2A-D, and GluN3A-B subunits and are involved in normal brain function and neurological disorders. We have identified a novel class of stereo-selective pyrrolidinone (PYD) positive allosteric modulators for GluN2C-containing NMDA receptors, exemplified by methyl 4-(3-acetyl-4-hydroxy-1-(2-(2-methyl-1H-indol-3-yl)ethyl)-5-oxo-2,5-dihydro-1H-pyrrol-2-yl)benzoate. Here we explore the site and mechanism of action of a prototypical analogue PYD-106, which at 30 μM does not alter responses of NMDA receptors containing GluN2A, GluN2B, and GluN2D, and has no effect on AMPA and kainate receptors. Co-application of 50 μM PYD-106 with a maximally effective concentration of glutamate and glycine increases the response of GluN1/GluN2C NMDA receptors in HEK-293 cells to 221% of that obtained in the absence of PYD (taken as 100%). Evaluation of the concentration-dependence of this enhancement revealed an EC$_{50}$ value for PYD of 13 μM. PYD-106 increased opening frequency and open time of single channel currents activated by maximally effective concentrations of agonist, but only had modest effects on glutamate and glycine EC$_{50}$. PYD-106 selectively enhanced the responses of diheteromeric GluN1/GluN2C receptors, but not triheteromeric GluN1/GluN2A/GluN2C receptors. Inclusion of residues encoded by GluN1-exon5 attenuated the effects of PYD. Three GluN2C residues (Arg194, Ser470, Lys470), at which mutagenesis virtually eliminated PYD function, line a cavity at the interface of the ligand binding and the amino terminal domains in a homology model of GluN1/GluN2C built from crystallographic data on GluN1/GluN2B. We propose that this domain interface constitutes a new allosteric modulatory site on the NMDA receptor.
Introduction

$N$-Methyl-$d$-aspartate receptors (NMDARs) are ligand-gated cation channels that mediate a slow $Ca^{2+}$-permeable component of excitatory synaptic transmission. NMDA receptors are involved in the development and normal function of the CNS. Dysfunction of NMDARs is associated with epilepsy, pain, depression, Parkinson’s disease, and schizophrenia, making these receptors an attractive therapeutic target (Hallett & Standaert, 2004; Kalia et al., 2008; Lisman et al., 2008; Preskorn et al., 2008; Wu & Zhuo, 2009; Endele et al., 2010; Traynelis et al., 2010; Balu & Coyle, 2011; Kostakis et al., 2011). The majority of NMDARs in the central nervous systems are heteromeric complexes formed by two GluN1 and two GluN2 subunits (Ulbrich & Isacoff, 2007), of which there are four subtypes (GluN2A-D) with temporal and spatial variation in expression (Watanabe et al., 1992; Ishii et al., 1993; Monyer et al., 1994). The development of GluN2-selective modulators provides a therapeutic opportunity to target NMDAR subtypes with anatomically restricted expression patterns, thereby minimizing potential side effects (Kalia et al., 2008; Ogden & Traynelis, 2011; Collingridge et al., 2013). Subunit-selective allosteric modulators exist for the GluN2A (TCN-201), GluN2B (ifenprodil), and GluN2C/GluN2D subunits (CIQ, QNZ, UBP, DQP analogues) (Williams et al., 1993; Bettini et al., 2010; Mullasseril et al., 2010; Acker et al., 2011; Hansen & Traynelis, 2011; Costa et al., 2012; Hansen et al., 2012; Monaghan et al., 2012). However, no modulators to date have been able to distinguish between GluN2C and GluN2D subunits.

The GluN2C subunit is expressed in the cerebellum, amygdala, olfactory bulb, retrosplenial cortex as well as in thalamic, cortical, and hippocampal interneurons (Farrant et al., 1994; Monyer et al., 1994; Wenzel et al., 1997; Binshtok et al., 2006;
Karavanova et al., 2007). Oligodendrocytes also express the GluN2C subunit (Karadottir et al., 2005; Salter & Fern, 2005; Micu et al., 2006). GluN2C-containing NMDARs have a lower sensitivity to voltage-dependent Mg$^{2+}$ block, reduced Ca$^{2+}$ permeability, and reduced conductance compared to GluN2A and GluN2B (Qian et al., 2005; Clarke & Johnson, 2006; Siegler Retchless et al., 2012). Behavioral studies evaluating the deletion of the GluN2C subunit suggest a possible role in working memory (Hillman et al., 2011). In addition, studies with the GluN2C/GluN2D-selective positive allosteric modulator CIQ suggest a possible role for enhancement of NMDAR function in emotional learning, working memory, and sensorimotor gating (Ogden et al., 2013; Suryavanshi et al., 2013). D-cycloserine (DCS), an antibiotic treatment used for tuberculosis, acts as a partial agonist relative to glycine at GluN1/GluN2A, GluN1/GluN2B, and GluN1/GluN2D receptors. By contrast, a maximally effective concentration of DCS produces more current at GluN1/GluN2C receptors than the endogenous agonist glycine, leading to selective enhancement of GluN1/GluN2C receptors when DCS replaces glycine at the GluN1 agonist binding site (Sheinin et al., 2001; Dravid et al., 2010). NMDAR hypofunction has been suggested to underlie some aspects of schizophrenia (Krystal et al., 1994; Olney et al., 1999; Lisman, 2012) and DCS has shown positive results in schizophrenic patients, suggesting increased occupancy of the agonist binding site on GluN1 and thereby enhancement NMDAR function (Goff et al., 1995; Goff et al., 2008; Gottlieb et al., 2011). The ability of DCS to enhance the function of the GluN2C-containing receptors raises the possibility that GluN2C modulation may contribute to clinically relevant actions of DCS in schizophrenia (Norberg et al., 2008; Kaplan & Moore, 2011). Thus, better pharmacological tools are
needed to evaluate the functional roles of GluN2C in neurological diseases such as schizophrenia.

We describe here the mechanism of action and structural determinants for a GluN2C-selective class of compounds exemplified by PYD-106, an analogue developed during the study of the structure-activity relationship around a pyrrolidinone identified from a high-throughput screen (Zimmerman et al., 2014). The PYD class of positive allosteric modulators is the only series that selectivity enhances the response to maximally effective concentrations of agonist for recombinant NMDARs containing two copies of the GluN2C subunit.
Material and Methods

Molecular biology

GluN1, GluN2A (D13211), GluN2B (U11419), GluN2C (M91563) and GluN2D (L31611) cDNAs were provided by Drs. Heinemann (Salk Institute for Biological Sciences, La Jolla, CA), Nakanishi (Kyoto University, Kyoto, Japan) and Seeburg (University of Heidelberg, Heidelberg, Germany). The GenBank accession numbers for GluN1-1a (hereafter GluN1), and splice variants GluN1-1b, -2a, -2b, -3a, -3b, -4a, -4b were U08261, U08263, U08262, U08264, U08265, U08266, U08267, U08268, respectively. The GluN2A-GluN2C chimeras and GluN2C point mutations were generated as previously described (Chen et al., 2008). Supplemental Table S1 lists the junctions for the chimeric receptors.

A GluN2C-2A C-terminal chimera was made from amino acids 1 - 851 of rat GluN2C and amino acids 841 - 1464 of rat GluN2A; these chimeric GluN2C-2A subunits will be referred to hereafter as GluN2C*. Constructs GluN2A-C1, GluN2A-C2, GluN2C*-C1, and GluN2C*-C2 were generated by adding a 23 residue synthetic linker followed by coiled-coil regions of the GABA\(_{B1}\) and GABA\(_{B2}\) receptor subunits (C1 and C2, respectively) plus a dilyssine ER retention signal (KKTN) at the 3' end of rat GluN2A and rat GluN2C*, as described previously (Hansen et al., 2014). Two point mutations were introduced into the agonist binding domains by site-directed mutagenesis using the Quikchange method and were GluN2A(R518K,T690I) and GluN2C*(R529K,T701I). All chimeric subunits and mutations were verified by DNA sequencing.

Two-electrode voltage clamp recordings
Xenopus laevis oocytes were obtained from EcoCyte (Austin, TX), injected with cRNA created by *in vitro* transcription using the mMessage mMACHINE kits according to the manufacturer’s instructions (Ambion), as previously described (Hansen et al., 2013). Plasmids containing the genes for the GABA<sub>A</sub> (α1β2γ2s), GABA<sub>C</sub> (ρ1), glycine (α1), serotonin (5-HT<sub>3A</sub>), nicotinic acetylcholine receptor (nAChR, α1β1δγ, α2β4, α4β3, α9α10) and purinergic (P2X<sub>2</sub> rat, P2X<sub>2</sub> human) receptors were provided by Drs. Heinemann (Salk), Weiss (Univ. of Texas, San Antonio), Papke (Univ. of Florida), and Hume (Univ. of Michigan), linearized and used to synthesize cRNA for these receptors. 1-4 days after cRNA injection (5-15 ng), oocytes were recorded at room temperature under two-electrode voltage clamp (V<sub>HOLD</sub> -30 to -60 mV) in a solution containing (in mM) 90 NaCl, 1 KCl, 10 HEPES, 0.5 BaCl<sub>2</sub> and 0.01 EDTA (pH 7.4). For triheteromeric receptor experiments, the cRNA was prepared at a ratio of 1:6:6 (GluN1:GluN2<sub>C1</sub>:GluN2<sub>C2</sub>), and approximately 5-10 ng of total cRNA was injected into each oocyte. Recordings were performed 3-4 days after injection. NMDAR currents were evoked by bath application of 50-100 μM glutamate and 30-100 μM glycine. Currents from the GluA1-4 and GluK1-2 receptors were evoked with 100 μM glutamate; GluK2 was incubated in 1 mg/ml concanavalin A for 10 minutes prior to recording. GluK2/GluK5 currents were evoked with 100 μM AMPA. Currents were evoked for following receptors using the agonist concentrations indicated: GABA<sub>C</sub> (1 μM GABA), GABA<sub>A</sub> (20 μM GABA), glycine α1 (50 μM glycine), 5-HT<sub>3A</sub> (1 μM serotonin), nicotinic acetylcholine α<sub>i</sub>β<sub>i</sub>δγ (1 μM acetylcholine), α<sub>3</sub>β<sub>4</sub> (10 μM acetylcholine), α<sub>4</sub>β<sub>2</sub> (10 μM acetylcholine), α<sub>9</sub>α<sub>10</sub> (1 μM acetylcholine), α<sub>7</sub> (300 μM acetylcholine), and the P2X<sub>2</sub> receptors (9 μM ATP).
Patch-clamp recordings

HEK-293 cells (ATCC CRL-1573, hereafter HEK cells) were maintained in DMEM with GlutaMAX, 110 mg/ml sodium pyruvate and 4.5 gm/L glucose (Invitrogen, Grand Island, NY) supplemented with 10% dialyzed fetal bovine serum, 10 U/ml penicillin, and 10 μg/ml streptomycin. HEK cells were maintained in a 5% CO₂ humidified 37°C incubator and transiently transfected using Fugene 6 with cDNAs encoding NMDAR subunits and green fluorescent protein (GFP) at a ratio of 1:1:5 for GluN1/GluN2A/GFP, 1:1:1 for GluN1/GluN2B/GFP, GluN1/GluN2C/GFP and GluN1/GluN2D/GFP. Voltage clamp recordings were performed 12-36 hrs after transfection (VHOLD -60 mV for whole cell, -80 mV for outside-out patches). The data was filtered at 8 kHz (8-pole Bessel filter, -3dB) and digitized at 20-40 kHz. The extracellular solution consisted of (in mM) 150 NaCl, 10 HEPES, 3 KCl, 0.5 CaCl₂, 0.01 EDTA and 30 D-mannitol (pH 7.4 for whole cell and 8.0 for outside-out patches). The intracellular solution contained (in mM) 110 d-gluconate, 110 CsOH, 30 CsCl₂, 5 HEPES, 4 NaCl, 0.5 CaCl₂, 2 MgCl₂, 5 BAPTA, 2 Na-ATP and 0.3 Na-GTP; the pH was adjusted to 7.35 with CsOH. Rapid solution exchange was achieved using a two-barrel theta glass pipette controlled by a piezoelectric translator. The open tip junction currents had a 10-90% rise time of less than 1 ms. The solution exchange around a whole cell had a 10-90% rise time of 3.4 ± 0.3 ms (n=9) as determined by exchanging the extracellular NaCl for KCl. Single channel recordings from outside-out patches were made in response to steady-state agonist application at pH 8.0. All recordings were performed at room temperature (23°C). The junction potential for HEK cell recording
solution was +5.4 mV (Vance et al., 2011); single channel chord conductance values were corrected for this value.

Recordings from outside-out patches were digitally filtered at 3-4 kHz (-3 dB) and idealized using time course fitting (SCAN; Dr. David Colquhoun, University College London; Colquhoun & Sigworth, 1983). An open resolution of 53 μs and shut resolution of 31 μs was imposed on the data and open periods were combined for different conductance levels. Both open and closed duration histograms were fitted with the sum of 2-5 exponential functions using maximum likelihood methods. The open probability was calculated as the total time the patch was in an open state divided by the total time of the recording. Most outside-out patches contained multiple channels since double and triple openings were occasionally observed. Multiple openings were excluded from analysis of channel dwell times, and thus the reported open probabilities are underestimates of the true open probability.

Molecular modeling

Amino acids are numbered with the initiating methionine set to 1. A protein sequence alignment of the different GluN2A-D sequences was generated with Muscle (Edgar, 2004) using the GluN1-1a (i.e. GluN1) and GluN2B sequences obtained from the resolved GluN1/GluN2B crystal structure (pdb 4PE5). Five GluN1/GluN2C homology models were generated with Modeler 9v12 (Sali & Blundell, 1993) using the GluN1/GluN2B crystal structure as template (PDB 4PE5; Karakas & Furukawa, 2014). No protein optimization was performed during model building. The models were subjected to protein quality analysis and the model with the lowest discrete optimized
protein energy (DOPE) score was selected. Side chain optimization and protonation
state assignment was performed with the protein preparation wizard (Sastry et al.,
2013) and monitored by visual inspection. This was followed by energy minimization
(heavy atom RMSD convergence of 0.3Å; Force field OPLS 2005) to relieve the
energetically unfavorable constraints. PYD-106 was prepared for docking using Ligprep
(Schrödinger Release 2014-2: LigPrep, version 3.0, Schrödinger, LLC, New York, NY,
2014). The docking grid was centered between the carbonyl oxygen of P428 and the
Cβ-atom of S472. The diameter midpoint of the docked ligands was required to remain
within a nested box (14 Å³) at the center of the grid. The extra precision (XP) scoring
algorithm of Glide (Friesner et al., 2006) was used to obtain the best scoring poses
during docking. PYD-106 was docked flexibly with docking poses being restricted to 10,
followed by post-docking minimization (OPLS 2005 force field) with an energy threshold
of 0.5 kcal/mol. A pdb file with PYD-106 docked into the homology model of
GluN1/GluN2C is included as supplemental data (see Data Supplement).

Synthesis of pyrrolidinone analogues

Compounds PYD-1 and PYD-106 were prepared as previously described (see
compounds in Zimmerman et al., 2014). Compounds were dissolved at 20 mM or 50
mM in 100% DMSO, and this stock was used for all solutions. Final DMSO
concentrations were between 0.005-0.5% (vol/vol). Both compounds were soluble up to
100 µM, as determined by nephelometry. Compound purity was greater than 95%.

Statistics
All results are presented as the mean ± standard error of the mean. Statistical significance was taken as p < 0.05 by t-test or one way ANOVA (Tukey, Bonferroni, or Dunnett’s post hoc), as appropriate. For all tables and figures, n is the number of observations.
Results

Pyrrolidinones are selective positive allosteric modulators of GluN1/GluN2C receptors

We have studied the actions of the pyrrolidinone PYD-106 (Zimmerman et al., 2014) on recombinant GluN1/GluN2C receptor function. Figure 1 shows concentration-effect curves for glutamate in the absence and presence of PYD-106 as determined in *Xenopus* oocytes injected with GluN1/GluN2C mRNA. PYD-106 prominently enhanced the maximal fitted current response by over two-fold. In addition, the glutamate EC_{50} was modestly increased by 70 μM PYD-106, being 0.72 ± 0.05 μM (n = 13, Hill slope 1.45 ± 0.03) in the absence and 1.17 ± 0.08 μM (n = 13, Hill slope 1.39 ± 0.02) in the presence of PYD-106 when studied in the same oocytes (p < 0.05; paired t-test; 30 μM glycine present in all solutions). We also determined the glycine concentration-effect curve independently in the absence or presence of 70 μM PYD-106. Coapplication of PYD-106 with glycine modestly reduced the EC_{50} from 0.23 ± 0.004 μM (n = 6) in the absence to 0.16 ± 0.006 μM (n = 5) in the presence of PYD-106 (p < 0.05, unpaired t-test; 100 μM glutamate present in all solutions). PYD-106 showed no agonist activity when applied alone, with the response amplitude being 1.1 ± 0.6% of control (n = 4). Co-application of PYD-106 with either glutamate alone or glycine alone did not produce inward currents (n = 5). These data suggest that PYD-106 is a positive allosteric modulator that increases agonist efficacy, and also reveal significant negative (glutamate) and positive (glycine) interactions with agonist potency.

We next evaluated the concentration-dependence of PYD-106 on recombinant NMDAR activated by maximally effective concentrations of glutamate (100 μM) and glycine (30 μM). The positive modulation produced by PYD-106 was both reversible and
repeatable, with an EC$_{50}$ value of 16 ± 0.5 μM and a Hill slope of 1.19 ± 0.03 in oocytes (n = 31, Figure 2A,B). Co-application of 100 μM PYD-106 and saturating concentrations of glutamate (100 μM) and glycine (30 μM) increased the maximal current response to 204 ± 3.9% of control (the response in the absence of PYD-106 was taken as 100%, n = 31). At sub-maximal concentrations of glutamate (1 μM) and glycine (0.3 μM), the GluN1/GluN2C receptor response was increased to 223 ± 6.3% of control by 100 μM PYD-106 with an EC$_{50}$ of 15 ± 1.9 μM (n = 5), which was not significantly different compared to the data obtained with saturating concentrations of glutamate and glycine (t-test, p > 0.05).

We subsequently tested the effect of PYD-106 at other recombinant ion channels expressed in oocytes. We used a concentration of PYD-106 (30 μM) that strongly enhanced the GluN1/GluN2C receptor response to maximally effective concentrations of glutamate and glycine. PYD-106 (30 μM) did not alter the amplitude for GluN1/GluN2A, GluN1/GluN2D, AMPA, kainate, GABA$_C$ and 5-HT$_{3A}$ receptor-mediated currents in response to saturating agonist concentrations (Figure 2E). Responses of GluN1/GluN2B, glycine $\alpha_1$, GABA$_A$ and nicotinic acetylcholine receptors were inhibited to 73 - 88% of control by 30 μM PYD-106 (Figure 2E, where the response in the absence of PYD is 100%). PYD-106 was also tested by the NIMH psychoactive drug screening program (PDSP) using a binding assay to assess interactions with G-protein coupled receptors, transporters, and voltage-gated ion channels. Out of 42 proteins that were tested by the NIMH-PDSP, 10 μM PYD-106 showed inhibition in excess of 50% of control for the kappa-opioid receptor, dopamine transporter (DAT) and the adrenergic $\alpha_{2C}$ receptor (Supplemental Table S2). A subsequent experiment determined the
binding affinity (Kᵢ) for PYD-106 to be 6.1 μM for the kappa-opioid receptor; the Kᵢ values for the α₂C receptor and DAT were greater than 10 μM. These data suggest that PYD-106 selectively enhances the GluN1/GluN2C response within the glutamate receptor family, but produces significant inhibition at multiple targets (including GluN2B at 100 μM).

Selectivity of pyrrolidinones for GluN1 splice variants

The GluN1 subunit RNA can be alternatively spliced with 8 different variants described (Hollmann et al., 1993). GluN1 alternative exon-5 encodes 21 amino acids, which are located near the ATD – S1 interface of GluN1. GluN1 exon5 is differentially expressed throughout the CNS (Laurie & Seeburg, 1994a; Laurie et al., 1995), with expression in cerebellar granule neurons, which also express GluN2C (Akazawa et al., 1994; Prybylowski et al., 2000). Exon-5 has been shown to alter the effects of several NMDA receptor modulators including neurosteroids, extracellular protons, Zn²⁺, and polyamines, in addition to altering glutamate potency and the deactivation time course (Traynelis et al., 1995; Traynelis et al., 1998; Rumbaugh et al., 2000; Kostakis et al., 2011; Vance et al., 2012). We therefore evaluated the effects of RNA splicing of GluN1 on the activity of the positive allosteric modulator PYD-106. GluN1 subunits that contain exon-5 are referred to as “b” and those lacking exon-5 as “a”; different combinations of C-terminal splice variants that lack exon-21 and/or exon-22 are denoted by the suffix 2,3,4 (Hollmann et al., 1993). Comparison of PYD-106 activity (100 μM) at the different GluN1 splice variants co-expressed with GluN2C revealed significant differences in the maximal level of modulation compared to GluN1-1a/GluN2C (200 ± 5.6% of control, n =
16). Modulation was significantly reduced for GluN1-1b (125 ± 2.8%, n = 18), GluN1-2b
(117 ± 0.6%, n = 7), GluN1-3a (173 ± 4.2%, n = 7), GluN1-3b (113 ± 0.4%, n = 9), and
GluN1-4b (118 ± 0.9%, n = 10) splice variants (one way ANOVA, Bonferroni’s post hoc,
p < 0.05), whereas there were no significant differences for GluN1-2a (202 ± 4.5%, n =
6) and GluN1-4a (188 ± 3.4%, n = 6). That is, PYD-106 produces a stronger
enhancement of the current response to maximal agonist concentrations for all GluN1
splice variants that lack exon-5 compared to those that contain exon-5. Concentration-
effect curves demonstrated that the PYD-106 \( EC_{50} \) for enhancement of the maximal
response of GluN1-1a (14 ± 1.1 \( \mu M \), n=25) was significantly different from the \( EC_{50} \) for
GluN1-1b-containing receptors (5.6 ± 0.4 \( \mu M \), n = 19, \( t \)-test, p < 0.05; Figure 2C).

To identify the structural determinants in the highly charged 21 amino acid
segment encoded by exon-5 that reduced positive allosteric modulation by PYD-106,
we first screened a series of triple charge neutralization mutations. The triple GluN1-1b
exon-5 mutation K207G, R208G, K211G restored the effects of PYD-106, which
enhanced the maximal current response to 206 ± 5.5% of control at 100 \( \mu M \) PYD-106
(\( EC_{50} \) of 21 ± 2.5 \( \mu M \), n = 8, Figure 1C). By contrast, the triple GluN1-1b mutant K192G,
K193G, R194G only partially rescued modulation by 100 \( \mu M \) PYD-106 to 145 ± 3.2% of
control (n = 4) with an \( EC_{50} \) of 9.6 ± 0.9 \( \mu M \) (n = 4) (Figure 1C). Mutation of three
negatively charged GluN1-1b residues encoded by exon-5 (E197A, D200A, D205A) did
not restore the effects of 100 \( \mu M \) PYD-106 (116 ± 2.3% of control; n = 4).

A number of effects of exon-5 on allosteric modulators as well as the deactivation
time course are controlled by Lys211 in exon-5 (Traynelis et al., 1995; Traynelis et al.,
1998; Vance et al., 2012). We therefore assessed whether mutation of Lys211 to Gly or
Arg restored the actions of PYD-106. The maximal current responses of GluN1-1b K211G and GluN1-1b K211R mutations were only modestly enhanced by 100 μM PYD-106 to 137 ± 2.3% (n = 9) and 129 ± 1.2% (n = 10), respectively (Figure 2C, Supplemental Figure S1). The degree of modulation observed with GluN1-1b K211G was significantly different compared to PYD-106 modulation of wild-type GluN1-1b (t-test, p < 0.05). The EC$_{50}$ values for PYD-106 enhancement of the maximal response of GluN1-1b K211G and GluN1-1b K211R were 9.5 ± 0.9 μM (n = 3) and 9.0 ± 1.9 (n = 6) μM, respectively, which were not significantly different compared to GluN1-1a (p > 0.05). These data suggest that the structural determinants within exon 5 responsible for its effects on protons, polyamines, and Zn$^{2+}$ are distinct from its effects on PYD-106.

**Pyrrolidinone activity on triheteromeric NMDARs**

NMDARs can form diheteromeric complexes that contain one type of GluN2 subunit or triheteromeric receptors containing two different GluN2 subunits. For example, triheteromeric receptors containing GluN1/GluN2A/GluN2C have been reported to form functional receptors in cerebellar neurons (Chazot et al., 1994; Cathala et al., 2000; Lu et al., 2006). To determine whether PYD-106 enhanced the responses of triheteromeric receptors that contain a single copy of GluN2C, we adapted a recombinant expression system that promotes the surface expression of triheteromeric receptors and limits the surface expression of diheteromeric receptors (Hansen et al., 2014) to control expression of GluN1/GluN2A/GluN2C receptors. The GluN2C C-terminus was replaced with that of GluN2A (referred to as GluN2C*), and heterodimeric coiled-coil regions with ER retention signals (C1 and C2) were added to the C-terminus...
of both GluN2A and GluN2C (hereafter named GluN2A<sub>C1</sub>, GluN2A<sub>C2</sub>, GluN2C<sup>*</sup><sub>C1</sub>, and GluN2C<sup>*</sup><sub>C2</sub>). NMDAR tetramers containing one C1-tagged GluN2 subunit and one C2-tagged GluN2 subunit are trafficked to the cell surface, whereas those complexes containing only a single C1 or C2 tag are retained in the ER (Hansen <i>et al.</i>, 2014). The C-terminal domain of GluN2C was replaced with that of GluN2A because differences between the C-terminal domains could lead to differing trafficking patterns or hinder the C1-C2 interaction. To test how PYD-106 affects triheteromeric NMDAR responses, <i>Xenopus</i> oocytes were co-injected with GluN1/GluN2A<sub>C1</sub>/GluN2A<sub>C2</sub>, GluN1/GluN2C<sup>*</sup><sub>C1</sub>/GluN2C<sup>*</sup><sub>C2</sub>, or GluN1/GluN2A<sub>C1</sub>/GluN2C<sup>*</sup><sub>C2</sub>, and receptors were activated with 100 µM glutamate and 30 µM glycine. PYD-106 application increased the maximal responses of GluN1/GluN2C<sup>*</sup><sub>C1</sub>/GluN2C<sup>*</sup><sub>C2</sub> receptors to 212 ± 7.8 % (n = 12) of control, whereas the responses of GluN1/GluN2A<sub>C1</sub>/GluN2A<sub>C2</sub> and GluN1/GluN2A<sub>C1</sub>/GluN2C<sup>*</sup><sub>C2</sub> receptors were modestly inhibited by PYD-106 (86 ± 0.9 % and 87 ± 2.2 % of control, respectively, n = 13-14; Figure 2D). To confirm that responses in oocytes expressing GluN1/GluN2A<sub>C1</sub>/GluN2C<sup>*</sup><sub>C2</sub> were mediated primarily by triheteromeric receptors, oocytes were co-injected as above with one double-mutated GluN2 that prevents glutamate binding, either GluN2C<sup>*</sup><sub>C2</sub>(R529K,T701I) or GluN2A<sub>C1</sub>(R518K,T690I) (Laube <i>et al.</i>, 1997; Hatton & Paoletti, 2005; Erreger <i>et al.</i>, 2007; Hansen <i>et al.</i>, 2014). The responses of oocytes co-injected with these mutated subunits to 100 µM glutamate and 30 µM glycine were 8.6 ± 1.6 % (n = 15, GluN2A<sub>C1</sub> escape) and 1.8 ± 1.1 % (n = 12, GluN2C<sub>C1</sub> escape) of GluN1/GluN2A<sub>C1</sub>/GluN2C<sup>*</sup><sub>C2</sub> responses, indicating that approximately 90% of the NMDAR current was mediated by
triheteromeric receptors. Together, these data suggest that PYD-106 selectively enhances diheteromeric GluN1/GluN2C receptors.

We subsequently tested 18 additional PYD analogues that were active at diheteromeric receptors containing GluN2C to determine whether the requirement of two GluN2C subunits was a feature of the entire class of PYD compounds. None of the PYD analogues with activity at diheteromeric GluN1/GluN2C receptors enhanced the responses of triheteromeric GluN1/GluN2A/GluN2C* receptors, suggesting that the ability to distinguish between the GluN2 composition of the receptors was a property of this class of modulator (Supplemental Table S3).

**Mechanism of action of pyrrolidinones in GluN2C modulation**

We evaluated the voltage-dependence of PYD-106 positive allosteric modulation to assess whether there are interactions with either the pore or process of ion permeation. Evaluation of the current-voltage curve showed that the reversal potential of GluN1/GluN2C receptor responses in oocytes was identical in the absence (-6.6 ± 1.9 mV, n = 4) and presence (-6.7 ± 1.9 mV, n = 4) of 100 μM PYD-106. Modulation was voltage-independent, being 195 ± 4.2% of control at -40 mV and 236 ± 44% of control at +40 mV (n = 4, p > 0.05, t-test). The EC₅₀ value and degree of modulation by PYD-106 were compared in the absence and presence of 1 mM Mg²⁺, which exerts a voltage-dependent block of the channel. The EC₅₀ for PYD-106 modulation in the absence of Mg²⁺ was 17 ± 1.2 μM (n = 7), only modestly different from the EC₅₀ observed in the presence of 1 mM Mg²⁺ (23 ± 1.2 μM, n = 7, p < 0.05, t-test). These data suggest that the actions of PYD-106 are largely voltage-independent.
In order to gain more insight into the functional mechanism by which PYD-106 enhances GluN1/GluN2C responses, we evaluated the time course of macroscopic current responses recorded from NMDARs transiently expressed in HEK cells. PYD-106 increased the GluN1/GluN2C whole cell current responses to maximally effective concentrations of glutamate (100 μM) and glycine (30 μM) with an EC50 of 13 ± 1.0 μM and a Hill slope of 1.30 ± 0.04 (n = 10, Figure 3A,D). Selective modulation of GluN2C-containing NMDARs activated by a maximally-effective concentration of glutamate and glycine was observed upon addition of 50 μM PYD-106 (224 ± 4.5% of control, n = 6), compared to weak inhibition of GluN1/GluN2A (88 ± 2.7% of control, n = 5), GluN1/GluN2B (81 ± 1.2% of control, n = 6), and GluN1/GluN2D (81 ± 1.0% of control, n = 6) NMDARs expressed in HEK cells. Following rapid removal of glutamate in the presence of 50 μM PYD-106, the time constants for a dual exponential function fitted to the current deactivation time course (τfast = 67 ± 17 ms, %fast = 83 ± 3%, τslow = 260 ± 32 ms, n = 5) were not detectably different from those observed in the absence of PYD-106 (τfast = 62 ± 15 ms, %fast = 74 ± 10%, τslow = 295 ± 2 ms, Figure 3B,C, n = 5, paired t-test, p > 0.05). There was no significant effect on the relative proportion of the two components. However, τweighted was slightly slower in the presence of PYD-106 (τweighted = 255 ± 9 ms) compared to control (τweighted = 204 ± 8 ms, n = 5, paired t-test, p < 0.05), perhaps reflecting a combined effect of slightly slower tau and more prominent slow component.

The time course for the onset of PYD-106 enhancement of GluN1/GluN2C was rapid (τONSET = 9.0 ± 1.6 ms at 100 μM, n = 6-9), and could be well-described by a single exponential function (Figure 3E). We determined the upper limit of the solution
exchange time around the cell by measuring the leak current during exchange of NaCl with KCl (Mott et al., 2001; Erreger & Traynelis, 2005). The time constant describing solution exchange was 2.3 ± 0.3 ms, 4-fold faster than the onset of PYD-106 effects at 100 μM (Figure 3E). This indicates that solution exchange was not a limiting step in assessing the association (k_{on}) and dissociation (k_{off}) rates of PYD-106. The reciprocal of the time constant for modulation of PYD-106 was linearly related to the concentration, with a slope corresponding to k_{on} of 9.84 x 10^{5} M^{-1}s^{-1}. The dissociation rate k_{off} was estimated from k_{off} = 1/τ_{recovery} to be 32 s^{-1}, which was similar to that determined from the intercept of the relationship between concentration and 1/τ_{ONSET} (k_{off-intercept} = 29 ± 0.6 s^{-1}). The dissociation constant (K_{D}) for PYD-106 was estimated from k_{off} / k_{on} to be 30 μM (n = 6-9, Figure 3F).

We subsequently evaluated the single channel mechanism of PYD-106 positive allosteric modulation of GluN1/GluN2C-containing NMDAR responses by analyzing individual single channel openings recorded from excised outside-out patches (Figure 4). Outside-out patches containing rat recombinant GluN1/GluN2C receptors were activated by 100 μM glutamate and 30 μM glycine in the absence and presence of 100 μM PYD-106. Because all patches contained more than one active channel, data analysis was restricted to openings of individual channels. Two sublevels for the unitary currents were observed under both the control conditions (2.58 ± 0.11 and 3.31 ± 0.16 pA) as well as in the presence of PYD-106 (2.40 ± 0.25 and 3.30 ± 0.16 pA; Table 1). The chord conductance levels in the absence and presence of drug were not significantly different (Table 1; n = 4, p < 0.05, paired t-test). The product of the number of channels in the patch and open probability (nP_o) under control conditions (0.032 ±
0.015) was significantly increased in the presence of 100 µM PYD-106 (0.067±0.024, n = 4, p < 0.05, paired t-test). The observed increase can be partly attributed to a significant increase in the mean open time (0.35 ± 0.07 ms to 0.48 ± 0.07 ms) in the presence of drug (n = 4, p < 0.05, paired t-test). This change in mean open time reflects a shift towards longer duration openings, rather than a change in the time constants describing the dual component open time histogram (Figure 4B, Table 1). These data suggest that PYD-106 stabilizes the open state of the receptor. Opening frequency was 0.086 ± 0.045 Hz in control and 0.159 ± 0.069 Hz in the presence of PYD-106 (n = 4, Table 1).

**Interaction of pyrrolidinones with known modulator sites**

We recently described three new classes of nonselective allosteric modulators, which also act on the GluN1/GluN2C receptor, exemplified by CIQ (Mullasseril *et al.*, 2010), QNZ-46 (Mosley *et al.*, 2010) and DQP-1105 (Acker *et al.*, 2011). CIQ enhances GluN1/GluN2C receptor function through actions at the GluN2 pre-M1/M1 helices (Ogden & Traynelis, 2013), whereas QNZ-46 and DQP-1105 inhibit GluN1/GluN2C function through actions that apparently involve residues within the GluN2 S2 region of the ligand binding domain (Acker *et al.*, 2011; Hansen & Traynelis, 2011). To assess whether PYD-106 might interact with either of these two sites or downstream mechanisms, we tested if positive allosteric modulation by PYD-106 would still be observed in the presence of maximally effective concentrations of CIQ, QNZ-46, or DQP-1105 on oocytes expressing the GluN1/GluN2C receptor. If the mechanisms of allosteric modulation for PYD and CIQ are independent, the effects of CIQ (8 µM) or
PYD-106 (100 μM) measured individually should add to give a combined enhancement of the maximal response of 318% of control. The measured modulation by co-application of CIQ and PYD-106 was 340 ± 14%, which was not significantly different from the estimated modulation of 318% (one way ANOVA), suggesting that CIQ and PYD-106 do not compete for the same molecular determinants or share downstream mechanisms of action (Supplemental Figure S2). To further evaluate the possibility that CIQ and PYD-106 might share a portion of their respective binding sites, we mutated T578I in the GluN2C M1 domain, which is equivalent to the GluN2D T592I mutation that eliminates CIQ modulation (Mullasseril et al., 2010). We found that the mutation GluN2C(T578I) eliminates the actions of CIQ on the GluN1/GluN2C receptor (104 ± 2.0%, n = 4), but has no effect on PYD-1 (100 μM) enhancement of the maximal response (249 ± 11.3% of control, n = 4, p < 0.05).

A similar approach was used to estimate the inhibition of QNZ-46 or DQP-1105 on oocytes when co-applied with PYD-106. The response when QNZ-46 (20 μM) or DQP-1105 (20 μM) were co-applied with PYD-106 (100 μM) as a percent of control should be 108% and 46% if these compounds do not interact. Consistent with this idea, we observed 108 ± 2.3% and 39 ± 3.5% modulation compared to control for QNZ-46 or DQP-1105 in the presence of PYD-106, respectively (p > 0.05 for both, one way ANOVA with Tukey’s post hoc test). Together, these data suggest that the structural determinants of PYD positive allosteric modulation are distinct from the elements required for CIQ, QNZ-46, and DQP-1105 modulation (Supplemental Figure S2).

Only one other ligand, D-cycloserine (DCS), is known to show selectivity in its actions as a co-agonist for NMDARs at the glycine site for GluN1/ GluN2C over
GluN1/GluN2A, GluN1/GluN2B, and GluN1/GluN2D (Sheinin et al., 2001; Dravid et al., 2010). A maximally effective concentration of DCS (100 μM) induces more current at GluN1/GluN2C than a maximally effective concentration of glycine (I_{DCS}/I_{GLYCINE}=180 ± 4.9%, n=6). By contrast, a maximally effective concentration of DCS produces a smaller response than glycine at GluN1/GluN2A, GluN1/GluN2B, GluN1/GluN2D receptors (Sheinin et al., 2001). We recently described structural determinants of the subunit-selective actions of DCS at the dimer interface between GluN1 and GluN2C ligand binding domains (Dravid et al., 2010). To assess whether DCS and PYD share either common structural determinants or similar mechanisms downstream of binding, we determined whether the actions of the PYD analogues and DCS at GluN1/GluN2C were additive. In this experiment, PYD-106 (100 μM) applied in the presence of both glutamate (100 μM) and DCS (100 μM, a saturating concentration) increased the maximal response to 172 ± 8.5% (n = 6). This reflects only a slight decrease of PYD-106 modulation in DCS compared to glycine evaluated in the same oocytes (207 ± 5.7% of control, n = 6, paired t-test, p < 0.05). Thus, the actions of PYD-106 and DCS are largely additive, suggesting they do not share an overlapping site or mechanism of action.

Molecular determinants of pyrrolidinones.

We constructed a series of GluN2A-GluN2C chimeric subunits in order to identify the regions of GluN2C necessary for pyrrolidinone modulation (Supplemental Table S1). For this extensive mutagenesis screen, we used a close analogue of PYD-106 that can be more readily synthesized (PYD-1, EC_{50} 19 ± 1.1 μM), which increased the maximal
response to 231 ± 10% of control (n=14). *Xenopus laevis* oocytes were used to express and test whether the chimeric receptors show altered responses to 100 μM PYD-1 compared to wild type receptors. Figure 5A shows that chimeras GluN2A(2C ATD), GluN2A(2C ATD-L0), and GluN2A(2C L0-S2) did not transfer modulation of PYD-1 to the GluN2A subunit. By contrast, the gain-of-function GluN2A(2C ATD-L0-S1) chimera was sensitive to PYD-1 (151 ± 2.2% of control, n = 8), suggesting that the ATD, L0, and S1 domains are required for PYD-1 modulation. To determine if all three regions are necessary, potential loss-of-function chimeras were generated individually by substituting the ATD, L0, and S1 of the GluN2A subunit into GluN2C. Replacement of the GluN2C ATD or S1 individually with the corresponding region from GluN2A eliminated modulation of GluN2C by PYD-1. Substitution of GluN2A L0 into GluN2C significantly decreased modulation (Figure 5B). These data suggest that the ATD and S1 domains of GluN2C-containing receptors might harbor a binding pocket for PYD-1.

To identify the residues necessary for modulation, 61 GluN2C point mutations were constructed in the ATD, L0 and S1 domains of the GluN2C subunit (Table 2). Sequence alignment of the GluN2A/C/D subunits was used to identify residues that were candidates for mutation on the basis of unique identity in GluN2C or a position near or within L0 (i.e. the ATD-S1 linker) (Figure 5C). Point mutations S393F, R401N and K467S significantly decreased modulation by 50 μM PYD-1 (123 ± 1.5% of control, 148 ± 3.2% and 164 ± 2.2%, respectively, ANOVA p < 0.05, n = 7-11). Interestingly, mutations K470G and S472T eliminated all effects of PYD-1. Several additional mutations (Q395D, V397E, V468R, V469I) significantly enhanced the effects of PYD-1, which increased the maximal responses up to 301-324% (n = 4-8, ANOVA, p < 0.0001,
Table 2). Evaluation of the PYD-1 concentration-effect curves showed that the S393F and R401N mutations decreased the potency to 90 ± 9.5 and 36 ± 0.7 μM and decreased the enhancement of the maximal current response to 117 ± 1.2 % and 136 ± 1.5 %, respectively.

The glutamate EC50 for wild-type GluN1/GluN2C receptors is 0.75 ± 0.08 (n = 5), which is not significantly different from that determined for the GluN2C mutations S393F (0.68 ± 0.08 μM, n = 3), R401N (0.90 ± 0.05 μM, n = 7), and S472T (1.0 ± 0.1 μM, n = 8), but is modestly different from K470G (1.2 ± 0.1 μM, n = 7, p < 0.05 ANOVA, Dunnett’s post hoc, Figure 5F). The glycine EC50 for wild-type GluN1/GluN2C receptors is 0.23 ± 0.01 (n = 8), which is not significantly different from S393F (0.29 ± 0.01 μM, n = 3), R401N (0.21 ± 0.01 μM, n = 7), K470G (0.22 ± 0.03 μM, n = 6), or S472T (0.26 ± 0.02 μM, n = 6, ANOVA post hoc Dunnett’s; Figure 5F). Thus, these mutations do not cause substantive changes to agonist potency or receptor function.

Because K470G and S472T eliminated the effects of PYD analogues, we substituted additional residues at these positions to further evaluate the effects of side chain composition, size, polarity and hydrogen bonding capability. The GluN2C mutations K470D, K470T, K470W, S472R and S472E all blocked PYD allosteric modulation completely (Table 2). Only one mutation (S472A) permitted minimal modulation (134 ± 2.8% of control, n = 6-8), suggesting that these two residues are critically positioned to control the actions of PYD analogues.

We subsequently evaluated this region in a homology model of GluN1/GluN2C built from the GluN1/GluN2B crystal structure, which is thought to represent an inhibitory conformational state of the receptor (see Data Supplement) (Karakas &
Furukawa, 2014; Lee et al., 2014). This suggests the structure of the modeled GluN1/GluN2C receptor may also represent a similar inactive conformation. The modeled structure shows extensive interactions between the ATD and the S1 region of the ligand binding domain, which our mutagenesis data suggest harbors the PYD binding site (Figure 6AB). Inspection of the structure between the ATD and S1 portion of the ligand binding domain revealed a small cavity of sufficient volume to act as a binding site for PYD analogues. A total of 35 residues form a pocket located between the ATD (R2) and LBD (S1) domains (Karakas and Furukawa, 2014), which shows 43% conservation between the GluN2A-D subunits. Interestingly, 10 of 24 of residues (44%, Table 2) that were found to markedly alter the modulation by PYD analogues in the mutagenesis screen formed part of the lining of this pocket (Figure 6C). Five additional mutations with significant effects resided in the nearby ATD-S1 linker within 12-14 Å of the cavity. These data support the idea that this cavity is involved in the actions of PYD.

To test whether PYD modulators can fit within this newly identified pocket, we docked PYD-106 into this site on the GluN2C subunit (Figure 6D). The resulting docking pose was then used to identify additional residues within the pocket that could be central to the binding of PYD-106. The docked pose suggested that the mutation GluN2C R194D should perturb PYD-106 binding, and thus we examined the effects of this mutation on PYD106 modulation. We observed that GluN1/GluN2C R194D nearly eliminated the actions of PYD-106 (112 ± 1.1%, n = 8). These data further support the identification of a binding pocket in the homology model of GluN2C based on the crystal structure of the GluN2B subunit (Karakas & Furukawa, 2014; Lee et al., 2014).
Discussion

We describe here the site and mechanism of action of the first class of selective positive allosteric modulators of GluN2C-containing NMDARs, which do not enhance the maximal response at GluN2A, GluN2B or GluN2D. We also show this class of modulator has the unique ability to sense the composition of the tetrameric assembly, acting on receptors with two copies of GluN2C but not receptors with a single copy of GluN2C. Furthermore, the compound series shows minimal activity when residues encoded by the alternative exon-5 are included in the GluN1 ATD. The subunit selectivity of this class of compounds suggests it should be useful in identifying NMDARs that lack GluN1 exon-5 and contain two copies of GluN2C throughout the CNS.

Recently, several new NMDAR modulators with novel candidate binding sites have been identified (Ogden & Traynelis, 2011; Monaghan et al., 2012). Among these, the GluN2C/D potentiator CIQ and related tetrahydroisoquinolines have been proposed to interact with the extracellular end of the M1 transmembrane domain and pre-M1 cuff helix (Ogden et al., 2013b). The GluN2C/D inhibitors QNZ46 and the DQP series appear to involve residues in the GluN2 S2 region of the agonist binding domain (Acker et al., 2011; Hansen & Traynelis, 2011). TCN-201 binds within the dimer interface between GluN1 and GluN2A agonist binding domains (Hansen et al., 2012). In addition, ifenprodil is a GluN2B-selective negative allosteric modulator that binds at the GluN1-GluN2B ATD heterodimer interface (Karakas & Furukawa, 2014). Several classes of positive and negative UBP ligands appear to interact with the ligand binding domain (Costa et al., 2010). The neurosteroid pregnanolone sulfate inhibits all NMDARs, with
the highest potency for GluN2C- and GluN2D-containing receptors (Malayev et al., 2002; Petrovic et al., 2005). By contrast, pregnenolone sulfate shows positive allosteric modulation of GluN2A- and GluN2B-containing NMDARs, and negative allosteric modulation of GluN2C- and GluN2D-containing NMDARs (Malayev et al., 2002).

Pregnenolone sulfate enhancement of GluN2B receptor function has been suggested to involve helices J/K in the S2 portion of the ligand binding domain in addition to residues near the M4 transmembrane helix (Jang et al., 2004). Negative allosteric modulation by pregnanolone sulfate has been suggested to involve the S2 region of the ligand binding domain (Petrovic et al., 2005). Thus, the molecular determinants of both positive and negative allosteric regulation of NMDAR function by neurosteroids are distinct from regions of the receptor proposed to interact with PYD (e.g. Figure 6). In addition, our data suggest that the selectivity and structural determinants for the PYD class of NMDAR modulators are distinct from other known modulators.

The potential site for PYD function at the S1 – ATD interface has several interesting features. For example, the ATD-S1 linker region is only weakly conserved among GluN2 subunits, and not previously known as a site of action for any modulator active within the glutamate receptor family. The site clearly exists in three crystal structures independently solved (Karakas & Furukawa, 2014; Lee et al., 2014), and may well be a feature of the subunit family, raising the possibility that new classes of ligands may act at this pocket in other GluN2 subunits. The relatively fast time course for the onset and recovery from positive allosteric modulation suggests that the extracellular binding site for PYD is readily accessible. Moreover, the region between the ATD and LBD is well positioned to influence a wide range of channel properties including agonist
EC$_{50}$, deactivation time course and open probability (Gielen et al., 2009; Yuan et al., 2009; Hansen et al., 2013).

Within this new potential site, our data suggest specific roles for several of the residues lining the pocket. The interactions described here are those proposed for the $R$ enantiomer of PYD-106, since its docking pose shows a more consistent correlation with the mutational data than the $S$ enantiomer (not shown). Whereas the two enantiomers show significantly different biological activities (Zimmerman et al., 2014), the absolute configuration of the most active enantiomer has yet to be determined. Our models show that Ser472 (GluN2C) forms a hydrogen bond with His402, which in turn hydrogen bonds with Asp474, perhaps stabilizing the ATD and LBD interface. Moreover, these interactions may allow hydrogen bond formation between adjacent residues Tyr473 and Ile475 and the acetyl and methyl ester moieties of PYD-106, respectively. The backbone of three residues at which mutations perturb PYD-106 modulation (Pro428, Asn429 and Thr430) form part of the binding pocket of the indole moiety in PYD-106. The latter is predicted to form a hydrogen bond with Pro428, which is retained in models of GluN2C P428R, a result that may explain the modest reduction in potency observed with this mutation. The similar modest decrease in modulation for GluN2C N429D might be explained by the loss of hydrogen bonding with Asp460 of the LBD. GluN2C A466S also reduces PYD modulation modestly, perhaps by influencing the orientation of the phenol ring of Tyr473. Interestingly, one of the 10 GluN2C mutations lining the pocket (V427I) increases the degree of modulation, which we speculate may alter the interaction between the ATD and LBD due to steric effects of the larger Ile side chain. The loss of PYD modulation by mutating Lys470 could be due
to a loss of its interaction with Asp220 (ATD), which we suggest may stabilize the ATD
and LBD interface. It will be important in the future to determine the absolute
configuration of the more active enantiomer of PYD-106 as well as details of its binding
interactions within the ATD-LBD domain interface of GluN2C.

In terms of mechanism, single channel analysis of the GluN1/GluN2C shows that
PYD-106 increases the open probability and mean open time. This suggests that the
compound increases receptor-mediated currents and agonist efficacy in part by
stabilization of the open state, which prolongs the channel open periods. Enhancement
of receptor open frequency and open duration by molecules binding to the ATD-LBD
interface is consistent with emerging understanding of control of receptor function by the
ATD (Hansen et al., 2010; Zhu et al., 2013). The strong influence that the ATD has on
NMDAR gating can also now be understood through the extensive contacts between
the ATD and ligand binding domain, as observed in the NMDAR crystal structure
(Karakas & Furukawa, 2014; Lee et al., 2014). It is intriguing that the PYD modulators
that act at this site require two GluN2C subunits to be present in the receptor complex.
It remains unclear whether this reflects the need for modification of the receptor
conformation driven by the binding of molecules at two sites across the region of two-
fold symmetry, or whether the nature of the pocket itself is perturbed when a GluN2
subunit other than GluN2C is present in the receptor complex. This observation
emphasizes the need to consider the actions of allosteric modulators on triheteromeric
receptors, and provides a new precedent illustrating the potential for new
pharmacological probes that sense receptor stoichiometry. Such probes could become
useful tools for evaluating receptor composition. For example, we predict that PYD will
not be active at cerebellar granule neurons, which express GluN1 exon-5 (Laurie & Seeburg, 1994b; Laurie et al., 1995) in addition to GluN1/GluN2A/GluN2C triheteromeric receptors (Chazot et al., 1994; Cathala et al., 2000; Lu et al., 2006). However, this probe could be used to assess subunit stoichiometry of receptors containing GluN2C in other neurons that lack GluN1 exon-5. In summary, the identification of the site and mechanism of action of this series at the ATD-LBD interface should spur new work evaluating whether this site in other NMDAR subunits can bind to allosteric modulators, and provides new tools with which to study neuronal NMDAR subunit composition.
Acknowledgements

We thank the Custom Cloning Core Facility at Emory University for constructing a subset of the chimeras and point mutations reported used in this study, and Phuong Le, Jing Zhang, and Anel Tankovic for excellent technical assistance.
Authorship Contributions

Participated in research design, Khatri, Burger, Swanger, Hansen, Zimmerman, Karakas, Liotta, Furukawa, Snyder, Traynelis

Conducted experiments, Khatri, Burger, Hansen, Swanger, Zimmerman, Karakas

Contributed new reagents or analytic tools, Zimmerman, Hansen, Liotta, Karakas, Furukawa

Performed data analysis, Khatri, Burger, Snyder, Traynelis

Wrote or contributed to the writing of the manuscript, Khatri, Burger, Swanger, Hansen, Zimmerman, Karakas, Liotta, Furukawa, Snyder, Traynelis
References


Footnotes

This work was supported by the National Institutes of Health [NS065371], National Institute of Mental Health [MH094525], National Institute of General Medicine [GM103546, GM105730], National Institute of Neurological Disorders and Stroke [NS078873], and the Michael J Fox Foundation.

Additional Information Competing financial interests: Several of the authors (DCL, SFT, SZ) are co-inventors on Emory University-owned intellectual property, board members (DCL) or paid consultants for companies developing NMDAR modulators (SFT, KBH).
Figure Legends

Figure 1. PYD-106 enhances the maximal current response of GluN1/GluN2C receptors. (A) Concentration-effect curves for glutamate are shown recorded in the same oocyte with and without 70 μM PYD-106 (n = 13 oocytes); 30 μM glycine was present in all solutions. All data in PYD-106 are expressed as a percentage of the response to 10 μM glutamate without PYD-106. The smooth curve is a fit of the Hill equation to the data

\[
Response(\%) = \frac{Maximal\ Response}{1 + \left(\frac{EC_{50}}{concentration}\right)^N}
\]

where EC_{50} is the concentration of agonist that produces half of the Maximal Response and N is the Hill slope. The fitted maximal response was increased by PYD-106 to 236%. (B) The concentration-effect curves were normalized to the responses at 10 μM glutamate for each and superimposed to more clearly illustrate the shift in glutamate EC_{50}, which was increased by PYD-106 from 0.72 ± 0.05 μM (Hill slope 1.45 ± 0.03) in the absence of PYD-106 to 1.17 ± 0.08 μM (Hill slope 1.39 ± 0.02) in the presence of 70 μM PYD-106 (p < 0.05; t-test).

Figure 2.

Subunit-selectivity of PYD-106. (A) Two-electrode voltage-clamp recordings are shown from Xenopus oocytes expressing recombinant NMDARs, which were activated by 100 μM glutamate and 30 μM glycine in the presence of PYD-106 (1, 3, 10, 30, 100 μM). (B) Composite concentration-effect curves are shown for PYD-106 at GluN1/GluN2A, GluN1/GluN2B, GluN1/GluN2C, and GluN1/GluN2D receptors. (C) PYD-106 is less effective for receptors expressing GluN1-exon5. GluN1-1b K211G and GluN1-1b
K192G,K193G,R194G (1b-KKR) did not fully restore PYD modulation of GluN1/GluN2C receptors, whereas the triple mutation GluN1-1b K207G,R208G,K211G (1b-KRK) fully restored PYD-106 modulation. (D) The ratio of the response to maximally effective concentrations of glutamate (100 µM) and glycine (30 µM) with 50 µM PYD-106 compared to the maximal response in the absence of PYD (expressed as percent) was increased in *Xenopus* oocytes expressing diheteromeric GluN1/GluN2C receptors (2C*C1 + 2C*C2, see Methods). Maximal responses were slightly reduced in oocytes expressing diheteromeric GluN1/GluN2A receptors (2A*C1 + 2A*C2) and triheteromeric GluN1/GluN2A/GluN2C receptors (2A*C1 + 2C*C2; *p* < 0.001, n = 6-8 cells, one-way ANOVA, post hoc Tukey's test. (E) 30 µM PYD-106 was tested for off-target effects at wild-type NMDA, AMPA (GluA), kainate (GluK), γ-aminobutyric acid (GABA), nicotinic acetylcholine (nACh), glycine (gly), and purinergic receptors (P2X), which were activated by their respective agonists (see Methods for concentrations). The ratio of the maximal current response in the presence and absence of PYD-106 is expressed as percent. Modest inhibition observed at some receptors was statistically significant. (F) The structure is shown for PYD-1(R=H) and PYD-106 (R=CH₃).

**Figure 3.** Mechanism of action of PYD modulation (A) Whole-cell recordings of HEK cells expressing GluN1/GluN2C receptors activated with 100 µM glutamate and 30 µM glycine showed concentration-dependent modulation by PYD-106. (B) Normalized current responses are superimposed to show the deactivation time course following removal of glutamate in the absence (black trace) and presence (gray trace) of PYD-106. (C) The deactivation time constant was fit with a dual exponential function. The
time constants describing the independent fast and slow component were not
significantly different in the presence or absence of PYD-106 (n = 5, t-test, p > 0.05),
although the mean tau weighted by the relative amplitude of each tau was significantly
faster in the presence of PYD-106 (n = 5, t-test, p < 0.05). (D) Composite
centration-effect curve for PYD-106 enhancement of maximal response in HEK cells
transiently expressing the GluN1/GluN2C receptor. (E) Expansion of the onset of PYD-
106 enhancement of the response at 10, 30, and 60 μM illustrates the normalized
centration-dependent time course. The dashed line is the fluid exchange rate around
the cell, determined by switching between 150 mM NaCl with 150 mM KCl. Exchange
time around a cell had a tau of 2.3 ± 0.3 ms with a 10-90% rise-time of 3.4 ± 0.3 ms (n =
5), 3.8-fold faster than the onset of current increase at 100 μM PYD-106. (F) τONSET and
τRECOVERY were used to estimate the k_on and k_off, and K_D for PYD-106 was determined as
k_off / k_on. The linear relationship between concentration and 1/τONSET suggest that the
compounds act directly on the receptor rather than, for example, through a multi-step
intracellular signaling pathway.

**Figure 4.** Representative excised outside-out patch recordings of HEK293 cells
expressing the GluN1/GluN2C NMDAR activated by 100 μM glutamate and 30 μM
glycine in the absence and presence of 100 μM PYD-106. The highlighted region is
expanded below demonstrating the open (o) and closed (c) levels of the recordings. (B)
The composite distributions of all the open and closed periods from four patches for the
control (black) and for PYD-106 (gray) were fitted with two (open times) and five (shut
times) exponentials components (Table 1).
Figure 5. Structural determinants of PYD modulation. (A) Diagram of the gain-of-function (GoF) and loss-of-function (LoF) chimeric GluN2 subunits that were evaluated. (B) The change in the response to 100 μM glutamate and 30 μM glycine by 100 μM PYD-1 is shown as a percent of the control response (taken as 100%). The data from the chimera subunits suggest that the ATD, L0, and S1 regions of the GluN2C subunit are required for PYD modulation (*p < 0.05 compared to GluN2A, ANOVA, Dunnett’s post hoc, number of oocytes in parentheses). (C) Sequence alignment of the GluN2C, GluN2A, and GluN2D subunits illustrates the identity between the three subunits. A total of 64 mutations were constructed in the ATD, L0, and S1 regions, and the effects of each mutation on PYD-1 modulation is indicated by a “+” (p < 0.05, ANOVA) or “−” (not statistically significant). All tested mutations except for R194D, E341D, G342N, and F345L are indicated. (D) The modulation by 100 μM PYD-1 of the response in oocytes to 100 μM glutamate and 30 μM glycine is shown as a percent of control for wild type GluN1/GluN2C and GluN2C mutants that showed the strongest effects (p<0.05 against wild type GluN2C, ANOVA, post hoc Dunnett’s). Table 2 gives the degree of PYD-1 modulation observed for all mutations. (E) PYD-1 concentration-effect curves show that S393F and R401N decreased the maximum modulation and PYD-1 EC$_{50}$, whereas K470G and S472T mutations caused a complete loss of modulation by PYD-1 at all concentrations tested. (F) Glutamate and (G) glycine concentration-effect curves for these same mutations show minimal or no changes in EC$_{50}$ values.
Figure 6. Site of action of PYD positive allosteric modulators. (A,B) A homology model of the GluN1/GluN2C receptor was based on the GluN1-1a/GluN2B crystal structure (see Data Supplement) (Karakas and Furukawa, 2014, see Methods). (C) A depiction of residues in close proximity to the proposed PYD binding pocket of GluN2C residing between the ATD and LBD. Residues in blue and green were identified using a mutational screen. Blue colored residues represent mutations that significantly decreased PYD modulation of the maximal current response. Residues colored green increased modulation by PYD of the maximal current response. The residue colored red was selected from PYD binding poses to test; mutations at this site significantly decrease the effect of PYD. (D) A docking pose of PYD-106 in the binding pocket is shown. Panels (C) and (D) are viewed from the same angle.

PDB. Homology Model of the GluN2C subunit based on the GluN2B crystal structure (See Karakas & Furukawa, 2014).
### Table 1. Effects of PYD-106 on single channel properties

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>+ 100 μM PYD-106</th>
</tr>
</thead>
<tbody>
<tr>
<td>nPo</td>
<td>0.032 ± 0.015</td>
<td>0.067 ± 0.024*</td>
</tr>
<tr>
<td>nPo (% control)</td>
<td>100</td>
<td>257 ± 45 %*</td>
</tr>
<tr>
<td>Opening frequency (Hz)</td>
<td>0.086 ± 0.045</td>
<td>0.159 ± 0.069</td>
</tr>
<tr>
<td>Opening frequency (% control)</td>
<td>100</td>
<td>217 ± 34 %*</td>
</tr>
<tr>
<td>Mean shut time</td>
<td>24.3 ± 10.6</td>
<td>10.0 ± 3.2</td>
</tr>
<tr>
<td>Mean open time</td>
<td>0.35 ± 0.07</td>
<td>0.48 ± 0.07*</td>
</tr>
<tr>
<td>Open tau 1, ms (%)</td>
<td>0.19 ± 0.07 (39)</td>
<td>0.25 ± 0.11 (42)</td>
</tr>
<tr>
<td>Open tau 2, ms (%)</td>
<td>0.46 ± 0.10 (61)</td>
<td>0.65 ± 0.16 (58)</td>
</tr>
<tr>
<td>Amplitude 1 (pA)</td>
<td>2.58 ± 0.11</td>
<td>2.40 ± 0.25</td>
</tr>
<tr>
<td>Amplitude 2 (pA)</td>
<td>3.31 ± 0.16</td>
<td>3.30 ± 0.16</td>
</tr>
<tr>
<td>Conductance 1 (pS)</td>
<td>32.3 ± 1.4</td>
<td>29.9 ± 3.1</td>
</tr>
<tr>
<td>Conductance 2 (pS)</td>
<td>41.4 ± 2.0</td>
<td>41.3 ± 2.0</td>
</tr>
</tbody>
</table>

All values are mean ± s.e.m, * p < 0.05, paired t-test. All patches contained 2 or more channels (n=4 patches). Intervals either adjacent to or containing a double opening were omitted from analysis. nPo is the product of the number of channels in the patch and the open probability for an individual channel, which is the probability of at least one channel being open.
Table 2. Structural determinants of PYD modulation

<table>
<thead>
<tr>
<th>GluN2C mutation</th>
<th>( \frac{I_{\text{TEST}}}{I_{\text{CONTROL}}} ) (%)</th>
<th>n</th>
<th>GluN2C mutation</th>
<th>( \frac{I_{\text{TEST}}}{I_{\text{CONTROL}}} ) (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>247 ± 2.3</td>
<td>63</td>
<td>P428R#</td>
<td>208 ± 6.0*</td>
<td>8</td>
</tr>
<tr>
<td>R194D#</td>
<td>112 ± 1.1*</td>
<td>8</td>
<td>N429D#</td>
<td>182 ± 8.0*</td>
<td>5</td>
</tr>
<tr>
<td>E341D</td>
<td>217 ± 4.7</td>
<td>4</td>
<td>T430S#</td>
<td>203 ± 3.2*</td>
<td>7</td>
</tr>
<tr>
<td>G342N</td>
<td>259 ± 4.2</td>
<td>4</td>
<td>R435K</td>
<td>244 ± 3.8</td>
<td>5</td>
</tr>
<tr>
<td>F345L</td>
<td>262 ± 1.4</td>
<td>3</td>
<td>Q436F</td>
<td>244 ± 8.1</td>
<td>6</td>
</tr>
<tr>
<td>M371V</td>
<td>263 ± 5.4</td>
<td>7</td>
<td>D537V</td>
<td>274 ± 16</td>
<td>4</td>
</tr>
<tr>
<td>R374S</td>
<td>267 ± 5.7</td>
<td>6</td>
<td>N438D</td>
<td>244 ± 9.5</td>
<td>5</td>
</tr>
<tr>
<td>D376E</td>
<td>239 ± 6.1</td>
<td>8</td>
<td>H377N</td>
<td>258 ± 3.9</td>
<td>8</td>
</tr>
<tr>
<td>H377N</td>
<td>276 ± 7.9*</td>
<td>6</td>
<td>T440N</td>
<td>242 ± 4.7</td>
<td>3</td>
</tr>
<tr>
<td>G378Q</td>
<td>238 ± 7.2</td>
<td>6</td>
<td>F441N</td>
<td>250 ± 4.0</td>
<td>5</td>
</tr>
<tr>
<td>V379T</td>
<td>229 ± 9.9</td>
<td>6</td>
<td>S443T</td>
<td>262 ± 6.7</td>
<td>6</td>
</tr>
<tr>
<td>Y381S</td>
<td>263 ± 11.2</td>
<td>6</td>
<td>G444N</td>
<td>238 ± 7.7</td>
<td>8</td>
</tr>
<tr>
<td>M382L</td>
<td>242 ± 5.4</td>
<td>4</td>
<td>D445N</td>
<td>257 ± 6.2</td>
<td>6</td>
</tr>
<tr>
<td>K383R</td>
<td>217 ± 6.9*</td>
<td>6</td>
<td>L446E</td>
<td>242 ± 6.5</td>
<td>6</td>
</tr>
<tr>
<td>S391K</td>
<td>277 ± 2.8</td>
<td>3</td>
<td>T447G</td>
<td>283 ± 6.9*</td>
<td>6</td>
</tr>
<tr>
<td>T392S</td>
<td>273 ± 7.1</td>
<td>7</td>
<td>P448M</td>
<td>254 ± 6.3</td>
<td>4</td>
</tr>
<tr>
<td>S393F</td>
<td>123 ± 1.5*</td>
<td>7</td>
<td>Y449N</td>
<td>246 ± 10.0</td>
<td>5</td>
</tr>
<tr>
<td>L394S</td>
<td>252 ± 4.9</td>
<td>6</td>
<td>A466S#</td>
<td>184 ± 4.3*</td>
<td>6</td>
</tr>
<tr>
<td>Q395D</td>
<td>314 ± 9.4*</td>
<td>6</td>
<td>K467S#</td>
<td>164 ± 2.2*</td>
<td>1</td>
</tr>
<tr>
<td>P396C</td>
<td>215 ± 8.8*</td>
<td>5</td>
<td>V468R</td>
<td>324 ± 8.6*</td>
<td>6</td>
</tr>
<tr>
<td>V397E</td>
<td>301 ± 11.1*</td>
<td>8</td>
<td>V469I</td>
<td>322 ± 4.3*</td>
<td>4</td>
</tr>
<tr>
<td>V398P</td>
<td>257 ± 3.4</td>
<td>7</td>
<td>K470G#</td>
<td>97 ± 1.3*</td>
<td>7</td>
</tr>
<tr>
<td>S400D</td>
<td>194 ± 4.9*</td>
<td>12</td>
<td>S472T#</td>
<td>96 ± 1.6*</td>
<td>7</td>
</tr>
<tr>
<td>R401N#</td>
<td>147 ± 3.2*</td>
<td>9</td>
<td>R486K</td>
<td>275 ± 4.9*</td>
<td>8</td>
</tr>
<tr>
<td>T404S</td>
<td>233 ± 8.2</td>
<td>8</td>
<td>R488N</td>
<td>276 ± 5.3*</td>
<td>6</td>
</tr>
<tr>
<td>V405I</td>
<td>255 ± 4.5</td>
<td>8</td>
<td>G489N</td>
<td>257 ± 3.1</td>
<td>5</td>
</tr>
<tr>
<td>A406V</td>
<td>262 ± 3.1</td>
<td>8</td>
<td>Y499V</td>
<td>265 ± 3.3</td>
<td>7</td>
</tr>
<tr>
<td>R411A</td>
<td>238 ± 6.6</td>
<td>6</td>
<td>K501Q</td>
<td>281 ± 3.6*</td>
<td>8</td>
</tr>
<tr>
<td>S418D</td>
<td>253 ± 8.2</td>
<td>7</td>
<td>K470D#</td>
<td>89 ± 2.0*</td>
<td>8</td>
</tr>
<tr>
<td>P419I</td>
<td>190 ± 3.5*</td>
<td>10</td>
<td>K470T#</td>
<td>102 ± 4.0*</td>
<td>8</td>
</tr>
<tr>
<td>G422L</td>
<td>237 ± 4.2</td>
<td>8</td>
<td>K470W#</td>
<td>102 ± 1.3*</td>
<td>6</td>
</tr>
<tr>
<td>G424E#</td>
<td>189 ± 2.8*</td>
<td>5</td>
<td>S472A#</td>
<td>134 ± 2.8*</td>
<td>7</td>
</tr>
<tr>
<td>G425T</td>
<td>240 ± 2.5</td>
<td>8</td>
<td>S472R#</td>
<td>90 ± 3.1*</td>
<td>7</td>
</tr>
<tr>
<td>V427I#</td>
<td>294 ± 5.2*</td>
<td>6</td>
<td>S472E#</td>
<td>98 ± 2.2*</td>
<td>6</td>
</tr>
</tbody>
</table>

This article has not been copyedited and formatted. The final version may differ from this version.
Response as a percent of control are shown for 100 μM PYD-1 co-applied with 100 μM glutamate and 30 μM glycine * p < 0.05 by ANOVA, Bonferroni post-hoc. # Residues that form part of the proposed PYD-106 binding pocket and have a significant effect on PYD-106 modulation.
Figure 1
Figure 2

A

GluN1/GluN2A
GluN1/GluN2B
GluN1/GluN2C
GluN1/GluN2D

0.2 μA
1 min
0.2 μA
1 min
0.25 μA
1 min
0.3 μA
1 min

B

Response (% control)

PYD-106 (μM)

C

Response (% control)

PYD-106 (μM)

D

E

F

Response (% control)

PYD-106 (μM)

*