

## Title Page

### **Mechanism for non-competitive inhibition by novel GluN2C/D NMDA receptor subunit-selective modulators**

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**Running Title Page: Non-Competitive NMDA GluN2C/D Subunit-Selective Antagonists**

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**ABBREVIATIONS:** AMPA,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester; cDNA, complementary DNA; DMSO, dimethyl sulfoxide; HPLC, high performance liquid chromatography, IC<sub>50</sub>, concentration of a test compound that produces half maximal inhibition, K-BAPTA, potassium 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; NMDA, N-methyl-D-aspartate; 7-CKA 7-chlorokynurenic acid; BCD, (2-hydroxypropyl)- $\beta$ -cyclodextrin; DQP, dihydroquinilone-pyrazoline containing.

## Abstract

The compound 4-(5-(4-bromophenyl)-3-(6-methyl-2-oxo-4-phenyl-1,2-dihydroquinolin-3-yl)-4,5-dihydro-1*H*-pyrazol-1-yl)-4-oxobutanoic acid (DQP-1105) is a representative member of a new class of NMDA receptor antagonists. DQP-1105 inhibited GluN2C- and GluN2D-containing receptors with  $IC_{50}$  values that were at least 50-fold lower than those for recombinant GluN2A-, GluN2B-, GluA1-, or GluK2-containing receptors. Inhibition was voltage-independent could not be surmounted by increasing concentrations of either co-agonists glutamate or glycine, consistent with a non-competitive mechanism of action. DQP-1105 inhibited single channel currents in excised outside-out patches without significantly changing mean open time or single channel conductance, suggesting that DQP inhibits a pre-gating step without changing the stability of the open pore conformation and thus channel closing rate. Evaluation of DQP-1105 inhibition of chimeric NMDA receptors identified two key residues in the lower lobe of the GluN2 agonist binding domain that control the selectivity of DQP-1105. These data suggest a mechanism for this new class of inhibitors and demonstrate that ligands can access, in a subunit-selective manner, a new site located in the lower, membrane-proximal portion of the agonist binding domain.

## Introduction

The N-Methyl-D-Aspartate (NMDA) receptors belong to the family of ionotropic glutamate receptors that mediate the majority of excitatory neuronal transmission within the central nervous system (Traynelis et al., 2010). NMDA receptors comprise four subunits that combine to form functional ion channels. Each subunit folds into four semi-autonomous domains, including the amino-terminal domain, the ligand-binding domain, the transmembrane domain and the carboxy-terminal domain. Functional NMDA receptors are formed by the assembly of two GluN1 subunits that bind the co-agonist glycine together with two GluN2 subunits that bind the co-agonist glutamate. Four independent genes encoding GluN2 subunits have been identified (GluN2A-D) and account for differences in functional properties of the NMDA receptors, including deactivation time course, agonist potency, mean open time, and open probability (Chen and Wyllie, 2006; Dingledine et al., 1999; Paoletti and Neyton, 2007; Traynelis et al., 2010). The GluN2 subunit also controls the sensitivity of the receptor to  $Mg^{2+}$  block, proton inhibition, modulation by  $Zn^{2+}$ , as well as potency and efficacy of the endogenous agonists glutamate and glycine (Traynelis et al., 2010).

The spatial and temporal expression of the various GluN2-containing NMDA receptors has led to hypotheses concerning specific roles for the various NMDA receptor subunits in certain pathophysiological conditions, including Parkinson's disease and schizophrenia (Chen and Lipton, 2006; Hallett and Standaert, 2004). Despite considerable progress in our understanding of ionotropic glutamate receptor structure and function, the lack of subunit-selective pharmacological tools targeting NMDA receptors has impeded advances in understanding the roles of different GluN2-containing NMDA receptor subtypes both in normal brain function and in various disease states. Here we report a new structural class of antagonists, typified by 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 (DQP-1105), which is approximately 50-fold selective for NMDA receptors containing GluN2C or GluN2D subunits over GluN2A- or GluN2B-containing receptors. We determined the mechanism of action and identified the structural determinants of subunit-selectivity for DQP-1105. Identification of the structure-activity

relationship for DQP-1105, the structural determinants of subunit selectivity, and the mechanism of action of this new class of NMDA receptor antagonist will facilitate the development of pharmacological probes with increased selectivity among GluN2 subunits.

## Materials and Methods

### *Molecular Biology*

cDNAs for rat wild type NMDA receptor subunits GluN1-1a (GenBank U08261; hereafter GluN1), GluN2A (GenBank D13211), GluN2B (GenBank U11419), GluN2C (GenBank M91563), and GluN2D (GenBank L31611, see Monyer et al., 1994), GluA1 (GenBank X17184), GluK2 (GenBank Z11548) were provided by Drs. S. Heinemann (Salk Institute), S. Nakanishi (Kyoto University), and P. Seeburg (University of Heidelberg). cDNAs encoding the full open reading frames of human NMDA receptor subunits were assembled from pieces of cDNA obtained from the I.M.A.G.E. Consortium and a commercial source (Origene, Rockville, MD). High GC-content in the open reading frames of human GluN2C and GluN2D subunits was reduced in order to increase expression in *Xenopus* oocytes. The final cDNAs encode protein sequences corresponding to wild type human NMDA receptor subunits GluN1-1a (GenBank NP\_015566), GluN2A (GenBank NP\_000824), GluN2B (GenBank NP\_000825), GluN2C (intermediate between GenBank NP\_000826.1 [EPP insertion at amino acid position 1048 is present] and GenBank NP\_000826.2 [serine residue at position 1212 in NP\_000826.1 is an arginine as in NP\_000826.2]), and GluN2D (GenBank NP\_000827.1). Chimeric receptor subunits were constructed as previously described (Table 5) (Hansen and Traynelis, 2011; Mullasseril et al., 2010). Site-directed mutagenesis was accomplished using the Quikchange approach (Agilent Technologies, Santa Clara, CA). All DNA constructs were verified by sequencing (SeqWright, Houston, TX).

### *Cell Culture*

HEK 293 cells (CRL 1573; ATCC, Rockville, MD; hereafter HEK cells) were plated on glass coverslips (5 mm diameter, Warner Instruments, Hamden, CT) coated with 0.1 mg/ml poly-D-lysine.

HEK cells were maintained in 5% humidified CO<sub>2</sub> at 37°C in DMEM (Dulbecco's Modified Eagle Medium, Cat. No. 11960; Invitrogen) supplemented with 10% fetal bovine serum, 10 units/ml penicillin, and 10 µg/ml streptomycin. HEK cells were transiently transfected using the Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) with cDNAs encoding green fluorescent protein (GFP), GluN1, and GluN2A or GluN2D at a ratio of 1:1:1 and 0.5 µg/well total cDNA for 16-24 hours before whole cell voltage-clamp recordings were conducted, as previously described (Yuan et al., 2009). Following transfection, cells were incubated in media supplemented with NMDA receptor antagonists D,L-2-amino-5-phosphonovalerate (200 µM) and 7-chlorokynurenic acid (200 µM, 7-CKA). BHK-21 cells (CCL-10; ATCC, hereafter BHK cells) were maintained in DMEM (Cat. No. 11960; Invitrogen), 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mg/ml G418, 10 µg/ml blasticidin, 200 µM 7-chlorokynurenic acid, and 200 µM D-AP5.

#### *Ca<sup>2+</sup> imaging from BHK cells*

Experiments were performed as described previously (Hansen et al., 2008) with the following modifications. One day prior to the experiment, the cells were seeded in 20 µl of media at 600,000 cells/ml in black Corning CellBind 384-well plates with clear bottom using a Multidrop 384 Reagent Dispenser (Thermo Scientific). On the day of the experiment, the media was gently aspirated by a 384-format Biomek liquid handler (Beckman Coulter). The cells were then loaded with Fluo-4 NW (Invitrogen) dissolved in HEPES-buffered saline (HBS, Gibco Cat. No. 14175-053) with 2.5 mM (1 %) Probenecid, and 30 µM 7-chlorokynurenic acid for 60 minutes at 37° C in the dark. Cells were gently washed again with 30 µl/well using the same buffer without Fluo-4 dye, and placed in 20 µl/well buffer. The cell plates were then loaded into a FDSS7000 instrument (Hamamatsu) and real time recordings of changes in Fluo-4 emission performed (excitation 480 nm and emission 540 nm) at room temperature (20-22° C). After 10 seconds of baseline recordings, 10 µl/well of 3x concentrated test compound, controls, or assay buffer in HBS (pH 7.4) and 1 mM glycine (final concentration) were added. The high

glycine concentration will displace the 7-chlorokynurenic acid (Hansen et al., 2010b). After 2 minutes, an additional 10  $\mu$ l/well of a 4x concentrated EC<sub>80</sub> solutions of NMDA (110  $\mu$ M for GluN2A, 100  $\mu$ M for GluN2C, 30  $\mu$ M for GluN2D) were added; an EC<sub>100</sub> concentration of NMDA (1 mM) was used for GluN2B. Changes in fluorescence were subsequently recorded for 2 minutes. For determination of the concentration-response relationships, test compounds were 3-fold serial diluted over 10 concentration steps. Responses (fluorescence units, FU) were normalized to the first recording and expressed as percent of NMDA activation (no inhibition) and NMDA plus 100  $\mu$ M MK801 as 100% inhibition according to

$$\text{Inhibition (\%)} = ( 1 - ( \text{FU}_{\text{TEST}} - \text{FU}_{\text{MK801}} ) / ( \text{FU}_{\text{NMDA}} - \text{FU}_{\text{MK801}} ) ) * 100). \quad (1)$$

The IC<sub>50</sub> value was determined by non-linear least squares fitting as described below.

#### *Whole-cell and single channel patch-clamp recordings*

Whole-cell voltage-clamp recordings were conducted on transiently transfected HEK cells using an Axopatch 200B amplifier (Molecular Devices, Union City, CA, USA). Current responses were digitized at 40 kHz by pClamp10 software (Molecular Devices). Recordings were filtered at 8 kHz using an eight-pole Bessel filter (-3 dB; Frequency Devices, Haverhill, MD, USA). Thin-walled borosilicate glass capillary tubes (World Precision Instruments, Cat. No. TW150F-4, Hamden, CT, USA) were used to form recording micropipettes for whole cell currents; thick-walled borosilicate capillary tubes (Warner Instruments, Cat. No. 64-0793) were used to form micropipettes for single-channel recording. All recording micropipettes were filled with an internal solution containing (in mM) 110 D-gluconic acid, 110 CsOH, 30 CsCl, 5 HEPES, 5 BAPTA, 4 NaCl, 2 MgCl<sub>2</sub>, 2 NaATP, 0.5 CaCl<sub>2</sub>, and 0.3 NaGTP (pH 7.35). Cells were bathed at 23°C in external solution that contained (in mM) 150 NaCl, 10 HEPES, 3 KCl, 0.5 CaCl<sub>2</sub>, 0.01 EDTA, and 30 D-mannitol at pH 7.4. All recording solutions were made from external solution and recordings were performed at holding potentials of -60 mV unless otherwise stated. Rapid solution exchange for macroscopic recordings was accomplished with a two-barrel theta glass pipette controlled by a piezoelectric translator (Burleigh Instruments, Fishers, NY); 10 to 90% open tip

solution exchange times were under 1 ms, and solution exchange around the whole cell was under 5 ms (Vance et al., 2011). Macroscopic currents were evoked by application of glutamate/NMDA, glycine, and DQP-1105 as described; between five and ten individual responses were recorded for each condition.

Outside-out patches containing multiple GluN1/GluN2D channels were excised from transfected HEK cells and recorded at a holding potential of -80 mV at pH 8.0. Recombinant NMDA receptors were activated by a maximally effective concentration of glutamate (1 mM) and glycine (50  $\mu$ M) for two minutes after which glutamate and glycine were co-applied with increasing concentrations (3 – 30  $\mu$ M) of DQP-1105 for two minutes. Response was also measured after removal of DQP-1105.

#### *Perforated patch-clamp recordings*

Perforated whole-cell patch-clamp recording from transfected HEK cells under voltage clamp (holding potential -60 mV) was performed with an Axopatch 200B amplifier. The recording chamber was continually perfused with the recording solution described above. Recording electrodes (3-4 M $\Omega$ ) were filled with (in mM) 150 CsMeSO<sub>4</sub>, 10 NaCl, 0.5 CaCl<sub>2</sub>, 10 HEPES, and 25–50  $\mu$ g ml<sup>-1</sup> gramicidin D (pH adjusted to 7.3 with CsOH and osmolality adjusted to 310 mosmol kg<sup>-1</sup> with sucrose). Once a gigaohm seal was achieved, the cell was lifted and held in the bath until electrical access to the intracellular compartment was achieved by gramicidin (Ebihara et al., 1995). It took 10–30 min to achieve perforation with series resistance ranging from 30 to 60 M $\Omega$ . Rapid solution exchange was achieved with a two-barreled theta-glass pipette controlled by a piezoelectric translator as described above. The cell was first exposed to 100  $\mu$ M glutamate and 100  $\mu$ M glycine, followed by a jump to 100  $\mu$ M glutamate and 100  $\mu$ M glycine with various concentrations of DQP-1105 (from 0.3  $\mu$ M to 30  $\mu$ M) for 3 seconds, and a subsequent wash-out of antagonist with 100  $\mu$ M glutamate and 100  $\mu$ M glycine. All electrophysiological data from HEK cells in this study were collected at room temperature (23°C).

#### *Two-electrode voltage-clamp recording from *Xenopus laevis* oocytes*

Two-electrode voltage-clamp recordings were performed in *Xenopus laevis* oocytes expressing recombinant rat GluN1/GluN2A, GluN1/GluN2B, GluN1/GluN2C, GluN1/GluN2D, GluA1, or GluK2. In some experiments, recordings were performed in oocytes expressing recombinant human GluN1/GluN2A, GluN1/GluN2B, GluN1/GluN2C, or GluN1/GluN2D. Oocytes were isolated from *Xenopus laevis* as approved by the Emory University IACUC, and treated according to methods previously described (Dravid et al., 2007). The pipettes for cRNA injection were filled with mineral oil and attached to an automatic injector (Nanoject II, Drummond Scientific). The cRNA was transcribed in vitro using the mMessage Machine kit (Ambion), diluted with nuclease free water, and injected at a ratio of 1:2 GluN1:GluN2 (5-10 ng total cRNA). The oocytes were kept in Barth's solution comprised of (in mM) 88 NaCl, 5 Tris-HCl, 2.4 NaHCO<sub>3</sub>, 1 KCl, 0.84 MgSO<sub>4</sub>, 0.41 CaCl<sub>2</sub>, 0.33 Ca(NO<sub>3</sub>)<sub>2</sub>, 0.1 mg/ml gentamycin sulfate, 1 U/ml penicillin, and 1 µg/ml /streptomycin at pH 7.4 at 15-17°C for two to five days before experiments.

Oocytes were placed into a perfusion chamber and continually washed with recording solution containing (in mM) 90 NaCl, 1.0 KCl, 0.5 BaCl<sub>2</sub>, 0.005 EDTA, and 10 HEPES at pH 7.4 (23°C). Glass electrodes with a tip resistance of 0.5-2.5 MΩ were pulled from thin-walled glass capillary tubes (World Precision Instruments, Cat. No. TW-150F-4, Sarasota, FL, USA). Voltage electrodes were filled with 0.3 M KCl and current electrodes were filled with 3.0 M KCl. Voltage clamp recordings were conducted at a holding potential of -40 mV using an OC-725 amplifier (Warner Instrument Co). For some experiments the current-voltage relationship was evaluated by stepping the membrane potential in 10 mV increments from -60 mV to +30 mV. All compounds were made as 20 mM stock solutions in DMSO and dissolved to reach the final concentration in recording solution. Final DMSO content was 0.05-0.5% (vol/vol). Oocytes expressing GluK2 were pre-treated with 10 µM concanavalin-A for 10 minutes prior to recording. Some oocytes expressing GluN1/GluN2A were pretreated with 50 µM BAPTA-AM (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester) for 10 minutes or

injected with 50 nl of 2 mM K-BAPTA (potassium 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) to prevent gradual run-up of the current response.

### Data analysis

Antagonist IC<sub>50</sub> values were determined by non-linear least squares fitting of the equation

$$Response = 100 / ( 1 + ( [inhibitor concentration] / IC_{50} )^N ) \quad (2)$$

to concentration-response data from individual experiments normalized to the response in the absence of inhibitor (100%), where IC<sub>50</sub> is the concentration that inhibits the response half-maximally and *N* is the Hill slope; inhibition was assumed to be complete at saturation. Some IC<sub>50</sub> values were determined by fitting equation (2) to mean composite data (HEK patch experiments, data describing the structure-activity relationship for multiple ligands). Only compounds that produced at least 25% inhibition of NMDA receptors at 30 μM were studied further. Agonist concentration-response data were fitted by the equation

$$Percent Response = 100 / ( 1 + (EC_{50} / [agonist concentration] )^N ), \quad (3)$$

where EC<sub>50</sub> is the concentration of agonist that produces a half-maximally effective response and *N* is the Hill slope. In patch recordings from transfected HEK cells, the GluN1/GluN2D current responses to co-application of agonist and DQP-1105 relaxed with a single exponential time course that we interpret to reflect the time course of association of drug with receptor. We fitted this response time course to an exponential function

$$Response = Amplitude \exp (-time / \tau_{ONSET}) \quad (4)$$

where τ<sub>ONSET</sub> describes the onset of DQP-1105 inhibition. The association rate (*k*<sub>ON</sub>) of DQP-1105 was calculated by plotting 1 / τ<sub>ONSET</sub> versus DQP-1105 concentration. Linear regression analysis was calculated for

$$1 / \tau_{ONSET} = k_{ON} [inhibitor concentration] + k_{OFF}, \quad (5)$$

where  $k_{ON}$  is the slope and describes association rate of the compound,  $k_{OFF}$  is the y-intercept and is the dissociation rate, and  $[inhibitor\ concentration]$  is the concentration of the DQP-1105 compound. The dissociation constant  $K_D$  was determined from  $k_{OFF} / k_{ON}$ .

Channel recordings were filtered after acquisition using a digital 4 kHz Bessel (8-pole) filter and idealized using time course fitting of openings (SCAN, provided by Dr. David Colquhoun, <http://www.ucl.ac.uk/Pharmacology/dcpr95.html>). Open and closed duration histograms were constructed using EKDIST (<http://www.ucl.ac.uk/Pharmacology/dcpr95.html>) with an open time resolution of 53  $\mu$ s and a shut time resolution of 31  $\mu$ s (Colquhoun and Sigworth, 1995). Open periods were defined as the duration of all contiguous openings, regardless of conductance level. That is, openings that showed direct transition between two conductance levels were treated as having a single duration. Although all patches contained several active channels, only a minority of openings (~1%) were due to simultaneous opening of two or more channels. Thus, multiple simultaneous channel openings were easily identified and excluded from open and closed duration histograms and for calculation of mean open time, mean shut time, and  $P_{OPEN}$ .  $P_{OPEN}$  was calculated according to

$$P_{OPEN} = (MOT \times Number_{OPEN}) / (MOT \times Number_{OPEN} + MST \times Number_{CLOSE}) \quad (6)$$

where  $MOT$  is the mean open time,  $MST$  is the mean shut time,  $Number_{OPEN}$  is the number of openings, and  $Number_{CLOSE}$  is the number of closed durations.

Similar results for  $P_{OPEN}$  were found by integrating the idealized trace to obtain the average current level of the recording and dividing by the average single channel current. Open period distributions, and shut duration distributions were fitted with mixtures of exponential components using maximum likelihood estimation with EKDIST. Open point amplitude distributions were fitted by the sum of 2 Gaussian components (EKDIST).

### *Computational Modeling*

The comparative homology modeling program MODELLER 9v1 (Sali and Blundell, 1993) was used to generate homology models for the GluN1/GluN2D and GluN1/GluN2A containing NMDA

receptors. A protein family alignment was generated using the rat sequences for the NMDA receptors (GluN1, GluN2A-D), AMPA receptors (GRIA1-4), and kainate receptors (GRIK1-5). The MUSCLE alignment program was used to align the sequences and was subsequently used in model construction (Edgar, 2004). The crystal structure of the AMPA receptor GluA2 (PDB entry: 3KG2; 3.60Å resolution) was used as template, which served as a scaffold to build an NMDA receptor that includes the amino terminal domain, the ligand binding domain (LBD), and the transmembrane domain. The symmetry used in the construction of the models is the same as that suggested by ((Sobolevsky et al., 2009); Chain A,C = GluN1; Chain B,D = GluN2). Due to a low overall homology between GluA2 and the NMDA receptors, multiple templates were used (19% sequence identity between GluA2 and GluN2D and 23% sequence identity between GluA2 and GluN1). The resolved structure for the monomeric ATD of GluN2B (Karakas et al., 2009; PDB entry; 3JPY; 3.21Å resolution) was used as a template to model the amino terminal domain of both GluN1 and GluN2 (sequence identities of 14% and 23%, respectively). The structure of the ligand binding domain of the GluN1 and GluN2A complex (PDB entry: 2A5T; 2.00Å resolution; GT-linker removed) was used as a template to model the respective domain in the GluN1/GluN2D models (Furukawa et al., 2005). The transmembrane region was modeled based on the AMPA coordinates with the unresolved regions being omitted. The model was subjected to quality analysis using PDB sum generator (<http://www.ebi.ac.uk/pdbsum>; (Laskowski, 2009)). The analysis revealed some discrepancy in the overall G factors between the crystal structure (0.32) and the GluN1/GluN2D (-0.29) model, which is expected for models built on low resolution structures with low homology; scores above -0.5 are considered acceptable.

### *Reagents*

The dihydroquinolone-pyrazoline analogue DQP-997 (**3**; 4-(3-(6-bromo-2-oxo-4-phenyl-1,2-dihydroquinolin-3-yl)-5-(4-bromophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-oxobutanoic acid) was obtained from Asinex (Moscow, Russia); DQP-1105 (**19**; 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), -1209 (**9**; 4-(3-(6-

bromo-2-oxo-4-phenyl-1,2-dihydroquinolin-3-yl)-5-(2-chlorophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-oxobutanoic acid), -1210 (**22**; 4-(3-(6-bromo-4-phenylquinolin-3-yl)-5-(4-bromophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-oxobutanoic acid), -1308 (**18**; 4-(3-(6-methyl-2-oxo-4-phenyl-1,2-dihydroquinolin-3-yl)-5-phenyl-4,5-dihydro-1H-pyrazol-1-yl)-4-oxobutanoic acid) and -1309 (**21**; 6-bromo-3-(5-(4-bromophenyl)-1-propionyl-4,5-dihydro-1H-pyrazol-3-yl)-4-phenylquinolin-2(1H)-one) were obtained from Pharmeks (Moscow, Russia); DQP-1177 (**25**; 4-(3-(4-bromophenyl)-5-(6-methyl-2-oxo-4-phenyl-1,2-dihydroquinolin-3-yl)-4,5-dihydro-1H-pyrazol-1-yl)-4-oxobutanoic acid), -1178 (**8**; 4-(3-(6-bromo-2-oxo-4-phenyl-1,2-dihydroquinolin-3-yl)-5-(2-fluorophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-oxobutanoic acid), -1183 (**1**; 4-(3-(6-bromo-2-oxo-4-phenyl-1,2-dihydroquinolin-3-yl)-5-phenyl-4,5-dihydro-1H-pyrazol-1-yl)-4-oxobutanoic acid), -1184 (**2**; 4-(3-(6-bromo-2-oxo-4-phenyl-1,2-dihydroquinolin-3-yl)-5-(4-fluorophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-oxobutanoic acid), -1185 (**5**; 4-(3-(6-bromo-2-oxo-4-phenyl-1,2-dihydroquinolin-3-yl)-5-(p-tolyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-oxobutanoic acid), and -2003 (**7**; 4-(3-(6-bromo-2-oxo-4-phenyl-1,2-dihydroquinolin-3-yl)-5-(2,5-dimethoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-oxobutanoic acid) were obtained from Life Chemicals Inc. (Kiev, Ukraine); DQP-1250 (**24**; 3-(1-acetyl-5-(4-bromophenyl)-4,5-dihydro-1H-pyrazol-3-yl)-6-methyl-4-phenylquinolin-2(1H)-one) and -2005 (**20**; 4-(5-(2-methoxyphenyl)-3-(6-methyl-2-oxo-4-phenyl-1,2-dihydroquinolin-3-yl)-4,5-dihydro-1H-pyrazol-1-yl)-4-oxobutanoic acid) were obtained from Enamine (Kiev, Ukraine); DQP-2033 (**4**; 4-(3-(6-bromo-2-oxo-4-phenyl-1,2-dihydroquinolin-3-yl)-5-(4-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-oxobutanoic acid) was obtained from Chembridge (Moscow, Russia); DQP-1128 (**6**; 4-(3-(6-bromo-2-oxo-4-phenyl-1,2-dihydroquinolin-3-yl)-5-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-oxobutanoic acid) was obtained from SPECS (Wakefield, RI). All compounds were more than 90% pure as determined by the chemical suppliers. Glutamate, glycine, and all other chemicals were from Sigma-Aldrich (St Louis, MO). DQP-1105 had a molecular weight of 558.42, a maximum solubility at 1 hour in oocyte recording buffer plus 0.1% DMSO of 27  $\mu$ M, and maximum solubility at 24 hours in phosphate buffered saline (no DMSO) of 6.5  $\mu$ M (*Supplementary Information*). DQP-1105 is

also known as DC060015, and was identified in a virtual screen as a low potency ( $K_D$  18.6  $\mu\text{M}$ ) inhibitor of SARS-Coronavirus 3CL, where it was reported to have a CLogP value of 6.55 (Chen et al., 2006).

## Results

### *Structure activity relationship for dihydroquinolone-pyrazolines*

The dihydroquinolone-pyrazoline scaffold was identified as an inhibitor of NMDA receptors from a multiwell  $\text{Ca}^{2+}$ -based screen of approximately 100,000 compounds from ChemDiv and Asinex diversity libraries against GluN1/GluN2C or GluN1/GluN2D expressing BHK cell lines (Hansen et al., 2010b). Evaluation of a subset of analogues sharing this scaffold lead to the identification of DQP-1105 (Fig. 1A), which is the primary focus of this study. Various substitution patterns of the dihydroquinolone-pyrazoline-containing scaffold (Table 1) affect potency and selectivity and reveal several key features of the ligand-receptor interactions. Beginning from the initial screening hit (compound **3**), we evaluated the  $\text{IC}_{50}$  value at four different recombinant NMDA receptors for analogs that altered substituents at five positions as a starting point for assessing the structure-activity relationship (SAR). The  $\text{IC}_{50}$  values of compound **3** ranged from 2.7  $\mu\text{M}$  at GluN2D containing receptors to >75  $\mu\text{M}$  at GluN2A when evaluated at recombinant receptors expressed in *Xenopus* oocytes. Evaluation of various substitutions on the C-ring while the A-ring  $\text{R}_1$  bromine was held constant showed that the  $\text{R}_4$ -bromo (compound **3**) is the most favorable configuration. Substituting  $\text{R}_1$  of the A-ring with a methyl group lead to compound **19**, referred hereafter to as DQP-1105, which shows the most favorable combination of potency and selectivity for GluN2D over GluN2A and GluN2B of all of the analogs tested. We also evaluated core structural perturbations to better understand the requirements of activity (Table 2). Beginning with analogs of the initial screening hit (compound **3**), truncation of the acyl-chain off of the pyrazoline nitrogen from an acid to an ethyl group (compound **21**) eliminated antagonist activity. Similarly, removal of the succinate group and substituting with  $\text{R}_1$ -methyl analog (compound **24**) abolishes activity. Removing the oxo-functionality from the quinoline (compound **22**), which removes a resonance form, also eliminates activity (Table 2). Thus, the position of the imine within the pyrazoline compound as well as the

connectivity, length and charge of the acyl chain are important features for potency and selectivity. We selected DQP-1105 (compound **19**) as the compound with the best combination of potency and selectivity to study in terms of mechanism of action.

#### *Subunit selectivity of DQP-1105 inhibition*

We have evaluated the actions of DQP-1105 against current responses from recombinant NMDA, AMPA, and kainate receptors expressed in *Xenopus laevis* oocytes (Fig. 1B, Table 3) to determine its potency and selectivity across the glutamate receptor ion channel family. When co-applied in solutions containing maximally effective concentrations of glutamate and glycine, DQP-1105 inhibited recombinant NMDA receptors containing the GluN1/GluN2C and GluN1/GluN2D subunits with IC<sub>50</sub> values that were lower than that for inhibition of GluN1/GluN2A (Table 3). For example, in *Xenopus* oocytes, DQP-1105 inhibited GluN1/GluN2D receptors with an IC<sub>50</sub> value of  $2.7 \pm 0.2 \mu\text{M}$  (mean  $\pm$  SEM; n=44), while it had minimal effects on GluN1/GluN2A. The IC<sub>50</sub> value at GluN1/GluN2A determined by fitting each concentration-effect curve was at a minimum  $206 \pm 36 \mu\text{M}$ , and might be higher as responses from some oocytes showed no inhibition and thus could not be fitted and included in this analysis (n=13; Fig. 1B, Table 3). IC<sub>50</sub> values determined at recombinant human and rat NMDA receptors were similar (Table 3). DQP-1105 had minimal effects on current responses of homomeric GluA1 AMPA receptors or homomeric GluK2 kainate receptors (Table 1), and no effect on the leak currents in uninjected *Xenopus* oocytes (n=4; data not shown), or on the leak currents from oocytes expressing NMDA receptors (n=4; data not shown).

In order to determine whether DQP-1105 is selective for GluN2C/D- over GluN2A/B-containing NMDA receptors expressed in mammalian cells, we evaluated the actions of this compound on recombinant NMDA receptor responses in BHK cells. IC<sub>50</sub> values for DQP-1105 inhibition of the fluorescent signal in BHK cell lines stably expressing GluN1/GluN2A, GluN1/GluN2B, GluN1/GluN2C, or GluN1/GluN2D loaded with the Ca<sup>2+</sup>-sensitive dye Fluo4 were determined (Hansen et al., 2008;

Hansen et al., 2010b). DQP-1105 inhibited responses of GluN1/GluN2C and GluN1/GluN2D receptors activated by an EC<sub>80</sub> concentration of NMDA plus glycine with IC<sub>50</sub> values that were slightly more potent than those obtained from oocyte recordings (Table 3, Figure 1C). GluN1/GluN2A-containing NMDA receptor responses were inhibited to only 65 ± 3% of control by 33 μM DQP-1105, which allowed us to estimate a mean IC<sub>50</sub>, whereas GluN1/GluN2B responses were negligibly inhibited (Table 3, Figure 1C). Thus, DQP-1105 exhibited a similar level of selectivity for GluN2C/D in intact mammalian cells as was observed in *Xenopus* oocytes.

We also recorded recombinant NMDA receptor responses from transiently transfected HEK cells using the whole-cell patch clamp recording configuration. These experiments confirmed that DQP-1105 inhibited the current response at GluN1/GluN2D receptors with similar potency as the other expression systems (Fig. 2, Table 3). Surprisingly, DQP-1105 showed little selectivity for GluN2D-containing receptors over GluN2A-containing receptors in HEK cells recorded in the whole-cell configuration (Fig. 2D; Table 3). Whereas DQP-1105 inhibited GluN2D with similar potency in all three preparations, it was more potent at inhibiting GluN2A in whole cell patch recordings than in oocytes or BHK cells studied using Ca<sup>2+</sup>-sensitive fluorescent dyes (Table 3). Because we had observed selectivity in both oocytes and BHK cells in which the intracellular milieu had not been disrupted by dialysis, we reasoned that the whole cell patch recording configuration, which is known to enhance NMDA receptor desensitization (Sather et al., 1990) (compare Fig. 2A to 2B), may have artificially increased the potency of DQP-1105 on GluN2A receptors. To test this idea, we utilized perforated patch recordings in which electrical access to the cell was obtained through gramicidin pores (Ebihara et al., 1995). This configuration allows measurements of whole cell currents without changing the intracellular composition. As predicted, we observed considerable selectivity between glutamate-activated GluN2D-containing (IC<sub>50</sub> 2.1 μM) over GluN2A-containing (IC<sub>50</sub> 54 μM) NMDA receptors when we recorded using perforated patches (Table 3). This observation suggests that dialysis of the cytoplasm and perhaps the associated enhancement of desensitization can increase the affinity of DQP-1105 for its binding site. We interpret the differences between the observed IC<sub>50</sub> values for GluN2A with the different patch configurations to be due to a

diffusible factor that is lost during dialysis of the intracellular compartment. We also interpret the lack of effect of dialysis on the DQP-1105  $IC_{50}$  for GluN1/GluN2D to suggest that this receptor is not sensitive to loss of the same diffusible factor. Table 3 summarizes the potency and selectivity of DQP-1105 for GluN2C/D-containing NMDA receptors under the various conditions used in this study.

### *Mechanism of action of DQP-1105*

To begin to determine the mechanism of DQP-1105 inhibition, we evaluated whether the actions of DQP-1105 were dependent on the agonist concentration for GluN1/GluN2D receptor responses recorded in *Xenopus* oocytes. Sub-maximal inhibition of GluN1/GluN2D receptor responses by 5  $\mu$ M DQP-1105 could not be surmounted by 10-fold increases in either glutamate or glycine concentration, suggesting that DQP-1105 acts by a non-competitive mechanism (Fig. 3A). Furthermore, the  $EC_{50}$  values for glutamate and glycine at GluN1/GluN2D receptors expressed in *Xenopus* oocytes were not significantly altered in the presence of an  $IC_{75}$  concentration of DQP-1105 (5  $\mu$ M). Glutamate  $EC_{50}$  values in 30  $\mu$ M glycine were  $0.30 \pm 0.02$  and  $0.27 \pm 0.03$   $\mu$ M in the absence and presence of DQP-1105, respectively ( $p > 0.05$ , unpaired t-test,  $n=10-11$ ). Glycine  $EC_{50}$  values in 100  $\mu$ M glutamate were  $0.11 \pm 0.01$  and  $0.11 \pm 0.01$   $\mu$ M in the absence and presence of DQP-1105, respectively ( $p > 0.05$ , unpaired t-test,  $n=4$ ). In order to determine whether DQP-1105 inhibition involved the permeation pathway, we evaluated the inhibition at various membrane potentials. The degree of inhibition produced by DQP-1105 was the same at all voltages tested ( $p=0.86$ , 1-way ANOVA;  $n=5$ ), suggesting that DQP-1105 inhibition is voltage-independent. In addition, the mean reversal potential ( $V_{REV}$ ) was not significantly different between control response to glutamate plus glycine ( $V_{REV} -0.31 \pm 1.4$  mV;  $n=5$ ) and the response to glutamate and glycine plus 3-5  $\mu$ M DQP-1105 ( $V_{REV} -0.81 \pm 3.3$  mV;  $n=5$ ;  $p = 0.76$ ; t-test; Fig. 3B). We interpret these data to suggest that DQP-1105 neither inhibits receptor function by competing with glutamate or glycine at the agonist binding domain, nor inhibits receptor function by interacting with the channel pore in a manner that is influenced by the transmembrane electric field.

We subsequently examined the effects of DQP-1105 on the NMDA receptor response time course in whole-cell voltage clamp recordings of GluN1/GluN2D receptors expressed in HEK cells. Current responses were evoked by rapid solution exchange from glycine alone to glycine plus glutamate or NMDA (Fig 4A). The current response of GluN1/GluN2D undergoes virtually no desensitization during agonist application, as previously described (Vicini et al., 1998; Wyllie et al., 1998). Co-application of agonist plus 0.3-30  $\mu\text{M}$  DQP-1105 caused concentration-dependent relaxation of the current response (Fig. 4), with virtually complete inhibition of GluN1/GluN2D responses at 30  $\mu\text{M}$ . Concentration-effect curves for the steady-state current responses to the agonist NMDA show that the  $\text{IC}_{50}$  for DQP-1105 inhibition on GluN1/GluN2D receptors was in excellent agreement with that obtained in the other expression systems (Fig. 4B, Table 3). Co-application of DQP-1105 with NMDA and glycine induced a relaxation of the current response that could be fitted by a single exponential function. Co-application of different concentrations of DQP-1105 with NMDA resulted in different time constants ( $\tau_{\text{ONSET}}$ ), which we interpret to reflect the time course of association of DQP-1105 with the receptor. In this case, the reciprocal of  $\tau_{\text{ONSET}}$  should be linearly related to DQP-1105 concentration with a slope equal to  $k_{\text{ON}}$  and an intercept equal to  $k_{\text{OFF}}$ . Consistent with this idea, values of  $1/\tau_{\text{ONSET}}$  were significantly correlated with DQP-1105 concentration ( $R=0.996$ ) for GluN1/GluN2D (Fig. 4C). Regression analysis of this relationship for NMDA-activated GluN1/GluN2D gives values of  $4.5 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$  for  $k_{\text{ON}}$  and  $0.65 \text{ s}^{-1}$  for  $k_{\text{OFF}}$ , from which  $K_{\text{D}}$  can be calculated as 1.4  $\mu\text{M}$ , in agreement with the fitted  $\text{IC}_{50}$  value (Fig. 4C, Table 3). Inhibition by DQP-1105 also was evaluated when GluN1/GluN2D receptors were activated by 100  $\mu\text{M}$  glutamate and 30  $\mu\text{M}$  glycine. Analysis of the glutamate-activated GluN1/GluN2D response relaxation time course as a function of DQP-1105 concentration yielded similar values for  $k_{\text{ON}}$  ( $4.4 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ ) and  $k_{\text{OFF}}$  ( $1.44 \text{ s}^{-1}$ ;  $n=6$  cells). The resulting  $K_{\text{D}}$  for DQP-1105 inhibition of glutamate-evoked currents (3.3  $\mu\text{M}$ ) was similar to the independently determined  $\text{IC}_{50}$  for glutamate-evoked responses (Table 3). In addition, the  $K_{\text{D}}$  and  $\text{IC}_{50}$  values for DQP-1105 inhibition were typically less than two-fold

lower for the partial agonist NMDA- compared to glutamate-evoked currents, suggesting the affinity of DQP-1105 is only modestly influenced by agonist efficacy.

We next evaluated whether the relaxation of the current response during DQP-1105 application was dependent upon the binding of NMDA or glycine. GluN1/GluN2D receptors expressed in HEK cells were recorded under voltage-clamp and activated by rapid 5 s application of 200  $\mu$ M NMDA in the continuous presence of 100  $\mu$ M glycine. The peak and steady-state current responses then were compared to the currents evoked by application of 200  $\mu$ M NMDA following pre-incubation with 100  $\mu$ M glycine and 3  $\mu$ M DQP-1105 (Fig. 5A). Even when DQP-1105 was pre-applied, the NMDA-evoked currents exhibited a pronounced relaxation of the current response, with a peak response of  $84 \pm 1.9\%$  compared to control and a steady-state response of  $21 \pm 1.6\%$  compared to control (Fig. 5A,C; n=7), suggesting that DQP-1105 did not bind with the same affinity in the absence of NMDA. The time course could be described by a single exponential function with a time constant ( $580 \pm 33$  ms) that was not significantly different than that obtained with no pre-application of DQP-1105 ( $530 \pm 27$  ms;  $p=0.72$ , unpaired t-test; compare Fig 4A and Fig 5A). By contrast, activation of GluN1/GluN2D by a rapid application of 300  $\mu$ M glycine following pre-incubation with 20  $\mu$ M NMDA and 3  $\mu$ M DQP-1105 (Fig. 5B) did not show a pronounced relaxation in current response time course. Rather, the peak response was inhibited to  $29 \pm 0.9\%$  of control, with a steady-state response of  $24 \pm 1.6\%$  of control (Fig. 5B,C; n=5), suggesting that DQP-1105 can access its binding site with a similar affinity when NMDA is present as is observed during steady-state responses to NMDA plus glycine. We interpret this result to suggest that the binding of DQP-1105 is dependent upon binding of NMDA but not glycine. This use-dependence upon activation of the GluN2D subunit is reflected in the significant difference in the peak responses of GluN1/GluN2D receptors activated by 5 s application of NMDA or glycine in the presence of DQP-1105 ( $p<0.05$ ; paired t-test). The maximal, steady-state inhibition by DQP-1105 is not significantly influenced by pre-incubation with either NMDA or glycine (Fig. 5C,D). These results are similar to that for quinazolin-4-one GluN2C/D modulators (Hansen and Traynelis, 2011).

We subsequently recorded the single channel activity of GluN1/GluN2D in excised outside-out patches in response to a maximally effective concentration of glutamate (1 mM) and glycine (50  $\mu$ M) plus DQP-1105 (3 and 30  $\mu$ M). Figure 6 shows typical channel activity from patches containing several channels in the absence and presence of DQP-1105. Time course fitting of the open and closed durations in 4 patches revealed that DQP-1105 increased the mean shut time in a concentration-dependent manner, but did not significantly affect the mean open time or either of the two time constants describing the open duration histogram (Table 4). The open probability was decreased to  $43 \pm 12\%$  (Table 4) by 3  $\mu$ M DQP-1105, a value that agrees well with the extent of inhibition of macroscopic currents in *Xenopus* oocytes by 3  $\mu$ M DQP-1105 ( $35 \pm 3.6\%$  of control,  $n=11$ ), suggesting inhibition of channels in excised outside-out patches quantitatively matches inhibition observed in intact cells. In addition, 30  $\mu$ M DQP-1105 decreased the open probability of GluN1/GluN2D receptors to  $2.0 \pm 0.6\%$  of control (Figure 6). DQP-1105 had minimal effects on chord conductance values (Table 4). These data suggest DQP-1105 does not affect the pore conductance or the stability of the open state of the receptor, but rather acts to prevent the channel from opening by raising the activation energy for a critical conformational change.

#### *Structural determinants of DQP-1105 activity*

DQP-1105 shows sufficient selectivity for GluN2D over GluN2A to allow use of a chimeric strategy in which domains of subunits are swapped to identify the divergent structural elements of the GluN2D subunit that might account for the observed selectivity. We previously constructed a series of GluN2A and GluN2D chimeric receptors that transferred different portions of GluN2D into GluN2A in order to test for gain of function for novel GluN2C/D preferring modulators (Hansen and Traynelis, 2011; Mullasseril et al., 2010) (Table 5). We used this set of chimeric receptors to evaluate the structural determinants of DQP-1105 action, and found that transferring the ATD, S1 region of the ligand binding domain, or the transmembrane elements by themselves (including two transmembrane helices and a reentrant loop) did not render GluN2A containing receptors sensitive to DQP-1105 (Fig 7A,B). By

contrast, transferring either the S1-S2 regions or the S2 domain alone from GluN2D to GluN2A completely transferred the DQP-1105 sensitivity to GluN2A (Fig. 7A, B), suggesting structural determinants that define subunit selectivity may be contained within the S2 region of GluN2D. This result is surprisingly similar to the structural determinants for neurosteroids (Horak et al., 2006; Jang et al., 2004) and the structurally unrelated quinazolin-4-ones (Hansen and Traynelis, 2011). Consistent with this result, experiments in which the GluN2D subunit ATD has been removed show that the ATD is not involved in the selectivity of DQP-1105 for GluN2D over GluN2A (Fig. 7A, B).

To identify specific divergent structural elements within the S2 region that might define the subunit selectivity, we utilized 12 additional chimeric receptors (Hansen and Traynelis, 2011) that revert non-conserved S2 residues within the GluN2A(2D-S2) chimera back to wild type GluN2A residues (see Fig. 7C,D). This strategy allowed us to examine whether clusters of residues that are divergent between GluN2D and GluN2A impact DQP-1105 selectivity. We found that the S2a, S2b, and S2c chimeric receptors exhibited reduced sensitivity to DQP-1105 when tested at a single concentration, suggesting that some of the nine divergent residues between GluN2A and GluN2D within these three regions control DQP-1105 potency at the receptor. Subsequent mutation of each of these divergent residue in GluN2D to that observed in GluN2A identified two amino acids (GluN2D Gln701 and Leu705) that significantly reduced inhibition (Fig. 7E). Concentration-effect curves showed that GluN2D(Q701Y) and GluN2D(L705F) decreased the potency of DQP-1105 more than 6-fold to  $19 \pm 2 \mu\text{M}$  (n=8) and  $17 \pm 2 \mu\text{M}$  (n=8), respectively. These data suggest that DQP-1105 shares structural determinants with quinazolin-4-ones (Hansen and Traynelis, 2011) and perhaps neurosteroids (Horak et al., 2006), identifying this region as a site at which structurally diverse endogenous and exogenous ligands can interact.

## Discussion

We have identified a novel class of GluN2C/D preferring non-competitive antagonists that inhibit NMDA receptors by blocking a conformational change necessary for channel opening. Inhibition occurs

without actions on the stability of the open state or the channel closing rate. Rather, binding of DQP-1105, presumably to the GluN2 subunit, reduces the frequency of channel openings without other actions. We also show, unexpectedly, that the structural determinants of selectivity for DQP-1105 reside within a portion of the S2 region of GluN2D that overlaps closely with previously identified structural determinants important in the selectivity of quinazolin-4-ones, another class of GluN2C/D preferring antagonists (Hansen and Traynelis, 2011). In addition, the shared reliance of quinazolin-4-ones, dihydroquinilone-pyrazolines, and neurosteroids on the lower lobe of the ligand binding domain clamshell indicate that this region is an important site at which channel gating can be controlled (Jang et al., 2004). These data also show that the allosteric binding site(s) in this region can accommodate a wide range of structurally diverse, negative modulators, which increases the likelihood that well-tolerated and potentially therapeutically useful subunit-selective NMDA receptor antagonists can be developed. Furthermore, the findings presented here provide support for a previously unknown antagonist binding site that does not involve the amino terminal domain, the well-conserved agonist recognition sites, or residues within the channel pore. That is, this new site is distinct from GluN2B-selective negative allosteric modulators (Hansen et al., 2010a), competitive antagonists, and channel blockers.

Several studies have recently identified positive and negative allosteric modulators that act with enhanced potency at the GluN2C/D subunits (Costa et al., 2010; Mosley et al., 2010; Mullasseril et al., 2010). Of these, only one other class of non-competitive GluN2C/D antagonist has been reported with potency in the low micromolar range and greater than 20-fold selectivity over NMDA receptors comprised of other GluN2 subunits, AMPA receptors, and kainate receptors. That class, which contains the previously identified AMPA antagonist quinazolin-4-one backbone (Mosley et al., 2010), is chemically distinct from DQP-1105 yet shares similar structural determinants of action, and a similar, mechanism in that glutamate/NMDA binding enhances antagonist potency (Hansen and Traynelis, 2011). Structural determinants within NMDA receptor subunits have not been defined for other GluN2C/D-preferring antagonists, apart from the suggestion that the S2 region of the ligand binding domain is important in their actions (Costa et al., 2010; Horak et al., 2006). Thus, it is presently unclear whether the

neurosteroids and phenanthrene derivatives share structural determinants with DQP-1105 and the quinazolin-4-ones.

It is known that the ligand binding domain undergoes a significant conformational change upon binding of agonist (Furukawa and Gouaux, 2003). The data from our single channel analysis, as well as the structural determinants that were identified, support the idea that DQP-1105 could interfere with a crucial gating step that leads to an increase in the energy barrier for the transition from the closed to the open state for DQP-1105-bound receptors. Moreover, we have previously proposed that the GluN2C/D ligand binding domain closure around glutamate can increase the potency for quinazolin-4-one antagonists (Hansen and Traynelis, 2011), which share structural determinants of selectivity with DQP-1105. In this model, residues implicated by site-directed mutagenesis as important for the actions of inhibitors, including the residues identified in this study, are in sufficiently close proximity to the linkers (S1-M1 and M3-S2) connecting the transmembrane helices to the proximal region of the ligand binding domain to influence their movement (Fig. 8). Numerous studies have shown that structural determinants near or within the linker regions modulate receptor gating (Krupp et al., 1998; Talukder et al., 2010; Yuan et al., 2005). Most recently, specific residues within the S1-M1, M3-S2 and S2-M4 linker regions were suggested to serve as potential targets for allosteric modulators (Talukder et al., 2010). Our identification of residues in the vicinity of the extracellular end of the transmembrane linkers raise the possibility that DQP-1105 and related analogues could interact with both the LBD and portions of the linker regions in order to negatively modulate the receptors.

While the residues implicated by mutagenesis data in DQP-1105 selectivity are not immediately adjacent on the polypeptide chain, homology models of GluN1/GluN2 place these residues close in three dimensional space (Fig. 8). However, no crystallographic data exists for GluN2C, and the region identified in chimeric receptors shows a high degree of chain flexibility in the crystallographic structure of the isolated GluN2D ligand binding domain (Vance et al., 2011). Moreover, whereas we conclude that GluN2D residues Gln701 and Leu705 control the selectivity between GluN2A and GluN2D, the determinants of GluN2C selectivity have yet to be explored in depth. One of the residues important for

GluN2D selectivity (Leu705) is a conserved Phe in GluN2A/B/C, suggesting this residue does not contribute to selectivity for GluN2C over GluN2A. Nevertheless, it is tempting to speculate that these residues may form or influence part of the binding pocket for DQP-1105 and related analogues. The finding that the DQP class of molecules shares structural determinants with the previously identified quinazolin-4-ones suggests that potent and selective molecules incorporating functionality from both groups might be developed through medicinal chemistry efforts.

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**Authorship Contributions.**

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*Wrote or contributed to the writing of the manuscript:* Acker, Burger, Hansen, Jensen, Ogden, Vance, Yuan, Snyder, Liotta and Traynelis.

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*Manuscript in press.*

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## Footnotes

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## Legends For Figures

**Figure 1.** Subunit selectivity of DQP-1105. *A*, Representative current response of GluN1/GluN2D receptors expressed in *Xenopus* oocyte recording during co-application of 100  $\mu$ M glutamate, 30  $\mu$ M glycine (Glu/Gly), and the designated concentration of DQP-1105 (structure shown in *inset*). *B*, Composite concentration-effect curves determined using two-electrode voltage-clamp electrophysiology for DQP-1105 against recombinant AMPA, kainate, and NMDA receptors expressed in *Xenopus* oocytes. *C*, Concentration-effect curves determined using fluorescence-based measurements of intracellular calcium expressed as a percent of baseline fluorescence ( $F/F_0$ ) for DQP-1105 inhibition of recombinant GluN1/GluN2A, GluN1/GluN2B, GluN1/GluN2C, or GluN1/GluN2D stably expressed in BHK cells (see *Methods*).  $IC_{50}$  values are listed in Table 3.

**Figure 2.** DQP-1105 inhibits recombinant NMDA receptors expressed in HEK cells

*A*, Representative traces of conventional whole cell voltage-clamp recordings of currents evoked from recombinant GluN1/GluN2A receptors by 3 s applications of 100  $\mu$ M glutamate and 100  $\mu$ M glycine. Co-application of 30  $\mu$ M DQP-1105 with glutamate and glycine attenuates the steady-state current response to a new steady-state level that is  $24 \pm 6\%$  ( $n = 5$ ) of control. *B*, Representative gramicidin perforated-patch whole cell voltage-clamp recordings from recombinant GluN1/GluN2A receptors of currents evoked by 3 s applications of 100  $\mu$ M glutamate and 100  $\mu$ M glycine. DQP-1105 (30  $\mu$ M) attenuated the steady-state current response to  $65 \pm 3.1\%$  ( $n = 6$ ) of control. *C*, Representative perforated whole cell voltage-clamp recordings of current responses from GluN1/GluN2D evoked by 3 s applications of 100  $\mu$ M glutamate and 100  $\mu$ M glycine. DQP-1105 (30  $\mu$ M) inhibited the steady-state current response upon co-application with glutamate and glycine, with a final steady-state response of  $0.2 \pm 0.1\%$  ( $n = 3$ ) compared to the glutamate and glycine control. Data are from 6-8 cells. *D*, DQP-1105 composite concentration-response curves of the steady-state current responses activated by glutamate and glycine from dialyzed whole cell patch recordings give a fitted  $IC_{50}$  value of 3.2  $\mu$ M GluN1/GluN2D receptors and 12  $\mu$ M for GluN1/GluN2A receptors (data are from 6 cells). *E*, Composite concentration-response

curves of the steady-state current responses to glutamate and glycine from perforated whole cell patch recordings give an  $IC_{50}$  of 2.1  $\mu$ M GluN1/GluN2D receptors and 54  $\mu$ M for GluN1/GluN2A receptors, suggesting that dialysis alters  $IC_{50}$  of DQP-1105 at GluN1/GluN2A (data are from 6-8 cells).

**Figure 3.** DQP-1105 inhibits recombinant GluN1/GluN2D receptors through a non-competitive and voltage-independent mechanism. *A*, GluN1/GluN2D responses to 100  $\mu$ M glutamate and 30  $\mu$ M glycine were inhibited by co-application of glutamate, glycine, and 5  $\mu$ M DQP-1105, and the glutamate concentration was subsequently increased 10-fold to 1000  $\mu$ M (*left panel*). GluN1/GluN2D responses elicited by 100  $\mu$ M glutamate and 30  $\mu$ M glycine were inhibited by co-application of 5  $\mu$ M DQP-1105, and the glycine concentration was subsequently increased 10-fold to 300  $\mu$ M glycine (*right panel*). Neither the increase of glutamate or glycine altered the level of inhibition, suggesting a non-competitive mechanism. *B*, The mean current-voltage relationship of recombinant GluN1/GluN2D receptors was determined in the absence and presence of 3-5  $\mu$ M DQP-1105 (10 mV steps from -60 mV to +30 mV, n=5). Error bars are SEM and shown when larger than symbol size.

**Figure 4.** Determination of DQP-1105  $K_D$  for inhibition of recombinant GluN1/GluN2D NMDA receptors expressed in HEK cells. *A*, Conventional whole cell voltage-clamp recordings of currents evoked from recombinant GluN1/GluN2D receptors by 2 s applications of 1000  $\mu$ M NMDA and 50  $\mu$ M glycine. Co-application of DQP-1105 with NMDA and glycine caused a concentration-dependent attenuation of the steady-state current response, with a final steady-state response of  $3.4 \pm 1.1\%$  (n=8) at 30  $\mu$ M DQP-1105 compared to control. *B*, The composite concentration-response relationship of the steady-state current responses to 1000  $\mu$ M NMDA plus 30  $\mu$ M glycine shows that DQP-1105 inhibits GluN1/GluN2D receptors with an  $IC_{50}$  value of 1.9  $\mu$ M. *C*, There is a linear relationship between  $1/\tau_{ONSET}$  and DQP-1105 concentration, suggesting that the time-dependent inhibition of NMDA-activated GluN1/GluN2D reflects the association of DQP-1105 with its binding site. From this linear relationship,

$K_D$  can be calculated from  $k_{ON}$  and  $k_{OFF}$ , and was 1.4  $\mu\text{M}$  for GluN1/GluN2D receptors, similar to the measured  $\text{IC}_{50}$  value.

**Figure 5.** Inhibition by DQP-1105 is dependent upon binding of NMDA to the GluN2D subunit. Current responses were evoked by agonist application to HEK cells transiently expressing GluN1/GluN2D receptors that were recorded under voltage-clamp at -60 mV. *A*, GluN1/GluN2D receptors were activated by a 5 s application of 200  $\mu\text{M}$  NMDA in the continuous presence of 100  $\mu\text{M}$  glycine and subsequently were activated by 5 s applications of 200  $\mu\text{M}$  NMDA in the continuous presence of both 100  $\mu\text{M}$  glycine and 3  $\mu\text{M}$  DQP-1105. These recordings displayed a pronounced relaxation of the current response following NMDA binding, with an  $84 \pm 1.9\%$  peak response compared to control and a steady-state response of  $21 \pm 1.6\%$  compared to control ( $n=7$ ). *B*, GluN1/GluN2D receptors were activated by a 5 s application of 300  $\mu\text{M}$  glycine in the continuous presence of 20  $\mu\text{M}$  NMDA, and subsequently activated by a 5 s application of 300  $\mu\text{M}$  glycine in the continuous presence of both 20  $\mu\text{M}$  NMDA and 3  $\mu\text{M}$  DQP-1105. These recordings did not show a prominent relaxation of response, with a peak response of  $29 \pm 0.9\%$  compared to control and a steady-state response of  $24 \pm 1.6\%$  of the control response amplitude ( $n=5$ ). *C*, Whereas the peak responses of GluN1/GluN2D receptors activated by NMDA or glycine following pretreatment with 3  $\mu\text{M}$  DQP-1105 were significantly different ( $p<0.05$ ; paired t-test), the steady-state responses (*D*) were not significantly different (paired t-test). These data suggest that DQP-1105 shows higher potency after NMDA (but not glycine) binding to the receptor.

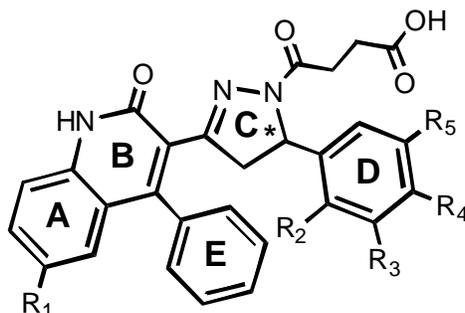
**Figure 6.** DQP-1105 increases mean shut time and decreases open probability of GluN1/GluN2D receptors. *A*, Unitary currents recorded from outside-out patches from HEK cells expressing GluN1/GluN2D were activated by glutamate (1 mM) and glycine (50  $\mu\text{M}$ ) at -80 mV *B*, After a 2 minute recording in control conditions, 3  $\mu\text{M}$  DQP-1105 was co-applied with agonists to the patch and currents were recorded for 2 minutes. *C*, 30  $\mu\text{M}$  DQP-1105 was then co-applied to the same patch and currents

recorded for 2 minutes. For A-C, the boxed region in the top trace is shown on an expanded time scale in the three lower traces. Individual openings and closings were idealized by time course fitting (see *Methods*) and composite open duration (*D*) and shut duration (*E*) histograms were constructed from idealized data from 4 patches. The open period distribution was fitted with the sum of two exponential components (Table 4) and the shut time distribution was fitted with the sum of five exponential components with taus in ms (area in %) of 0.026 (27%), 0.16 (11%), 1.4 (9%), 7.2 (43%), and 22 (10%) for control (7547 intervals), 0.016 (36%), 0.13 (12%), 3.3 (9%), 14 (34%), and 55 (9%) for 3  $\mu$ M DQP-1105 (5617 intervals), and 0.026 (27%), 0.23 (13%), 11 (22%), 85 (18%), and 1500 (20%) for 30  $\mu$ M DQP-1105 (952 intervals).

**Figure 7.** Identification of structural determinants of GluN2D-selective inhibition using chimeric GluN2A-GluN2D receptors. *A*, Indicated regions of the polypeptide were exchanged between wild type GluN2A and GluN2D. Concentration-effect curves at GluN1/GluN2D, GluN1/GluN2A, GluN1/GluN2A(2D-S1S2), GluN1/GluN2A(2D-S1), GluN1/GluN2A(2D-S2), *B*, GluN1/GluN2A(2D-M1M2M3), GluN1/GluN2A(2D-ATD), and GluN1/GluN2A( $\Delta$ ATD) receptors were analyzed, and the mean IC<sub>50</sub> values ( $\pm$ SEM) are shown. These data suggest that the S2 region transfers DQP-1105 sensitivity to GluN2A. *C*, Linear representation of the S2 regions of GluN2A(2D-S2) in which regions of GluN2D-S2 have been reverted to that of GluN2A to probe for loss of inhibition of DQP-1105. *D*, The inhibition produced by 5  $\mu$ M DQP-1105 in the presence of 1000  $\mu$ M glutamate and 300  $\mu$ M glycine is shown as a percent of control. Three chimeric receptors show significantly reduced inhibition as compared to GuN2A(S2) chimera ( $p < 0.05$ , 1-way ANOVA with Tukey's test,  $n = 5-13$  oocytes per chimeric receptor). *E*, Site-directed mutagenesis of residues within GluN2D S2 chimeric regions identified Gln701 and Leu705 (asterisks in *B*) as key structural determinants for antagonist activity of DQP-1105 at GluN2D receptors ( $p < 0.05$ , 1-way ANOVA with Tukey's test;  $n = 4-11$  oocytes per mutant receptor).

**Figure 8.** Homology models of GluN1-GluN2 receptors shows that residues identified in lower lobe of the clamshell shaped ligand binding domain are localized near each other in three-dimensional space, and in close proximity to the linkers that connect the ligand binding domain to the transmembrane helices. *A*, The *left panel* shows a GluN1-GluN2D receptor homology model (ATD omitted); the *right panel* shows a GluN1-GluN2A homology model (ATD omitted). *B*, The GluN2D-subunit is expanded (*left panel*) to show the lower portion of the ligand binding domain containing residues Q701 and L705 (red), which are critical divergent structural determinants for the antagonist activity of DQP-1105. The S1-M1 and M3-S2 linker regions are also shown in red. A GluN2A-subunit is expanded (*right panel*) to show the same region with the residues corresponding to GluN2D-Gln701 (Tyr in GluN2A, yellow) and GluN2D-Leu705 (Phe in GluN2A yellow); linkers are also shown in yellow. Compound DQP-1105 is shown in the lower panel on the same scale.

**Table 1: Structure-activity relationship for DQP analogues**



DQP-	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	GluN2A IC <sub>50</sub> (μM)	GluN2B IC <sub>50</sub> (μM)	GluN2C IC <sub>50</sub> (μM)	GluN2D IC <sub>50</sub> (μM)
1183(1)	Br	H	H	H	H	NE	NE	50	19
1184(2)	Br	H	H	F	H	NE	85	35	21
997(3)	Br	H	H	Br	H	78	19	5.4	2.7
2033(4)	Br	H	H	NO <sub>2</sub>	H	NE	43	8.4	16
1185(5)	Br	H	H	CH <sub>3</sub>	H	NE	59	15	8.9
1128(6)	Br	H	H	OCH <sub>3</sub>	H	NE	NE	NE	NE
2003(7)	Br	OCH <sub>3</sub>	H	H	OCH <sub>3</sub>	NE	NE	75	51
1178(8)	Br	F	H	H	H	NE	NE	NE	23
1209(9)	Br	Cl	H	H	H	79	35	22	13
1308(18)	CH <sub>3</sub>	H	H	H	H	NE	NE	63	31
1105(19)	CH <sub>3</sub>	H	H	Br	H	NE	113	7.0	2.7
2005(20)	CH <sub>3</sub>	OCH <sub>3</sub>	H	H	H	NE	NE	NE	NE

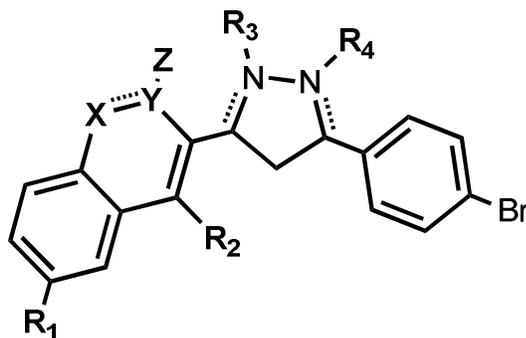
Fitted IC<sub>50</sub> values were determined for the indicated combinations of recombinant GluN1/GluN2 receptors expressed in *Xenopus* oocytes and activated by 100 μM glutamate plus 30 μM glycine.

Compounds that produced less than 25% inhibition at 30 μM were considered to have no effect (NE).

IC<sub>50</sub> values were determined from composite concentration-effect curves as described in the *Methods*.

Hill slopes were in the range 0.6-1.2. Experiments were performed on 6-22 oocytes from 2-5 frogs for all receptors. The asterisk (\*) in the molecule indicates the chiral center.

**Table 2: Backbone Modification DQP analogues**



DQP-	R <sub>1</sub>	X-Y-Z	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	GluN2A IC <sub>50</sub> (μM)	GluN2B IC <sub>50</sub> (μM)	GluN2C IC <sub>50</sub> (μM)	GluN2D IC <sub>50</sub> (μM)
<b>997(3)</b>	Br	-NH-C(O)-	C <sub>6</sub> H <sub>5</sub>	-	-COCH <sub>2</sub> CH <sub>2</sub> CO <sub>2</sub> H	78	19	5.4	2.7
<b>1309(21)</b>	Br	-NH-C(O)-	C <sub>6</sub> H <sub>5</sub>	-	-COCH <sub>2</sub> CH <sub>3</sub>	NE	NE	NE	NE
<b>1210(22)</b>	Br	-N=C(CH <sub>3</sub> )-	C <sub>6</sub> H <sub>5</sub>	-	-COCH <sub>2</sub> CH <sub>2</sub> CO <sub>2</sub> H	NE	NE	26	17
<b>1105(19)</b>	CH <sub>3</sub>	-NH-C(O)-	C <sub>6</sub> H <sub>5</sub>	-	-COCH <sub>2</sub> CH <sub>2</sub> CO <sub>2</sub> H	NE	113	7.0	2.7
<b>1250(24)</b>	CH <sub>3</sub>	-NH-C(O)-	C <sub>6</sub> H <sub>5</sub>	-	-COCH <sub>3</sub>	NE	NE	NE	NE
<b>1177(25)</b>	CH <sub>3</sub>	-NH-C(O)-	-	-COCH <sub>2</sub> CH <sub>2</sub> CO <sub>2</sub> H	-	NE	NE	NE	NE

The fitted IC<sub>50</sub> values were determined for the indicated combinations of recombinant GluN1/GluN2 receptors expressed in *Xenopus* oocytes and activated by 100 μM glutamate plus 30 μM glycine.

Compounds that produced less than 25% inhibition at 30 μM were considered to have no effect (NE).

IC<sub>50</sub> values are given to two significant digits, and were determined from composite concentration-effect curves as described in the *Methods*. Experiments were performed on 6-22 oocytes from 2-5 frogs for all receptors. Compounds **3** and DQP-1105 in Table 2 are shown here to facilitate comparison.

**Table 3: Concentration-response data for DQP-1105 at ionotropic glutamate receptors**

	Agonist	GluN2A IC <sub>50</sub> (μM)	GluN2B IC <sub>50</sub> (μM)	GluN2C IC <sub>50</sub> (μM)	GluN2D IC <sub>50</sub> (μM)	GluA1 IC <sub>50</sub> (μM)	GluK2 IC <sub>50</sub> (μM)
Oocytes TEVC (rat)	Glutamate	206 ± 36	121 ± 32	8.5 ± 1.2	2.7 ± 0.2	198 ± 44	153 ± 9.1
Oocytes TEVC (rat)	NMDA	135 ± 66	43 ± 4	2.4 ± 0.2	1.5 ± 0.2	--	--
Oocytes TEVC (human)	Glutamate	NE	206 ± 137	5.4 ± 1.0	2.2 ± 0.1	--	--
BHK Ca <sup>2+</sup> Imaging (rat)	NMDA	85 ± 24	NE	1.4 ± 0.5	1.6 ± 0.5	--	--
HEK perforated patch (rat)	Glutamate	54	--	--	2.1	--	--
HEK dialyzed patch (rat)	Glutamate	12	--	--	3.2	--	--
HEK dialyzed patch (rat)	NMDA	7.4	--	--	1.9	--	--

Mean IC<sub>50</sub> values (± SEM) for inhibition of responses in oocytes to 100 μM glutamate and 30 μM glycine were determined as described in the *Methods*. Oocyte experiments were performed in 3-44 oocytes from 1-4 frogs. Oocytes assayed using NMDA as agonist were activated using 50 – 1000 μM NMDA and 50 μM glycine. BHK cell imaging results are from 3 or more independent experiments (*Methods*). For HEK perforated patch experiments, responses were evoked by 100 μM glutamate and 30 μM glycine in 6-8 cells. From HEK whole-cell experiments using conventional dialyzed patches, data are from 6-8 cells activated by 100 μM glutamate or 1000 μM NMDA plus 30 μM glycine. Mean fitted Hill slopes for GluN1/GluN2C and GluN1/GluN2D were between 0.87-1.14; Hill slope was fixed to 1.0 for GluN1/GluN2A, GluN1/GluN2B, GluA1, and GluK2. NE indicates that there was no detectable effect at the concentrations evaluated; -- indicates that conditions were not assayed.

**Table 4. DQP-1105 decreases open Probability of GluN1/GluN2D receptor channels**

	<b>Control</b>	<b>3 <math>\mu</math>M DQP-1105</b>	<b>30 <math>\mu</math>M DQP-1105</b>
<b>P<sub>OPEN</sub></b>	0.10 $\pm$ 0.03	0.038 $\pm$ 0.008	0.0018 $\pm$ 0.0005
<b>P<sub>OPEN</sub> (%)</b>	100	43 $\pm$ 12*	2.0 $\pm$ 0.6*
<b>Mean Shut Time (ms)</b>	6.9 $\pm$ 1.4	17 $\pm$ 3*	460 $\pm$ 130*
<b>Mean Open Time (ms)</b>	0.61 $\pm$ 0.03	0.62 $\pm$ 0.02	0.64 $\pm$ 0.018
<b><math>\tau</math>1, ms (Area, %)</b>	0.038 (31%)	0.032 (32%)	0.030 (42%)
<b><math>\tau</math> 2, ms (Area, %)</b>	0.62 (69%)	0.64 (68%)	0.65 (58%)
<b><math>\gamma</math>1 (pS)</b>	36.4 $\pm$ 1.5	40.8 $\pm$ 1.2*	38 $\pm$ 2
<b>Area <math>\gamma</math>1 (%)</b>	32 $\pm$ 3	30. $\pm$ 6	26 $\pm$ 3
<b><math>\gamma</math> 2 (pS)</b>	63 $\pm$ 2	65 $\pm$ 2	65 $\pm$ 2
<b>Area <math>\gamma</math>2 (%)</b>	68 $\pm$ 3	70. $\pm$ 7	74 $\pm$ 3

GluN1/GluN2D receptors were expressed in HEK cells and activated by glutamate (1 mM) and glycine (50  $\mu$ M) in outside-out patches held under voltage clamp at -80 mV. Responses were recorded first to glutamate plus glycine (7862 open periods), and subsequently to glutamate plus glycine co-applied with 3  $\mu$ M (5724 open periods) or 30  $\mu$ M DQP-1105 (954 open periods) to the same patch. All values are mean  $\pm$  SEM (n=4 patches). \*p<0.05 versus control (One-way ANOVA with Dunnett's post test). Since all patches contained multiple channels, P<sub>OPEN</sub> was normalized to the value for the patch in the absence of DQP-1105 for statistical testing.

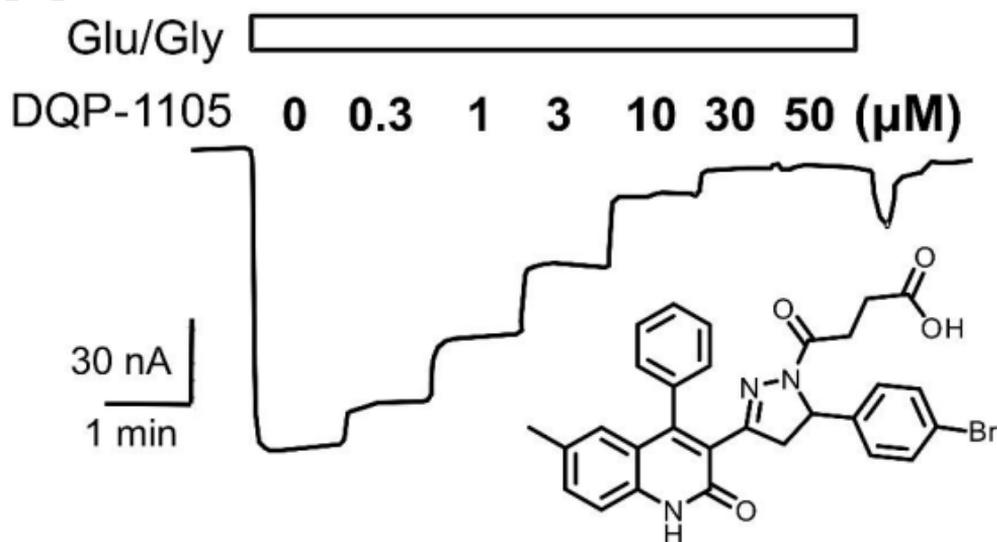
**Table 5: Amino acid numbering and composition for chimeric receptors**

GluN2A	1-1464	
GluN2D		1-1323
2A(2D-ATD)	405-1464	1-427
2A(2D-S1)	1-388; 539-1464	412-563
2A(2D- S2)	1-660; 812-1464	686-836
2A(2D-S1S2)	1-388; 539-660; 812-1464	412-563; 686-836
2A(2D- M1M2M3)	1-552; 661-1464	578-685
2D(2A- ATD)	1-404	428-1323
2D-ΔATD		428-1323
2A(2D-S2a)	1-673; 798-1464	699-822
2A(2D-S2b)	1-660; 674-679; 798-1464	686-698; 705-822
2A(2D-S2c)	1-660; 680-691; 798-1464	686-704; 717-822
2A(2D-S2d)	1-660; 692-699; 798-1464	686-716; 725-822
2A(2D-S2e)	1-660; 700-707; 798-1464	686-724; 733-822
2A(2D-S2f)	1-660; 708-714; 798-1464	686-732; 740-822
2A(2D-S2g)	1-660; 715-737; 798-1464	686-739; 763-822
2A(2D-S2h)	1-660; 738-753; 798-1464	686-762; 779-822
2A(2D-S2i)	1-660; 754-765; 798-1464	686-778; 791-822
2A(2D-S2j)	1-660; 766-782; 798-1464	686-790; 808-822
2A(2D-S2k)	1-660; 783-789; 798-1464	686-807; 815-822
2A(2D-S2l)	1-660; 790-1464	686-814

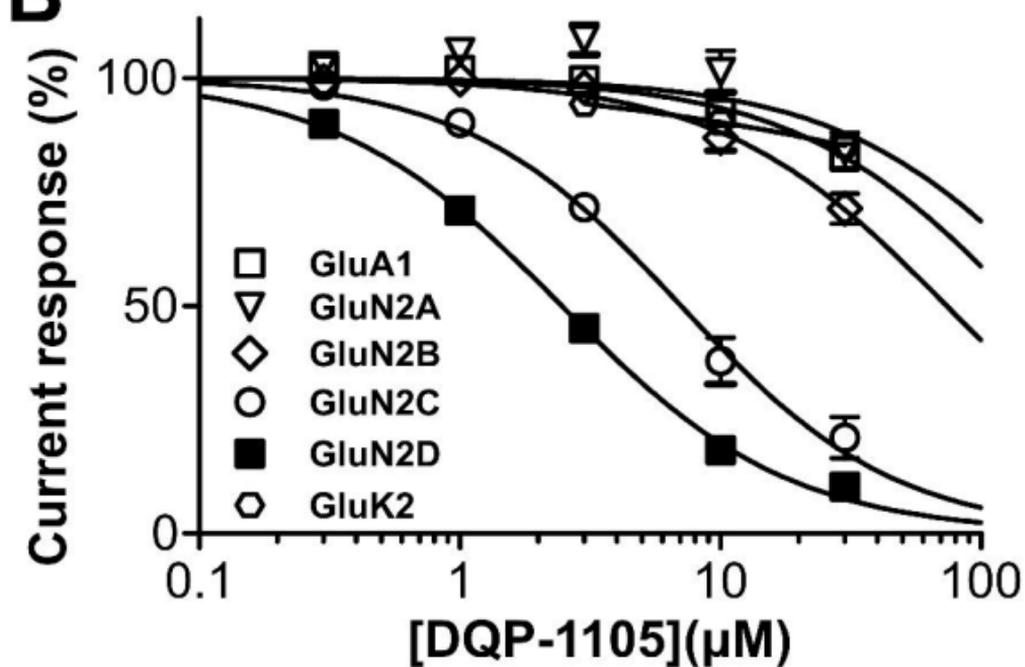
Amino acid numbering and composition for chimeric GluN2A-GluN2D subunits. Amino acid numbering is according to the full-length GluN2 subunits, including the signal peptide (initiating methionine is 1).

FIGURE 1

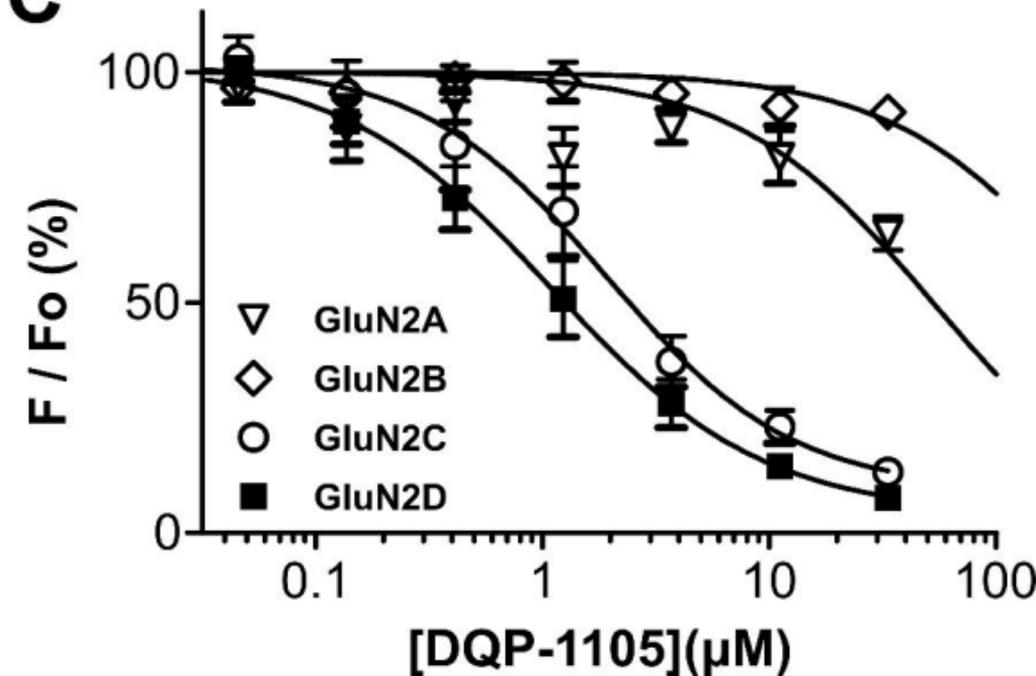
**A**



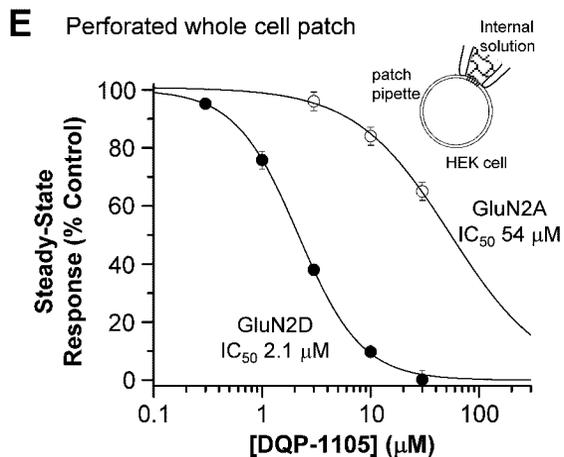
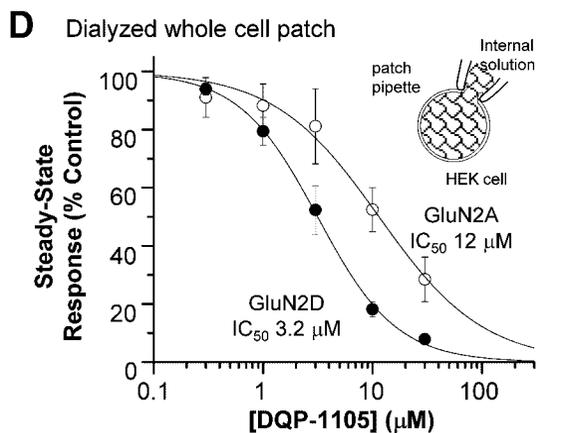
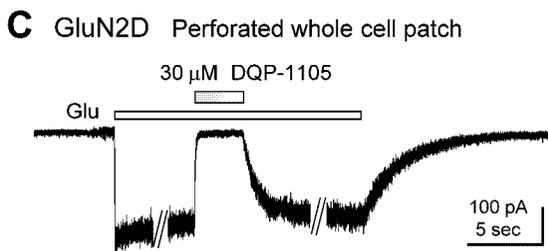
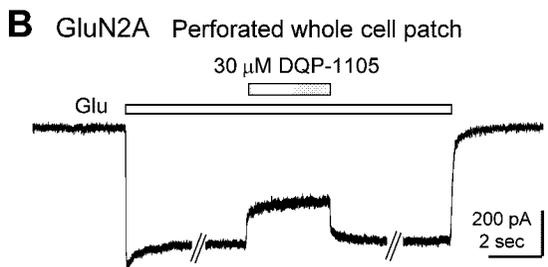
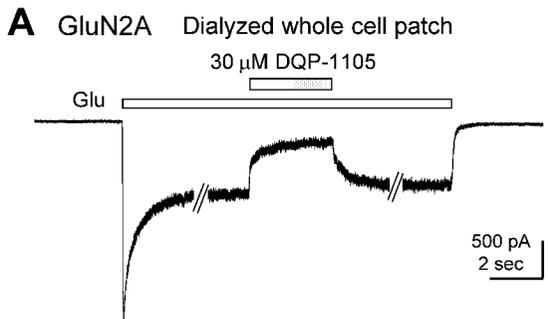
**B**



**C**

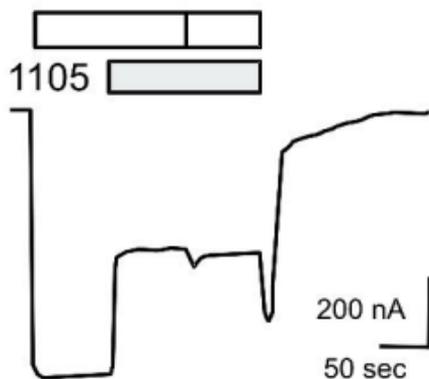


**FIGURE 2**

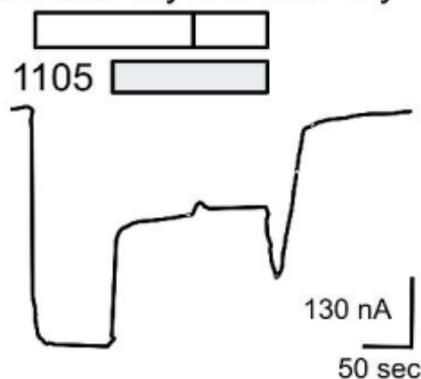


**FIGURE 3****A**

0.1 mM Glu 1 mM Glu



0.03 mM Gly 0.3 mM Gly

**B**

GluN1/GluN2D

Holding potential (mV)

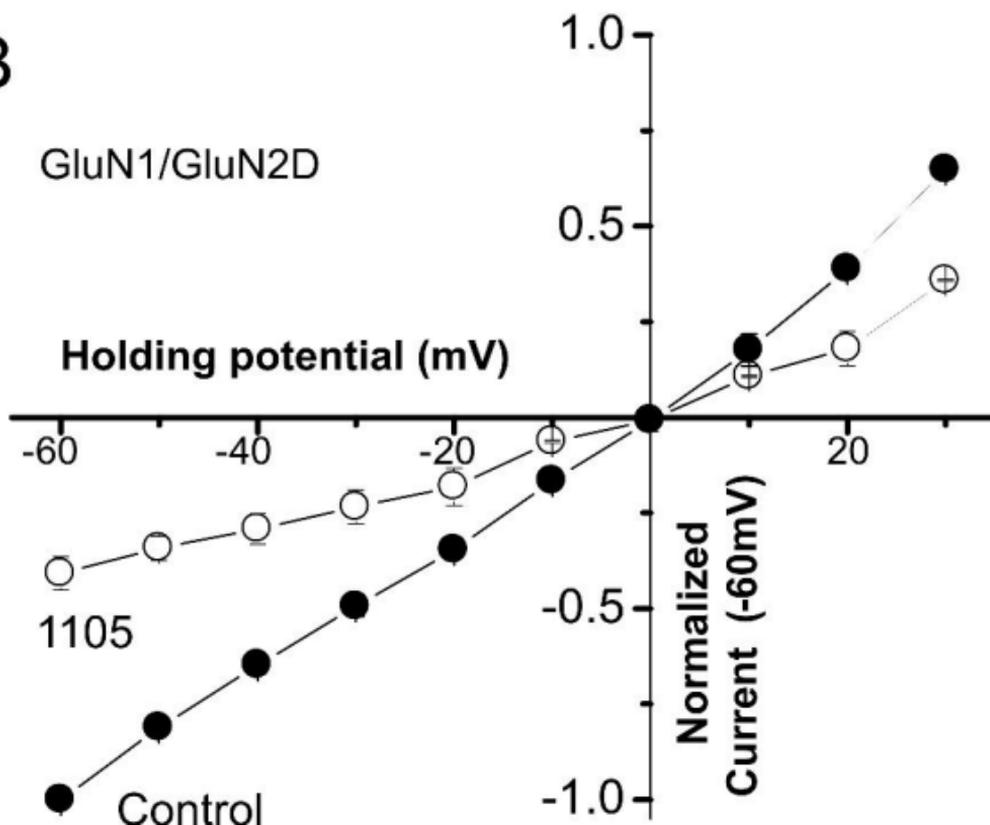
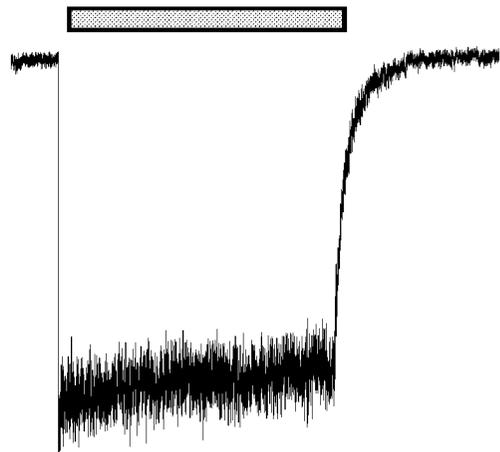


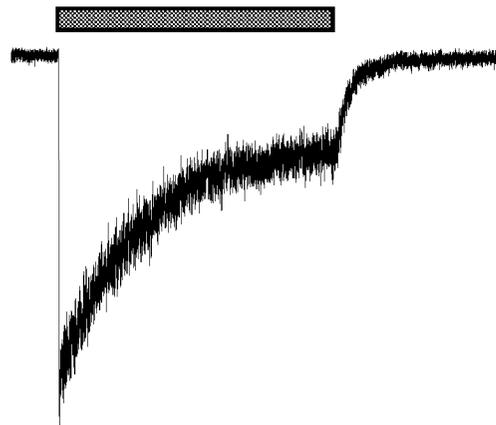
FIGURE 4

**A**

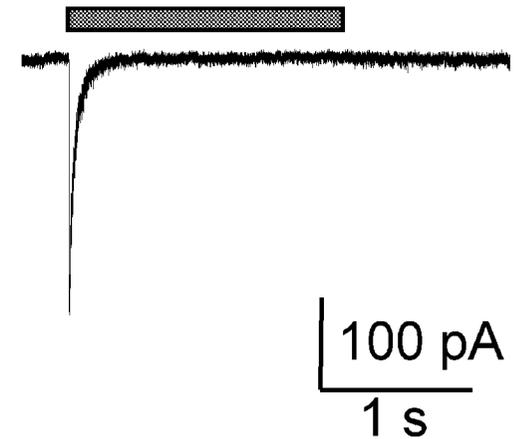
1000  $\mu\text{M}$  NMDA



1000  $\mu\text{M}$  NMDA  
3  $\mu\text{M}$  DQP-1105



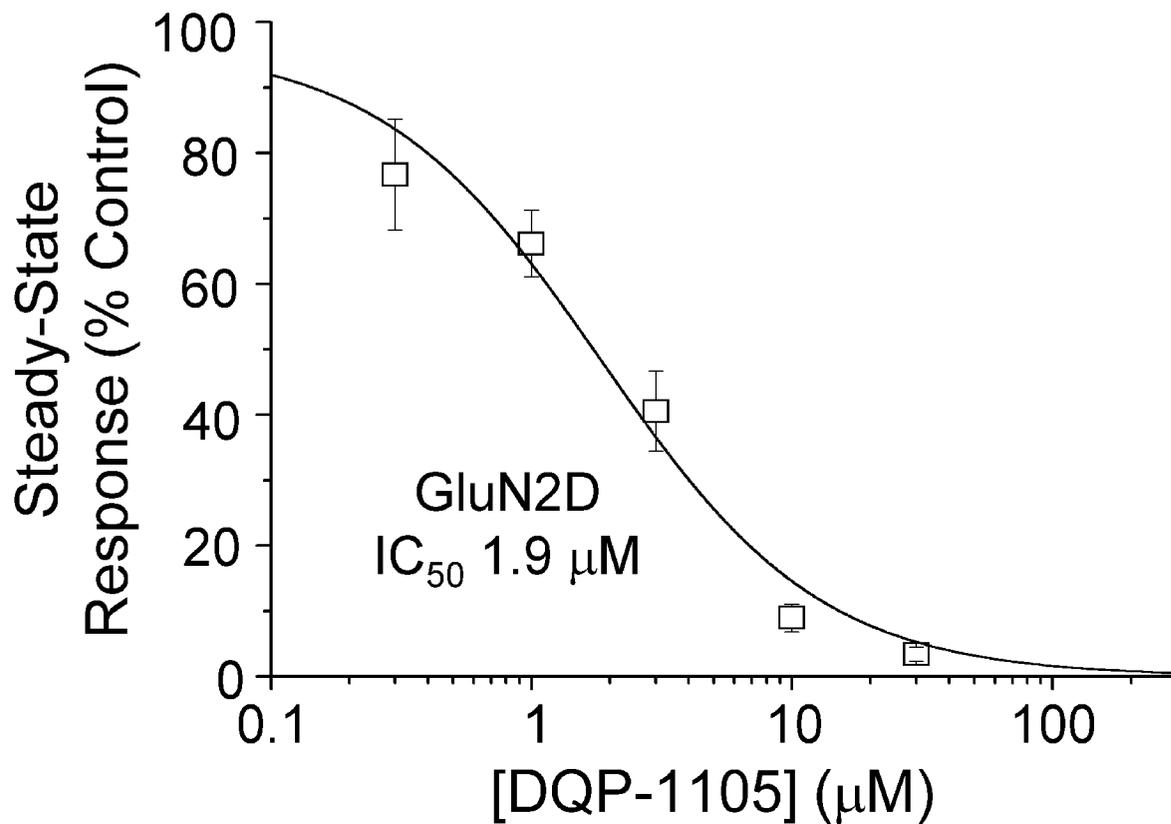
1000  $\mu\text{M}$  NMDA  
30  $\mu\text{M}$  DQP-1105



100 pA  
1 s

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2014

**B**



**C**

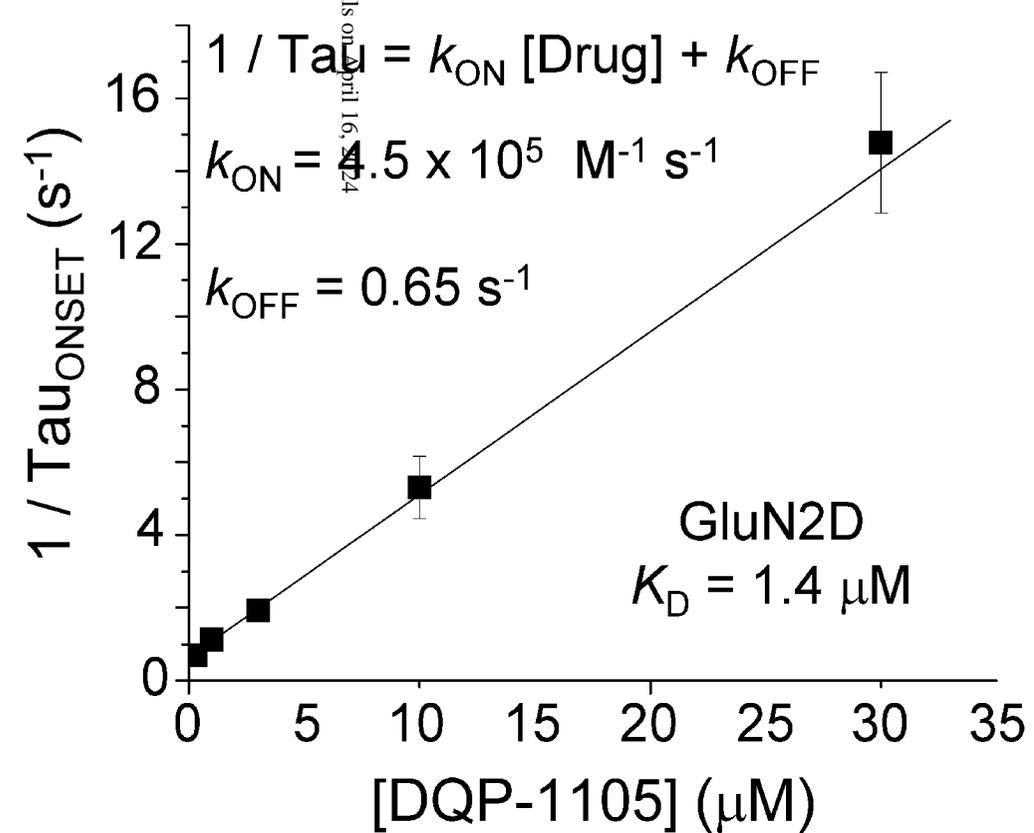


FIGURE 5

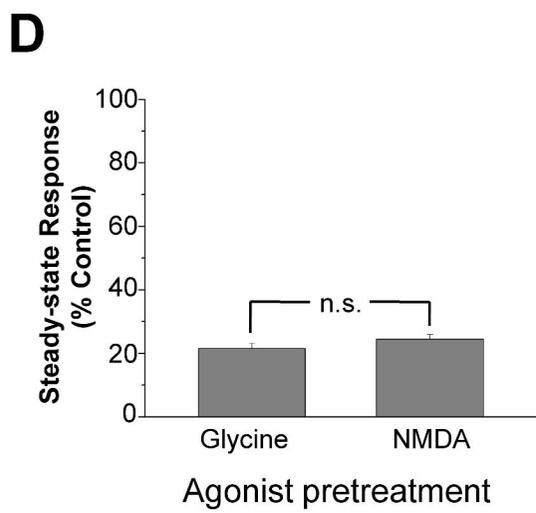
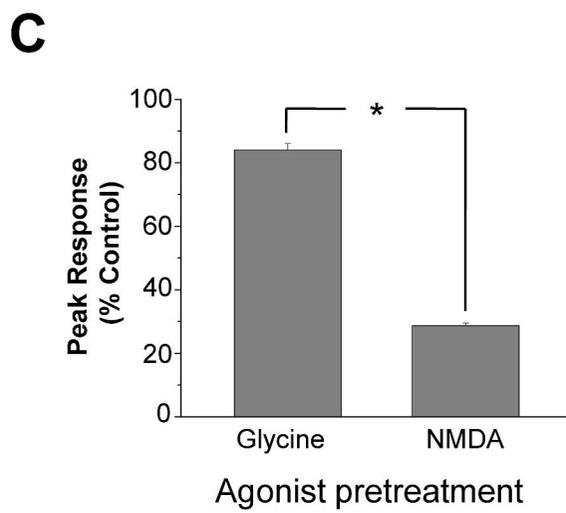
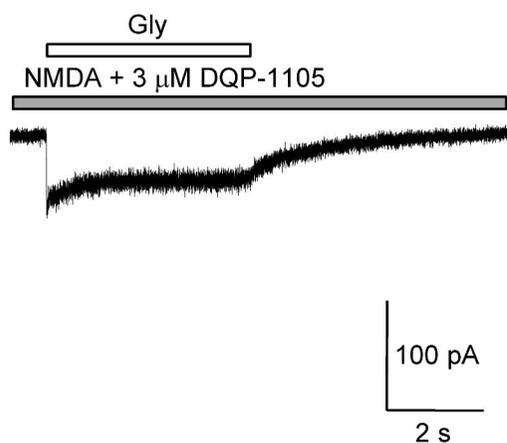
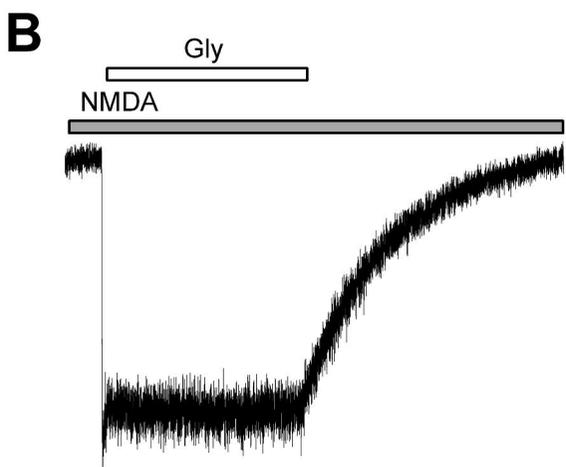
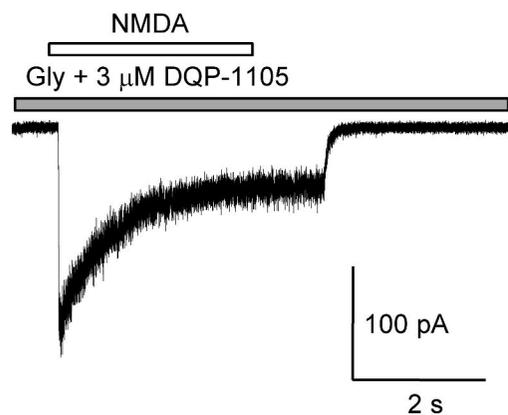
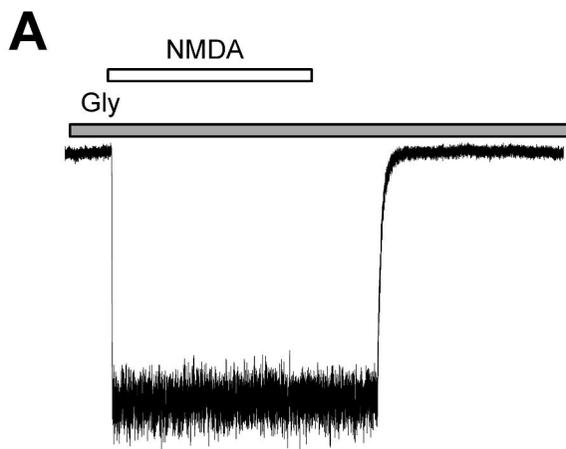


FIGURE 6

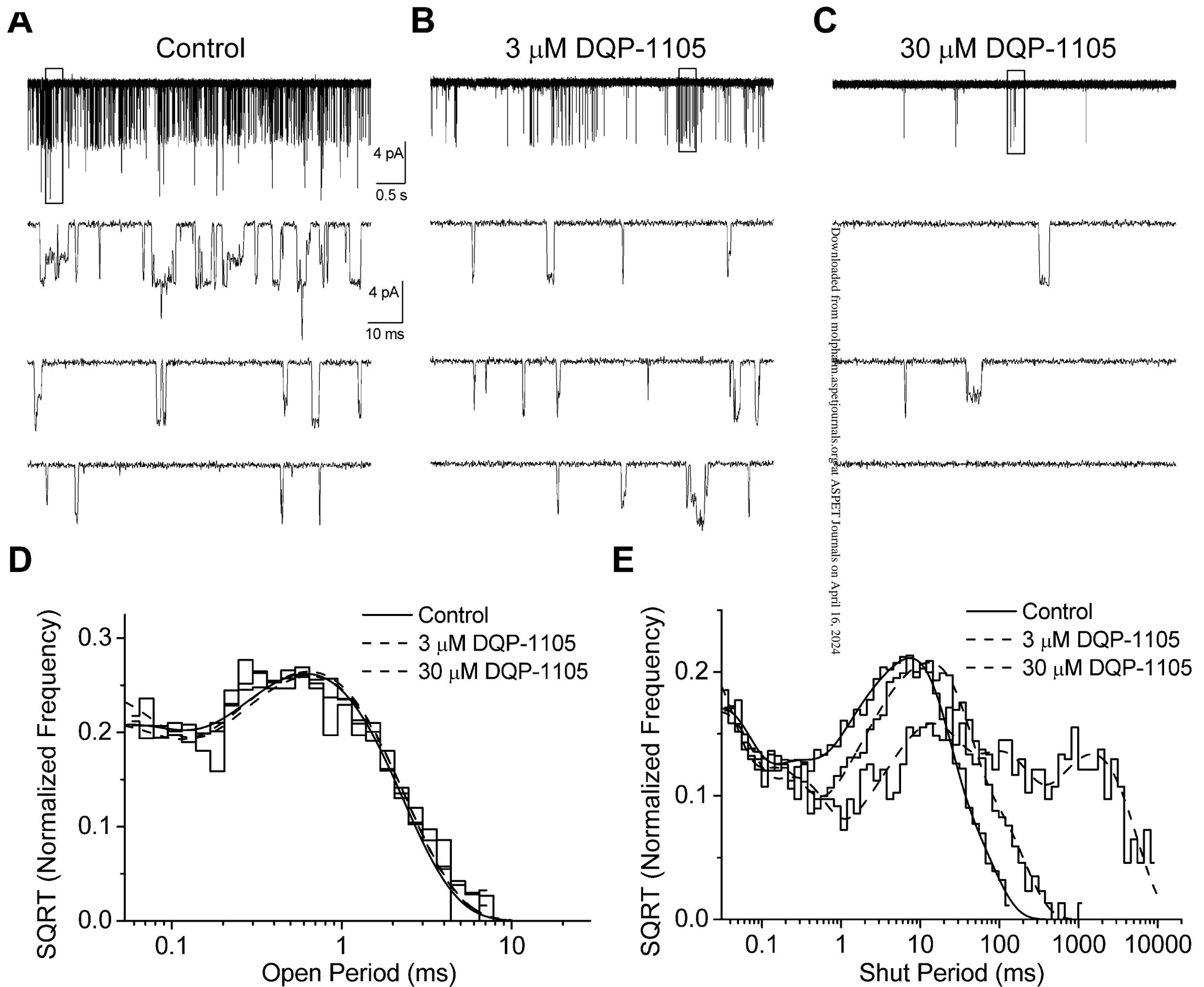
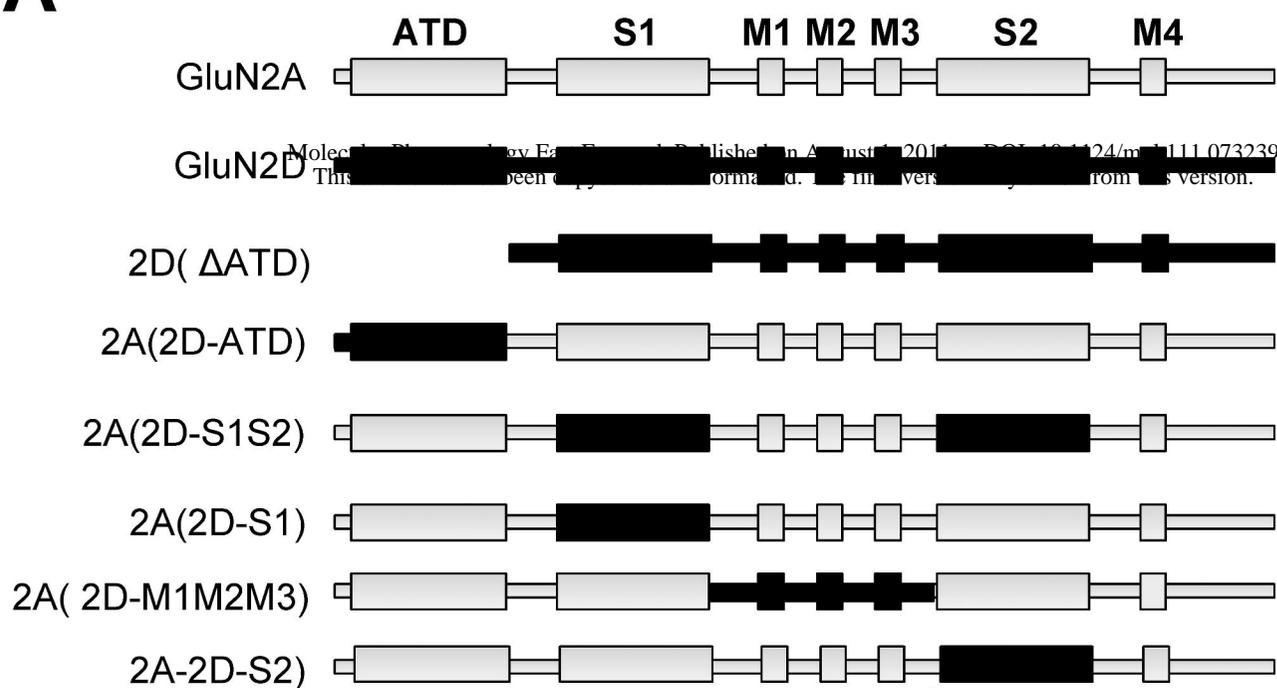
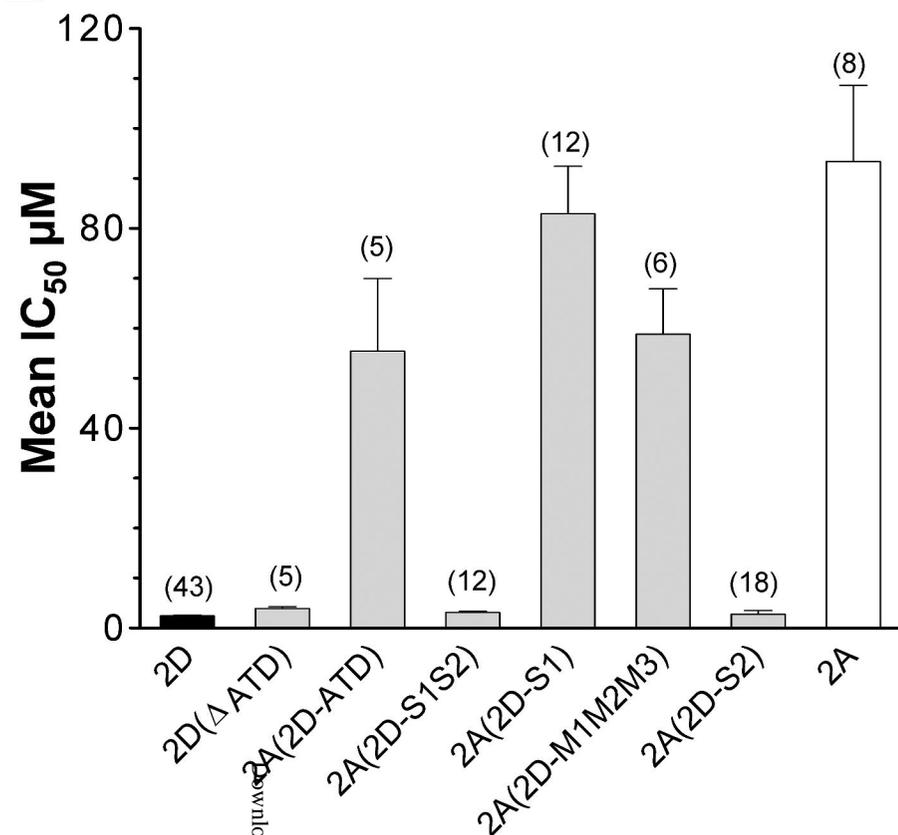


FIGURE 7

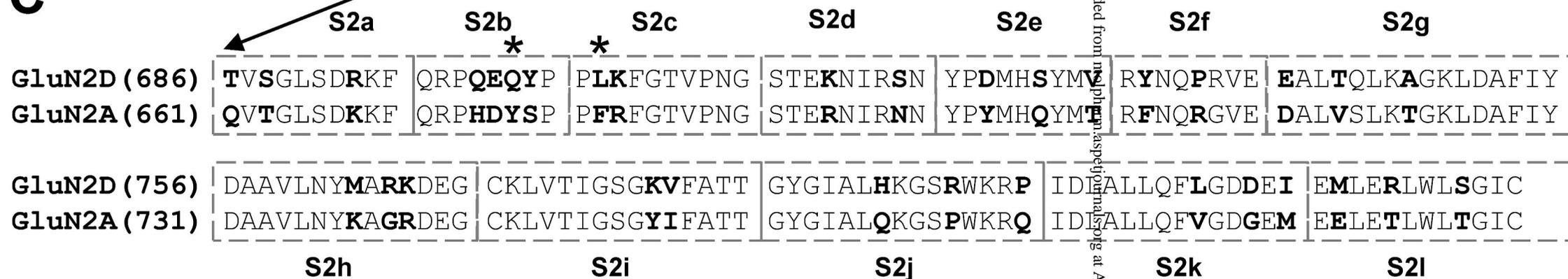
**A**



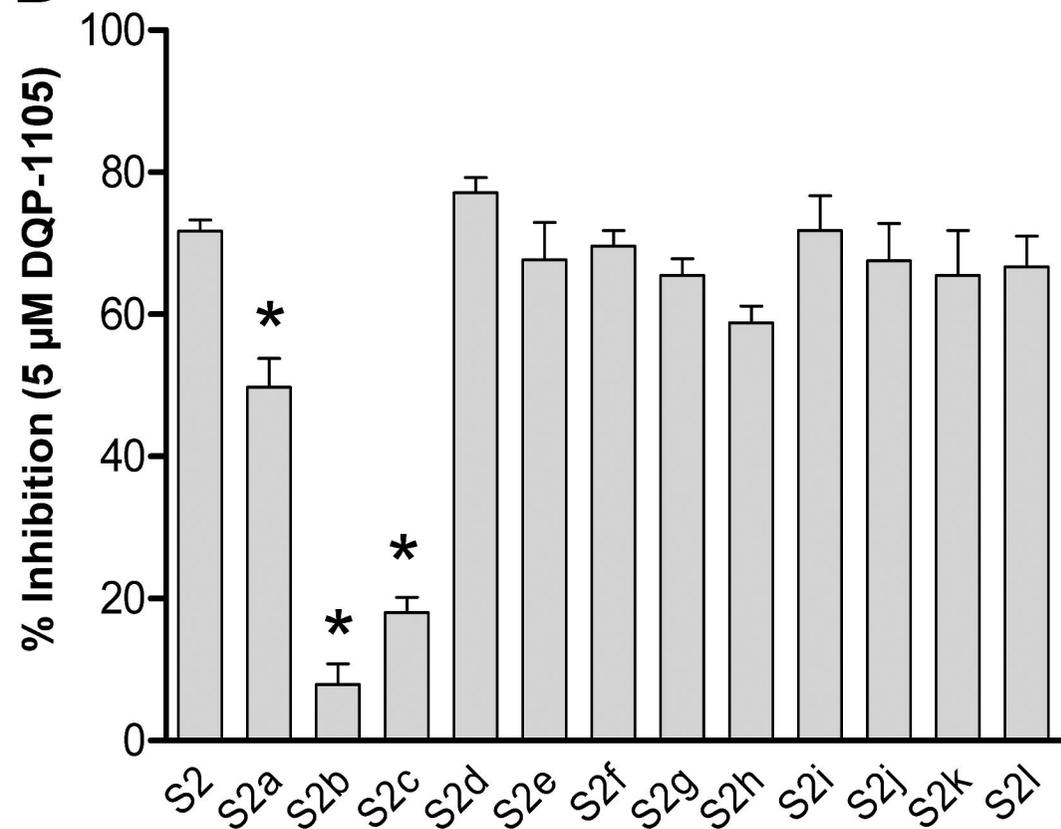
**B**



**C**



**D**



**E**

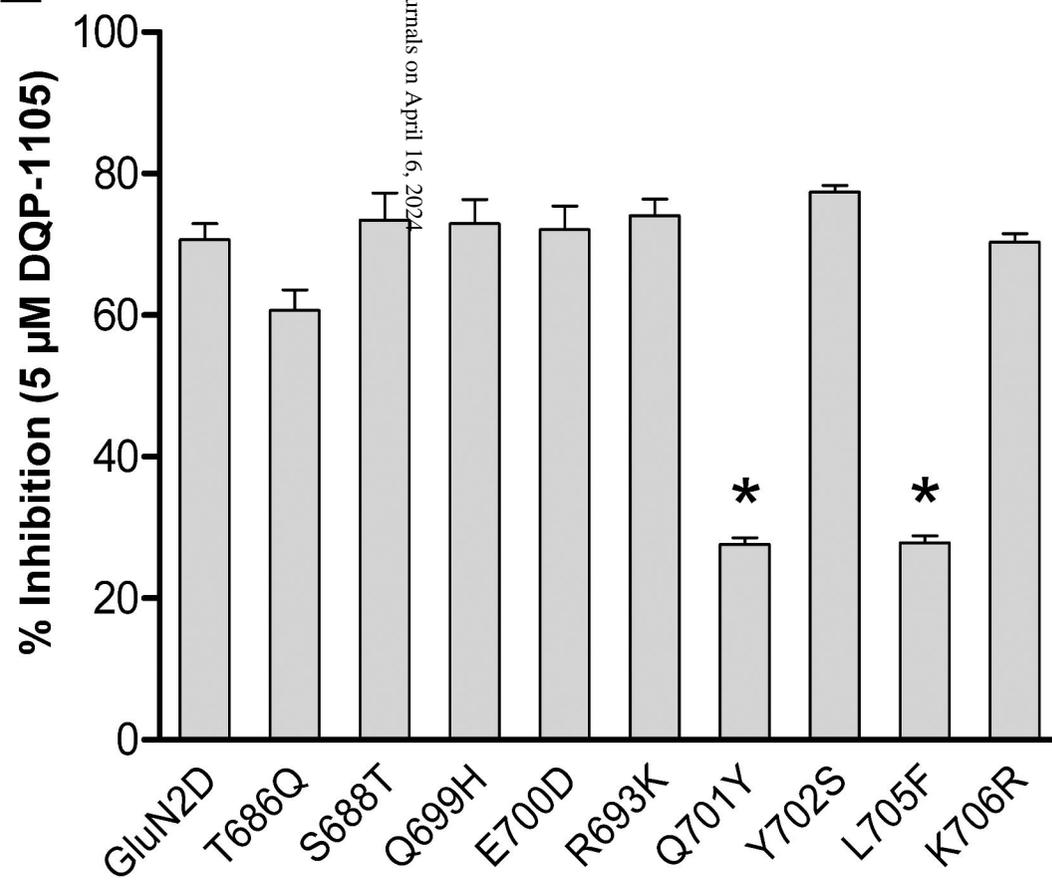
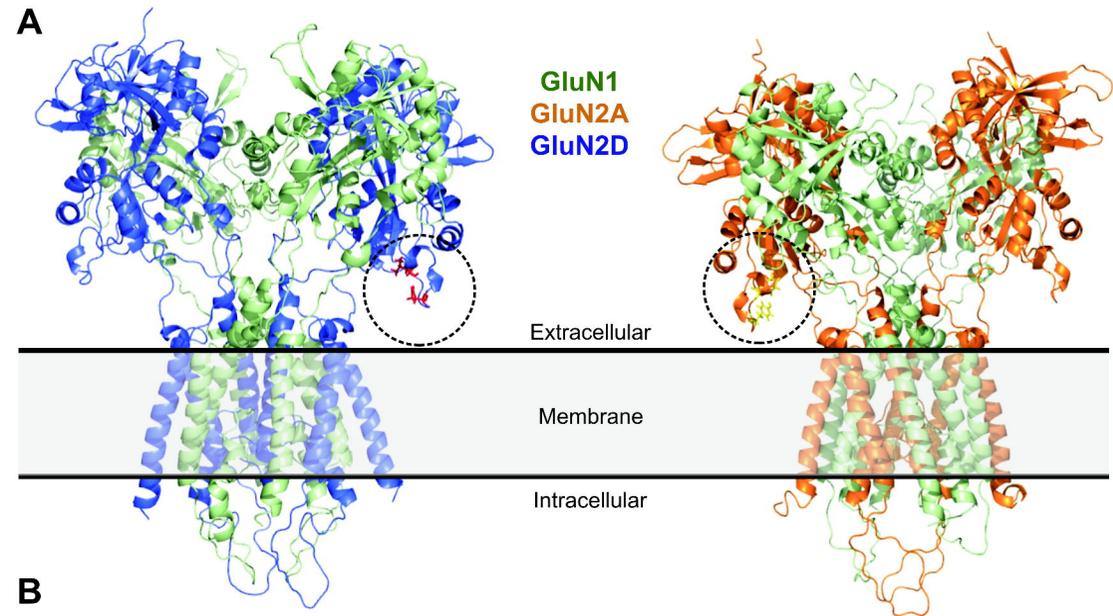


FIGURE 8



**B**

