N-Methyl-D-aspartate (NMDA) Receptor NR2 Subunit Selectivity of a Series of Novel Piperazine-2,3-dicarboxylate Derivatives: Preferential Blockade of Extrasynaptic NMDA Receptors in the Rat Hippocampal CA3-CA1 Synapse

Blaise Mathias Costa, Bihua Feng,¹ Timur S. Tsintsadze, Richard M. Morley,² Mark W. Irvine, Vera Tsintsadze, Natasha A. Lozovaya,³ David E. Jane, and Daniel T. Monaghan

Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, Nebraska (B.M.C., B.F., D.T.M.); Department of Cellular Membranology, Bogomoletz Institute of Physiology, Kiev, Ukraine (T.S.T., V.T., N.A.L.); and Department of Physiology & Pharmacology, Medical Research Council Center for Synaptic Plasticity, University of Bristol, Bristol, United Kingdom (R.M.M., M.W.I., D.E.J.)

Received May 28, 2009; accepted August 13, 2009

ABSTRACT

N-Methyl-D-aspartate (NMDA) receptor antagonists that are highly selective for specific NMDA receptor 2 (NR2) subunits have several potential therapeutic applications; however, to date, only NR2B-selective antagonists have been described. Whereas most glutamate binding site antagonists display a common pattern of NR2 selectivity, NR2A > NR2B > NR2C > NR2D (high to low affinity), (2*S**,3*R**)-1-(phenanthrene-2-carbonyl)piperazine-2,3-dicarboxylic acid (PPDA) has a low selectivity for NR2C- and NR2D-containing NMDA receptors. A series of PPDA derivatives were synthesized and then tested at recombinant NMDA receptors expressed in *Xenopus laevis* oocytes. In addition, the optical isomers of PPDA were resolved; the (–) isomer displayed a 50- to 80-fold greater potency than the (+) isomer. Replacement of the phenanthrene moiety of PPDA with naphthalene or anthracene did not improve selectivity. However, phenylazobenzoyl (UBP125) or phenylethynylbenzoyl (UBP128) substitution significantly improved selectivity for NR2B-, NR2C-, and NR2D-containing receptors over NR2A-containing NMDA receptors. Phenanthrene attachment at the 3

position [(2*R**,3*S**)-1-(phenanthrene-3-carbonyl)piperazine-2,3-dicarboxylic acid (UBP141); (2*R**,3*S**)-1-(9-bromophenanthrene-3-carbonyl)piperazine-2,3-dicarboxylic acid (UBP145); (2*R**,3*S**)-1-(9-chlorophenanthrene-3-carbonyl)piperazine-2,3-dicarboxylic acid (UBP160); and (2*R**,3*S**)-1-(9-iodophenanthrene-3-carbonyl)piperazine-2,3-dicarboxylic acid (UBP161)] displayed improved NR2D selectivity. UBP141 and its 9-brominated homolog (UBP145) both display a 7- to 10- fold selectivity for NR2D-containing receptors over NR2B- or NR2A-containing receptors. Schild analysis indicates that these two compounds are competitive glutamate binding site antagonists. Consistent with a physiological role for NR2D-containing receptors in the hippocampus, UBP141 (5 μ M) displayed greater selectivity than PPDA for inhibiting the slow-decaying component of the NMDA receptor-mediated CA3-CA1 synaptic response in rat hippocampal slices. UBP125, UBP128, UBP141, and UBP145 may be useful tools for determining the function of NMDA receptor subtypes.

N-Methyl-D-aspartate (NMDA) receptors are a family of glutamate-gated ion channel receptors that play important roles in synaptic transmission and neuronal plasticity; they are also

involved in a wide variety of pathological conditions, such as epilepsy (Meldrum, 2002), neuropathic pain (Childers and Baudy, 2007), and neuronal loss following stroke (Choi, 1998). NMDA receptors are multimeric complexes composed of subunits from at least two families, NR1A–H and NR2A–D (Nakanishi, 1992; Monyer et al., 1994; Mori and Mishina, 1995). In addition, some NMDA receptors may contain an NR3 subunit (Tong et al., 2008). The NR2 subunits contain the glutamate-binding site of the receptor complex (Laube et al., 1997). Accordingly, the NR2 subunit determines the glutamate-site pharmacological properties of the NMDA receptor complex (Ishii et al., 1993; Buller et al., 1994; Laurie and Seeburg, 1994; Buller and Monaghan, 1997).

This work was supported by the National Institutes of Health [Grant MH60252] (to D.T.M. and D.E.J.).

¹ Current affiliation: Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts.

² Current affiliation: Tocris Cookson Ltd., Avonmouth, Bristol, United Kingdom.

³ Current affiliation: INMED-INSERM U29, Université de la Méditerranée, Marseille, France.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.109.156752.

It is now apparent that the different NR2 subunits underlie differing physiological and cell-signaling properties in NMDA receptor complexes. However, in the absence of antagonists that are highly selective for each of the individual NR2 subunits, progress has been slow in identifying their respective roles in synaptic and brain function. NR2 subtype-selective NMDA receptor antagonists are also likely to have novel therapeutic/adverse effect profiles because these subunits differ significantly in their anatomical and physiological properties (Ishii et al., 1993; Monyer et al., 1994; Vicini et al., 1998). For example, a NR2D-selective antagonist would be expected to be effective in some cases of neuropathic pain (Minami et al., 2001), and NR2C-selective antagonists may be useful in blocking ischemic white matter injury (Kárádóttir et al., 2005).

A major difficulty in developing subunit-specific glutamate binding site antagonists is the highly conserved structure of the glutamate binding pocket (Kinarsky et al., 2005). Although there is significant amino acid sequence variation between NR2 subunits within the glutamate binding site domain (segments S1 and S2), receptor-modeling studies have shown that the variable amino acids are located distant to the glutamate binding site. As a consequence, the commonly used NMDA receptor antagonists that occupy the core of the glutamate binding pocket [e.g., (*R*)-AP5 and (*R*)-CPP] display a similar pattern of NR2 selectivity, high to low affinity, in the order of NR2A > NR2B > NR2C > NR2D (Ikeda et al., 1992; Buller et al., 1994; Laurie and Seeburg, 1994; Feng et al., 2005). Although small competitive antagonists display some structure-activity relationships that vary the degree of this selectivity pattern (Feng et al., 2005), compounds with bulky aromatic substituents, such as NVP-AAM077 (Auberson et al., 2002), EAB-515 (Urwiler et al., 1996), PPDA (Morley et al., 2005), and 4-propyl *N*-hydroxy-pyrazol-5-yl glycine (Clausen et al., 2008) have been shown to display an atypical pattern of NMDA receptor subunit selectivity. The altered subunit-specificity of large competitive antagonists may be due to antagonist interactions with subunit-specific amino acid residues at the edge of the glutamate binding pocket (Kinarsky et al., 2005; Clausen et al., 2008).

The compound PPDA is distinct in having a 2- to 5-fold higher affinity for NR2C- or NR2D-containing receptors than that for receptors containing either NR2A or NR2B (Feng et al., 2004; Morley et al., 2005). Although PPDA has a low degree of selectivity, studies comparing its action to other competitive antagonists have been able to identify differing

physiological actions for NMDA receptors containing different NR2 subunits. By contrasting the actions of PPDA with (*R*)-(*E*)-4-(3-phosphonoprop-2-enyl)piperazine-2-carboxylic acid [(*R*)-CPPene] and (*R*)-AP5, it was possible to first demonstrate that long-term potentiation (LTP) and long-term depression (LTD) are mediated by pharmacologically distinct NMDA receptor populations (Hrabětová et al., 2000). Likewise, comparing PPDA with (*R*)-CPP reveals two pharmacologically distinct NMDA receptor-mediated components of the CA3-CA1 hippocampal synapse response (Lozovaya et al., 2004). PPDA causes a small, preferential block of the slow-decaying NMDA receptor synaptic current, whereas (*R*)-CPP selectively blocks the fast-decaying peak response of the NMDA receptor synaptic current. In the initial studies, we found that the PPDA derivative, UBP141, displayed an improved NR2D and NR2C selectivity over PPDA (Morley et al., 2005). In the present study, we extend the structure-activity analysis around the PPDA and UBP141 structures and demonstrate that UBP141 and UBP145 are modestly selective competitive glutamate site antagonists. We also find that UBP125 and UBP128 are modestly selective for NMDA receptors that do not contain NR2A.

Materials and Methods

Compounds. Structures of compounds synthesized and tested for this report are shown in Fig. 1. Compounds were synthesized by the reaction of the appropriately substituted acid chloride with (2*S**,3*R**)-piperazine-2,3-dicarboxylic acid (Morley et al., 2005) under modified Schotten-Bauman conditions (full details will be reported elsewhere). Following synthesis and purification, compound structure was verified by ¹H NMR and mass spectroscopy. All compounds had elemental analyses where the determined percentage C, H, and N were less than 0.4% different from theoretical values.

NR Subunit Expression in *Xenopus laevis* Oocytes. cDNA encoding the NMDAR1a and NMDAR1g (NR1-14b) subunits were a generous gift of Dr. Shigetada Nakanishi (Kyoto, Japan). cDNA encoding the NR2A, NR2C, and NR2D were kindly provided by Dr. Peter Seeburg (Heidelberg, Germany), and the NR2B (5'-untranslated region) cDNA was the generous gift of Drs. Dolan Pritchett and David Lynch (Philadelphia, PA). Plasmids were linearized with NotI (NR1a), EcoRI (NR2A, NR2C, and NR2D), or SalI (NR2B) and transcribed in vitro with T3 (NR2A and NR2C), SP6 (NR2B), or T7 (NR1a and NR2D) RNA polymerase using the mMessage mMachine Transcription Kits (Ambion, Austin, TX).

Oocytes were removed from mature female *X. laevis* (*Xenopus* One, Ann Arbor, MI) and prepared as described previously (Buller et al., 1994). All animal procedures were performed in accordance with

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; AMPA, 2-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; NR2, NMDA receptor subunit 2; NVP-AAM077, [(*R*)-[(*S*)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid; (*R*)-AP5, (*R*)-2-amino-5-phosphonopentanoate; (*R*)-CPP, (*R*)-4-(3-phosphonopropyl)piperazine-2-carboxylic acid; (*R*)-CPPene, (*R,E*)-4-(3-phosphonoprop-2-enyl)piperazine-2-carboxylic acid; PPDA, (2*R**,3*S**)-1-(phenanthrene-2-carbonyl)piperazine-2,3-dicarboxylic acid; UBP125, (2*R**,3*S**)-1-(4-phenylazobenzoyl)piperazine-2,3-dicarboxylic acid; UBP128, (2*R**,3*S**)-1-(4-phenylethynylbenzoyl)piperazine-2,3-dicarboxylic acid; UBP129, (2*R**,3*S**,*E*)-1-(3-naphthalen-2-ylacryloyl)piperazine-2,3-dicarboxylic acid; UBP133, (2*R**,3*S**)-1-(9-bromophenanthrene-2-carbonyl)piperazine-2,3-dicarboxylic acid; UBP136, (2*R**,3*S**)-1-(anthracene-2-carbonyl)piperazine-2,3-dicarboxylic acid; UBP141, (2*R**,3*S**)-1-(phenanthrene-3-carbonyl)piperazine-2,3-dicarboxylic acid; UBP143, (2*R**,3*R**)-1-(anthracene-2-carbonyl)piperazine-2,3-dicarboxylic acid; UBP144, (2*R**,3*R**)-1-(9,10-dioxo-9,10-dihydro-anthracene-2-carbonyl)piperazine-2,3-dicarboxylic acid; UBP145, (2*R**,3*S**)-1-(9-bromophenanthrene-3-carbonyl)piperazine-2,3-dicarboxylic acid; UBP148, (2*R**,3*S**)-1-(3-oxo-3*H*-benzo[*f*]chromene-2-carbonyl)piperazine-2,3-dicarboxylic acid; UBP150, (–)-*cis*-1-(phenanthrene-2-carbonyl)piperazine-2,3-dicarboxylic acid; UBP151, (+)-*cis*-1-(phenanthrene-2-carbonyl)piperazine-2,3-dicarboxylic acid; UBP152, (2*R**,3*S**)-1-(3-naphthalene-2-ylpropionyl)piperazine-2,3-dicarboxylic acid; UBP160, (2*R**,3*S**)-1-(9-chlorophenanthrene-3-carbonyl)piperazine-2,3-dicarboxylic acid; UBP161, (2*R**,3*S**)-1-(9-iodophenanthrene-3-carbonyl)piperazine-2,3-dicarboxylic acid; EAB-515, (2-amino-5-(phosphonomethyl)[1,1'-biphenyl]-3-propanoic acid; (*R*)-AP7, (*R*)-2-amino-7-phosphonoheptanoate; CGS-19755, (2*R**,4*S**)-4-(phosphonomethyl)piperidine-2-carboxylic acid; QX-314, *N*-(2,6-dimethyl-phenylcarbamoylmethyl)-triethylammonium bromide; EPSC, excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; ADC, analog to digital converter; KSCN, potassium thiocyanate.

Fig. 1. Structures of PPDA derivatives. UB143* and UB144* indicate that UB143 and UB144 have the *trans*-(2*R**,3*R**) configuration; all other compounds have a *cis*-(2*R**,3*S**) structure.

institutional and federal animal care guidelines. NMDA receptor subunit RNAs were dissolved in sterile distilled H₂O. NR1 and NR2 RNAs were mixed in a molar ratio of 1:1 to 1:3, and 50 nl of the final RNA mixture was microinjected (15–30 ng total) into the oocyte cytoplasm. Oocytes were incubated in ND-96 (Buller et al., 1994) solution at 17°C prior to electrophysiological assay (1–5 days).

Oocyte Electrophysiology. Electrophysiological responses were measured using a standard two-microelectrode voltage clamp as described previously (Buller et al., 1994), with an oocyte clamp amplifier (model OC-725B; Warner Instruments, Hamden, CT). The recording buffer contained 116 mM NaCl, 2 mM KCl, 2 mM BaCl₂, and 5 mM HEPES, pH 7.4. Ambient Zn²⁺ levels were estimated to be approximately 10 nM. Response magnitude was determined by the plateau response elicited by bath application of 10 μM L-glutamate plus 10 μM glycine at a holding potential of –60 mV. Response amplitudes for the NR1/NR2-heteromeric complexes were generally between 50 and 200 nA. Attempts were made to keep response magnitudes within this range to minimize activation of the endogenous Cl[–] current. The lack of significant activation of the endogenous Cl[–] current by Ba²⁺ in these cells was indicated by the presence of a plateau response.

Antagonist inhibition curves were fit to a single site with variable slope (Prism; GraphPad Software Inc., San Diego, CA), using a nonlinear regression to calculate IC₅₀. Apparent K_i values were determined by correcting for agonist affinity according to the equation $K_i = IC_{50}/(1 + ([agonist]/EC_{50}))$ (Cheng and Prusoff, 1973). For K_i value calculations from IC₅₀ values and Schild analysis, the L-glutamate K_d values used were averages of those obtained from the literature (Ishii et al., 1993; Frizelle et al., 2006; Erreger et al., 2007;

Hansen et al., 2008) and our laboratory (NR1a/NR2A, 2.92 ± 0.27 μM; NR1a/NR2B, 1.93 ± 0.25 μM; NR1a/NR2C, 1.11 ± 0.20 μM; and NR1a/NR2D 0.44 ± 0.04 μM). NR1-4b/NR2 L-glutamate affinity values used for K_i determination were: NR2A, 6.8 ± 1.0 μM; NR2B, 5.8 ± 0.4 μM; NR2C, 1.63 ± 0.03 μM; and NR2D, 1.60 ± 0.12 μM. Splice form NR1-4b (Hollmann et al., 1993), corresponding to NR1g (Sugihara et al., 1992) and NR1₁₀₀ (Durand et al., 1993), contains only the first of three alternatively spliced cassettes. The predominant NR1 isoform, NR1a (NR1-1a or NR1₀₁₁), has only the last two alternatively spliced cassettes. For Schild analysis, at each of five antagonist concentrations, NMDA receptors were activated by two concentrations of L-glutamate in combination with 10 μM glycine. L-Glutamate response at each antagonist concentration was fitted by nonlinear regression analysis using Prism 5 (GraphPad Software, La Jolla, CA). For each individual experiment, Schild slope was allowed to vary but held to be common for the family of curves. The bottoms of the curves were set to zero response, and the maximal responses were set to be a shared value for each set of curves.

Preparation of Hippocampal Slices. This study was carried out on 21-day-old Wistar rats (WAG/GSto, Moscow, Russia). After decapitation, rat brains were immediately transferred to the chilled (4°C) solution of the following composition: 120 mM NaCl, 5 mM KCl, 26 mM NaHCO₃, 2 mM MgCl₂, 0.5 CaCl₂, and 10 mM glucose. The solution was constantly equilibrated with 95% O₂/5% CO₂ gas mixture to maintain pH 7.4. During the preincubation, the slices (300–400 μm thick) were kept fully submerged in the extracellular solution: 135 mM NaCl, 5 mM KCl, 26 mM NaHCO₃, 1.5 mM CaCl₂, 1.5 mM MgCl₂, and 10 mM glucose (pH 7.4, equilibrated with 95% O₂/5% CO₂).

Electrophysiological Recordings in Hippocampal Slices. A standard whole-cell patch clamp technique was used to record EPSCs from CA1 pyramidal neurons in situ in response to stimulation of Schaffer collateral/commissural pathway. To prevent the spread of electrical activity from area CA3, mini-slices were prepared by making a cut orthogonal to the stratum pyramidale and extending to the mossy fiber layer. Intracellular solution for patch pipettes contained 100 mM TrisPO₄ or CsF, 40 mM NaH₂PO₄, 10 mM HEPES-CsOH, and 10 mM Tris-Cl, pH 7.2. QX-314 (2–3 mM) was routinely added to the intracellular solution to block voltage-gated sodium conductance. Patch pipettes were pulled from soft borosilicate glass on a two-stage horizontal puller. When fire-polished and filled with the intracellular solution, they had a resistance of 2 to 3 MΩ. Currents were digitally sampled at 400-μs intervals by a 12-digit ADC board and filtered at 3 kHz. Access resistance was monitored throughout the experiments and ranged typically from 6 to 9 MΩ. When the access resistance was changed by more than 25% during the experiment, the data were discarded. To stimulate Schaffer collateral/commissural pathway, a bipolar Ni/Cr electrode was positioned on the surface of the slice. The current intensity of test stimuli (25–50 μA) was set to produce half-maximal EPSPs. Current pulses were delivered through the isolated stimulator (HG 203; Hi-Med, London, UK) at 0.066 to 0.2 Hz.

Pharmacologically isolated EPSP_{NMDA} were recorded in a modified extracellular solution containing 135 mM NaCl, 5 mM KCl, 26 mM NaHCO₃, 0.5 mM Mg²⁺, 2.5 mM Ca²⁺, and 10 mM glucose (pH 7.4, equilibrated with 95% O₂/5% CO₂) in the presence of 10 μM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) to block AMPA receptor currents and 20 μM bicuculline to suppress inhibitory activity of interneurons.

[³H]AMPA and [³H]Kainate Binding Assays. Compound potency at non-NMDA ionotropic glutamate receptors was tested by incubating rat brain tissue sections with radiolabeled [³H]AMPA or [³H]kainate [³H](2*S*,3*S*,4*S*)-2-carboxy-4-isopropenyl-3-pyrrolidineacetate as described previously (Monaghan, 1993). Following halothane anesthesia, brains were removed from adult male Sprague-Dawley rats (200–250g) and immediately frozen under powdered dry ice. Horizontal sections (8 μm) were thaw-mounted onto gelatin-subbed

slides and stored at -20°C overnight. Slides were preincubated for 20 min at 0°C in assay buffer ($[^3\text{H}]\text{AMPA}$ binding assay: 50 mM Tris acetate, pH 7.2, with 100 mM $[^3\text{H}]\text{kainate}$ binding assays: 50 mM Tris citrate, pH 7.0) followed by two additional preincubations for 10 min at 30°C in fresh buffer. Sections were then incubated with 50 nM $[^3\text{H}]\text{AMPA}$ (45 Ci/mM; PerkinElmer Life and Analytical Sciences, Boston, MA) or with 100 nM $[^3\text{H}]\text{kainate}$ (41.2 Ci/mM; PerkinElmer Life and Analytical Sciences) in assay buffer in the presence or absence of 100 μM of test compound. Nonspecific binding was determined with 100 μM AMPA or 100 μM kainate, respectively. Sections were washed for 30 s in ice-cold buffer and quickly dried by an airstream at room temperature. Liquid scintillation spectrophotometry was then used to determine radioligand binding.

Results

Compounds (Fig. 1) were tested for their ability to block agonist-evoked currents in *X. laevis* oocytes injected with NMDA receptor NR1 and NR2 subunit cRNA. Each of the putative NMDA receptor antagonists was able to block recombinant NMDA receptor responses. Inhibition constants for each of the NR1/NR2 receptors are shown in Table 1. The synthesis and initial biological characterization of UBP129 and UBP141 have been reported elsewhere (Morley et al., 2005); their K_i values are reported here for comparison with structural homologs. In Fig. 2A, an example of a dose-response analysis is shown; increasing concentrations of UBP145 inhibit NR1-4b/NR2D responses to the application of 10 μM L-glutamate and 10 μM glycine. The averaged UBP145 dose-response results are shown in Fig. 2B for each of the NR2 subunit-containing receptors in complex with NR1-4b. UBP145 inhibited NR2C- and NR2D-containing receptors with a lower IC_{50} than NR2A- and NR2B-containing receptors, despite the higher potency of L-glutamate at NR2C- and NR2D-containing receptors. Increasing concentrations of antagonist were applied in the presence of agonists, as shown in Fig. 2A. Consistent with the results of Frizelle et al. (2006), preapplication of the antagonist did not change the level of steady-state inhibition by the antagonist.

Although the widely used NMDA receptor antagonists, such as (*R*)-AP5, (*R*)-AP7, CGS-19755, and (*R*)-CPP, display a common selectivity pattern among the different NR1a/NR2-containing receptors of $\text{NR2A} > \text{NR2B} > \text{NR2C} >$

Fig. 2. A, a representative recording of antagonist blockade of NMDA receptor-mediated responses in *X. laevis* oocytes. NR1-4b/NR2D RNA-injected oocytes were voltage-clamped at -60 mV, and inward currents were evoked by bath application of 10 μM L-glutamate plus 10 μM glycine (heavy bar). Increasing concentrations of the bath-applied antagonist UBP145 reduced the inward currents. B, averaged dose-response curves for UBP145 inhibition of NR1-4b/NR2A, NR1-4b/NR2B, NR1-4b/NR2C, and NR1-4b/NR2D receptor complexes.

NR2D (high to low affinity) (Feng et al., 2005), none of the PPDA derivatives displayed this pattern of selectivity (Table 1; Fig. 3). Of the 15 compounds tested, 12 displayed higher affinity at both NR2C- and NR2D-containing receptors than at either of the NR2A- or NR2B-containing receptors. Resolution and testing of the optical isomers of PPDA revealed that the (–) isomer (UBP150) is 50- to 80-fold more potent than the (+) isomer (UBP151) and has a subunit selectivity that is similar to that of the racemic mixture (2–3-fold selective for NR2C or NR2D over NR2A or NR2B). UBP150 was

TABLE 1

Average antagonist K_i values (micromolar) $\pm$ S.E.M. for inhibiting agonist-evoked responses at recombinant NMDA receptors expressed in *X. laevis* oocytes

K_i values were statistically different from NR1/NR2B (b, B, **B**), NR1/NR2C (c, C, **C**), and NR1/NR2D (d, D, **D**), with $P < 0.05$ (b, c, d), $P < 0.01$ (B, C, D), and $P < 0.001$ (**B**, **C**, **D**). Number of experiments (*n*) is also indicated. Receptors contained NR1-1a subunits unless indicated otherwise.

Antagonist	NR1/NR2A	NR1/NR2B	NR1/NR2C	NR1/NR2D
UBP125	99.3 $\pm$ 7.0 BCD (<i>n</i> = 5)	12.9 $\pm$ 1.4 C (<i>n</i> = 5)	28.0 $\pm$ 1.0 D (<i>n</i> = 5)	10.8 $\pm$ 0.3 (<i>n</i> = 5)
UBP128	138 $\pm$ 9 BCD (<i>n</i> = 6)	24.1 $\pm$ 1.1 (<i>n</i> = 5)	21.4 $\pm$ 0.4 (<i>n</i> = 5)	10.5 $\pm$ 1.3 (<i>n</i> = 5)
UBP129	0.85 $\pm$ 0.08 BcD (<i>n</i> = 10)	0.32 $\pm$ 0.07 CD (<i>n</i> = 4)	1.14 $\pm$ 0.09 D (<i>n</i> = 5)	1.95 $\pm$ 0.14 (<i>n</i> = 5)
UBP133	6.00 $\pm$ 0.48 bCD (<i>n</i> = 6)	8.05 $\pm$ 0.93 CD (<i>n</i> = 6)	1.54 $\pm$ 0.23 (<i>n</i> = 5)	1.59 $\pm$ 0.29 (<i>n</i> = 6)
UBP136	10.5 $\pm$ 2.6 BCD (<i>n</i> = 5)	5.15 $\pm$ 0.54 (<i>n</i> = 5)	3.44 $\pm$ 0.37 (<i>n</i> = 5)	2.49 $\pm$ 0.30 (<i>n</i> = 6)
UBP141	22.0 $\pm$ 1.4 BCD (<i>n</i> = 5)	17.2 $\pm$ 1.2 CD (<i>n</i> = 4)	5.24 $\pm$ 0.54 d (<i>n</i> = 5)	2.36 $\pm$ 0.12 (<i>n</i> = 5)
UBP143	37.8 $\pm$ 3.2 BCD (<i>n</i> = 4)	19.8 $\pm$ 0.4 CD (<i>n</i> = 4)	11.7 $\pm$ 1.1 d (<i>n</i> = 6)	5.7 $\pm$ 0.6 (<i>n</i> = 5)
UBP144	>400 (<i>n</i> = 3)	227 $\pm$ 62 (<i>n</i> = 3)	186 $\pm$ 39 (<i>n</i> = 3)	147 $\pm$ 113 (<i>n</i> = 3)
UBP145	11.53 $\pm$ 0.79 BCD (<i>n</i> = 5)	7.99 $\pm$ 0.35 CD (<i>n</i> = 4)	2.79 $\pm$ 0.07 D , 4	1.19 $\pm$ 0.06 5
UBP145 (NR1-4b/NR2)	16.47 $\pm$ 2.79 BCD (<i>n</i> = 5)	10.89 $\pm$ 0.73 CD (<i>n</i> = 4)	1.70 $\pm$ 0.27 (<i>n</i> = 4)	1.53 $\pm$ 0.28 (<i>n</i> = 5)
UBP148	>100 (<i>n</i> = 4)	>100 (<i>n</i> = 4)	>100 (<i>n</i> = 4)	>100 (<i>n</i> = 4)
UBP150	0.21 $\pm$ 0.02 CD (<i>n</i> = 5)	0.22 $\pm$ 0.04 CD (<i>n</i> = 5)	0.068 $\pm$ 0.010 (<i>n</i> = 4)	0.093 $\pm$ 0.008 (<i>n</i> = 4)
UBP150 NR1-4b/NR2	0.27 $\pm$ 0.02 CD (<i>n</i> = 4)	0.16 $\pm$ 0.01 CD (<i>n</i> = 4)	0.045 $\pm$ 0.007 (<i>n</i> = 4)	0.093 $\pm$ 0.003 (<i>n</i> = 4)
UBP151	17.6 $\pm$ 2.1 BCD (<i>n</i> = 5)	13.5 $\pm$ 1.0 CD (<i>n</i> = 6)	3.38 $\pm$ 0.30 (<i>n</i> = 5)	4.64 $\pm$ 0.21 (<i>n</i> = 4)
UBP152	5.10 $\pm$ 0.53 BD (<i>n</i> = 7)	2.27 $\pm$ 0.23 c (<i>n</i> = 4)	4.14 $\pm$ 0.29 d (<i>n</i> = 4)	2.68 $\pm$ 0.22 (<i>n</i> = 4)
UBP160	7.16 $\pm$ 0.69 Bd (<i>n</i> = 4)	15.2 $\pm$ 2.6 CD (<i>n</i> = 4)	3.17 $\pm$ 0.16 (<i>n</i> = 4)	1.68 $\pm$ 0.09 (<i>n</i> = 4)
UBP161	7.41 $\pm$ 0.92 BCD (<i>n</i> = 4)	3.80 $\pm$ 0.86 d (<i>n</i> = 4)	1.86 $\pm$ 0.15 (<i>n</i> = 4)	1.045 $\pm$ 0.11 (<i>n</i> = 4)

Fig. 3. NMDA receptor subunit selectivity of PPDA derivatives. To compare subunit selectivity profiles, the inhibition constants (K_i) at NR1a/NR2 receptors for each compound were normalized by dividing by the drug's corresponding K_i value for its inhibition of NR1a/NR2D receptors. Normalized results for (R)-CPP are from Feng et al. (2005) and are presented for comparison.

also tested with the NR1-4b subunit coexpressed with NR2 subunits. L-Glutamate had 2- to 3-fold lower affinity at NMDA receptors containing the NR1-4b splice variant compared with the NR1-1a variant (NR1-4b/NR2A, 6.8 ± 1.0 ; NR1-4b/NR2B, 5.8 ± 0.4 ; NR1-4b/NR2C, 1.63 ± 0.03 ; and NR1-4b/NR2D, 1.60 ± 0.12). By use of these values for L-glutamate affinity, the estimated K_i values for UBP150 for receptors containing NR1-4b were very similar to those obtained for the NR1a/NR2 receptors (Table 1).

Modeling studies suggest that the phenanthrene ring of PPDA projects directly out of the ligand binding cavity (Kinarsky et al., 2005); thus, longer, linear structures might be tolerated. In two compounds, UBP125 and UBP128, the phenanthrene ring of PPDA was replaced with an extended, linear structure with two benzene rings separated by three covalent bonds (Fig. 1). In both cases, this modification was tolerated but was associated with a large reduction in overall affinity compared with PPDA, especially at NR2A-containing receptors. Of all of the compounds tested, UBP125 and UBP128 most strongly distinguished between NR2A- and NR2B-containing receptors.

Previously, we found that replacing the first aromatic ring in PPDA with an ethenyl linker (generating UBP129) resulted in a 12- and 15-fold reduction in NR2C and NR2D affinity, respectively, but had little effect on NR2A or NR2B affinity. This results in the selectivity pattern of NR2B > NR2A, NR2C > NR2D (high to low affinity). The ethenyl double bond of UBP129 seems important for maintaining high affinity at NR2A- and NR2B-containing receptors; sat-

uration of the ethenyl linker to generate UBP152 further reduced antagonist affinity, especially at NR2A- and NR2B-containing receptors.

In UBP141, attaching the carbonyl-piperazine group of PPDA at the 3-position of phenanthrene, instead of the at the 2-position, reduced receptor affinity overall while increasing selectivity for NR2C- and NR2D-containing receptors. Furthermore, UBP141, in contrast to PPDA, displays a weak selectivity for NR2D- over NR2C-containing receptors. With compound UBP133, bromination at the 9-position of the phenanthrene ring in PPDA also increases selectivity for NR2C- and NR2D-containing receptors over NR2B. In UBP145, we tested whether the selectivity gained by bromination at the 9-position in PPDA was additive, with the selectivity gained by attaching the phenanthrene ring to the carbonyl-piperazine at the 3-position as in UBP141. From IC_{50} analysis, this modification increased affinity over UBP141 2-fold but did not significantly alter selectivity. However, Schild analysis (see below) suggests that the bromination of UBP141 to make UBP145 may have led to a small increase in NR2D > NR2B selectivity as hypothesized. Because bromination at this position in PPDA (resulting in UBP133) significantly reduces affinity, whereas bromination of UBP141 (resulting in UBP145) increases affinity, it appears that the phenanthrene group in UBP141 occupies a different space in the receptor than it does in PPDA.

Halogenation at the 9-position of UBP141 was further explored by adding a chloro (UBP160) or iodo (UBP161) group. In general, the addition of a halogen group increased

receptor affinity. Furthermore, for NR2B, NR2C, and NR2D receptors, increasing halogen size was associated with increases in receptor affinity. NR2A-containing receptors did not display this pattern: the 9-chloro derivative had a 2-fold higher affinity for NR2A-containing receptors than for those with NR2B; the 9-bromo derivative displayed equivalent affinities at these two receptors; and the 9-iodo derivative had a 2-fold higher affinity for NR2B-containing receptors. As discovered with UBP141 and in contrast to PPDA, all of the UBP141 halogenated derivatives displayed higher affinity for NR2D-containing receptors than for those with NR2C.

Substitution of the phenanthrene ring of PPDA with UBP144 greatly reduced receptor affinity, with minimal effects on receptor selectivity. Likewise, substitution of phenanthrene with the heterocyclic 3-oxo-3*H*-benzo[*f*]chromene structure in UBP148 also greatly reduced receptor affinity.

Presently, UBP141 and UBP145 are the most selective antagonists for preferentially blocking NR2C- and NR2D-containing NMDA receptors. To determine whether these antagonists are competitive antagonists at the glutamate binding site and to provide an alternative measure of receptor affinity, a Schild analysis was performed (see example in Fig. 4). As expected for a glutamate binding site antagonist, increasingly higher concentrations of L-glutamate were required to obtain similar receptor responses in the presence of increasing concentrations of antagonist. Nonlinear fitting of these results indicate a Schild slope of approximately 1 but statistically different from 1. For the average of all experi-

ments, UBP141 Schild slopes were above 1 (1.14 ± 0.02 , $n = 29$, different from 1 with $p < 0.0001$ two-tailed t test), whereas UBP145 Schild slopes were below 1 (0.73 ± 0.03 , $n = 16$, $p < 0.0001$). As shown in Table 2, Schild slopes for each NR2-containing receptor subtype were above 1 for UBP141 and below 1 for UBP145. Each of these was statistically different from 1, with the exception of UBP141 at NR1/NR2C ($p = 0.12$).

Schild analysis estimates of affinity (K_B values) are fairly close to the K_i values determined by the IC_{50} /Cheng-Prusoff estimation. Schild analysis suggests that UBP141 may have a 2-fold lower affinity for NR2A-containing receptors than estimated by IC_{50} -derived K_i values, a 60% higher affinity for NR2B- and NR2C-containing receptors and essentially identical affinity at NR2D-containing receptors. Schild K_B values for UBP145 were also fairly similar to the IC_{50} -derived estimates; UBP145 K_B values for NR1a/NR2A were 40% larger (lower affinity) than the IC_{50} -derived K_i estimates, whereas the K_B values for the other receptors indicated that each was fairly similar to the IC_{50} -derived K_i estimates. Thus, for both UBP141 and UBP145, the largest deviation from the K_i estimates was the lower affinity at NR2A-containing receptors. Thus, these two antagonists may be better in distinguishing NR2C and NR2D from NR2A subunits than previously estimated by IC_{50} determination. Schild analysis suggests that UBP145 may be better at discriminating between NR2D and NR2B than UBP141.

To determine the effects of these antagonists on native AMPA and kainate receptors, 100 μ M concentrations of UBP125, UBP128, UBP141, and UBP145 were tested as inhibitors of [^{3} H]AMPA and [^{3} H]kainate binding to their respective receptors in rat brain tissue. As shown in Table 3, these compounds display weak activity at AMPA and kainate receptors. UBP128 inhibited approximately half of the binding at AMPA receptors but only 20% binding at kainate receptors. The structurally related compound UBP125 displayed weaker AMPA receptor activity (37% inhibition) and weaker kainate receptor activity (10% inhibition). UBP141 and UBP145 were relatively weak at both AMPA and kainate receptors, with less than 20% inhibition.

Previous studies have shown that the NMDA receptor-mediated synaptic currents of the CA3-CA1 hippocampal synapse has multiple components following a short train of high frequency stimulation (Lozovaya et al., 2004). After a few stimulations, the NMDA receptor-mediated EPSC develops an additional slow-decaying current that is pharmacologically distinct from the fast, early peak response. (*R*)-CPP, which is moderately selective for NR2A, was able to inhibit much of the early peak response while having a significantly less effect on the slow-decaying current. In contrast, PPDA (10 μ M), which has a weak selectivity pattern of NR2D > NR2B > NR2A, caused a small but proportionally greater inhibition of the slow-decaying NMDA receptor-mediated EPSC than the early peak response. This effect was most apparent after superimposing normalized traces after partial inhibition. These and other observations led to the conclusion that NR2B and NR2D subunits contribute to the slow-decay response and that NR2A and NR2B subunits contribute to the fast-decaying response (Lozovaya et al., 2004). Because UBP141 is better than PPDA at distinguishing NR2D from NR2A and NR2B, we tested this compound on the early peak and the slowly decaying NMDA receptor EPSCs to see

Fig. 4. Schild analysis of UBP141 inhibition of NMDA receptor responses. Top, NR1a/NR2B receptors were activated by bath applied L-glutamate in the presence of different concentrations of UBP141 as indicated. A family of two-point dose-response curves was fitted by nonlinear regression analysis (Prism) to determine the Schild slope value (1.14) and K_B (5.9 μ M). Bottom, Schild plot of the same data yields a Schild slope of 1.14 and pK_B of -5.24 ($K_B = 5.8$ μ M).

TABLE 2

Schild analysis of UBP141 and UBP145

	$K_B \pm \text{S.E.M.}$	n	Schild Slope $\pm \text{S.E.M.}$	Hill Slope $\pm \text{S.E.M.}$	$R^2 \pm \text{S.E.M.}$
μM					
UBP141					
NR1/NR2A	44.1 ± 1.7	9	1.18 ± 0.03	1.79 ± 0.05	0.998 ± 0.001
NR1/NR2B	10.4 ± 2.1	9	1.14 ± 0.04	1.66 ± 0.14	0.975 ± 0.005
NR1/NR2C	3.22 ± 0.31	4	1.05 ± 0.02	1.66 ± 0.24	0.937 ± 0.017
NR1/NR2D	2.57 ± 0.16	4	1.09 ± 0.03	1.53 ± 0.05	0.949 ± 0.022
UBP145					
NR1/NR2A	16.1 ± 2.3	4	0.83 ± 0.05	1.65 ± 0.19	0.985 ± 0.005
NR1/NR2B	6.32 ± 0.20	4	0.79 ± 0.04	1.41 ± 0.05	0.995 ± 0.003
NR1/NR2C	2.22 ± 0.55	4	0.66 ± 0.01	1.41 ± 0.08	0.995 ± 0.002
NR1/NR2D	0.94 ± 0.34	4	0.66 ± 0.02	1.25 ± 0.08	0.982 ± 0.004

TABLE 3

Inhibition of [^3H]AMPA and [^3H]kainate binding to rat brain AMPA and kainate receptors by select compoundsValues represent mean percentage inhibition ($\pm \text{S.E.M.}$, $n = 3$) by compound (100 μM).

Compound	[^3H]AMPA	[^3H]Kainate
UBP125	37 ± 2	10 ± 7
UBP128	53 ± 3	20 ± 2
UBP141	15 ± 8	9 ± 9
UBP145	13 ± 5	15 ± 10

whether it could better discriminate between these two EPSC components.

As shown in Fig. 5, when a single stimulation pulse is used to evoke an NMDA receptor synaptic current, 5 μM UBP141 does not inhibit the peak response but causes a small acceleration of the decay. However, after 3 pulses, when the slow-decay current becomes expressed, 5 μM UBP141 clearly inhibits a significant portion of the slow component while having little effect on the fast peak response. After 7 pulses when the slow current is more fully developed, UBP141 now inhibits approximately 40% of the slow-decaying NMDA receptor current while still having little effect on the early peak response. Although both of these components are fully blocked by NMDA receptor antagonists (Lozovaya et al., 2004), it appears that the early and late components are mediated by pharmacologically distinct subtypes of NMDA receptors. UBP141, unlike the other NMDA receptor antagonists, (*R*)-CPP, ifenprodil [4-[1-hydroxy-2-[4-(phenylmethyl)piperidin-1-yl]propyl]phenol], and PPDA (Lozovaya et al., 2004) or (*R*)- α -aminoadipate (Diamond, 2001; N. A. Lozovaya, unpublished observations), has little activity on the peak NMDA receptor current at a concentration that significantly inhibits the slow-decaying NMDA receptor current.

Discussion

Presently, there are few pharmacological tools available for distinguishing NMDA receptor subtypes (Jane et al., 2000; Neyton and Paoletti, 2006). Ifenprodil-like antagonists are available for blocking NR2B-containing receptors (Jane et al., 2000; Neyton and Paoletti, 2006); however, compounds that are highly selective for NR1, NR2C, or NR2D have not been identified. At low nanomolar concentrations, Zn^{2+} selectively inhibits NR2A-containing receptors (Neyton and Paoletti, 2006). Most well characterized competitive NMDA receptor antagonists display the subunit-selectivity pattern of NR2A > NR2B > NR2C > NR2D (high to low affinity) (Ikeda et al., 1992; Ishii et al., 1993; Buller et al., 1994;

Fig. 5. A, pharmacologically isolated EPSC_{NMDA} evoked by a single pulse (EPSC_{single}_{NMDA}) (a), a 3-pulse long train (EPSC_{train3}_{NMDA}) (b, 200 Hz), and a 7-pulse long train (EPSC_{train7}_{NMDA}) (c) in the control and with UBP141 (5 μM); stimulation protocols are schematically represented over the traces. Holding voltage was -100 mV . B, the charge transfer of the EPSC_{single}_{NMDA} (n1), EPSC_{train3}_{NMDA} (n3), and EPSC_{train7}_{NMDA} (n7) normalized to the corresponding peak current amplitude (defined here as Q); recordings in control solution and in the presence of UBP141 (5 μM). The Q value for the control EPSC_{single}_{NMDA} was taken as 100%. C, the contribution of the UBP141-sensitive component ($Q_{\text{CONTROL}} - Q_{\text{UBP141}}$) to the EPSC_{single}_{NMDA} and the EPSC_{train3}_{NMDA}, calculated as: $1 - (Q_{\text{UBP141}}/Q_{\text{CONTROL}})$. D, the late component of the EPSC_{train7}_{NMDA} has greater sensitivity to UBP141 compared to the early component. The contribution of the UBP141-sensitive component ($Q_{\text{CONTROL}} - Q_{\text{UBP141}}$) to the early (peak $\sim 700 \text{ ms}$) and late (peak $700\text{--}3000 \text{ ms}$) component of the EPSC_{train7}_{NMDA} [calculated as $1 - (Q_{\text{UBP141}}/Q_{\text{CONTROL}})$].

Laurie and Seeburg, 1994; Buller and Monaghan, 1997; Feng et al., 2005). The compound PPDA was significant in that it was the first compound reported to have a slightly higher

affinity for NMDA receptors containing NR2C or NR2D subunits than for those containing NR2A or NR2B (Feng et al., 2004; Morley et al., 2005). However, it is not a selective compound. After considering the higher affinity that L-glutamate displays for NR2C- and NR2D-containing receptors, the IC_{50} values for PPDA at NMDA receptors with various NR2 subunits are fairly similar. We have generated and tested several new PPDA derivatives and further characterized the selectivity of UBP141. These results indicate that UBP141 and UBP145 have an improved selectivity that may be useful for characterizing NR2C- and NR2D-containing receptors.

In this study, we found that the (–) isomer of PPDA (UBP150) is 50- to 80-fold more potent than the (+) isomer (UBP151). Although it is of lower affinity, the (+) isomer was slightly more selective than the (–) isomer. All of the new compounds, with the exception of UBP129, retained selectivity for NR2C/NR2D over NR2A/NR2B. Compared with the more potent isomer of PPDA (UBP150), 10 of the tested compounds have improved selectivity for receptors containing NR2C or NR2D subunits over those with NR2A subunits, and seven compounds had improved selectivity for NR2C and NR2D over NR2B-containing receptors. No compounds displayed a higher overall affinity for NMDA receptors than UBP150. Of these compounds, UBP125 and UBP128 are potentially useful because of their low affinity for NR2A-containing receptors, and UBP141 and UBP145 may be useful for their improved selectivity for NR2C- and NR2D-containing receptors.

Schild analysis was used to determine whether UBP141 and UBP145 are competitive glutamate site antagonists. It is clear that, in the presence of increasing antagonist concentration, higher concentrations of L-glutamate were required to achieve a similar receptor response. Thus, these antagonists seem to be competitive at the glutamate binding site. Schild slopes for UBP141 and UBP145 were near one but not identical to one. This suggests that the assumptions necessary for ideal behavior in a Schild analysis (Colquhoun, 2007) may not all be completely valid for NMDA receptors. In the case of a tetrameric NMDA receptor complex with two NR2 glutamate binding sites, it is possible that 1) the antagonist alters the conformation of the receptor or 2) the binding of the first antagonist does not have an identical affinity to the binding of the second antagonist molecule or 3) there is a weak agonist response with just one glutamate bound that is not identical to the response with one glutamate and one antagonist bound.

Receptor molecular modeling studies (Kinarsky et al., 2005) suggest that the phenanthrene ring of PPDA binds in the S1/S2 cleft along a groove found in S2 at the base of the “H” helix (Armstrong et al., 1998). Along this groove in NR2D, there is a nonconserved arginine residue (Arg737). The selectivity for NR2D by PPDA, and especially UBP141/UBP145, may be due in part to hydrophobic contact of the phenanthrene ring, with the hydrophobic hydrocarbon side chain of NR2D Arg737 as predicted in our modeling studies (Kinarsky et al., 2005). Modeling studies, together with the results of this study, also suggest that the long, linear antagonists, such as UBP125 and UBP128, are allowed due to the projection of the distal benzene ring directly out of the ligand binding pocket.

Relative to PPDA and its active isomer (UBP150), UBP141 displays an increase in selectivity for NR2C- and NR2D-

containing receptors. This magnitude of selectivity is similar to that seen for the NR2A/NR2C-preferring antagonist NVP-AAM077 (Feng et al., 2004). To test the usefulness of UBP141 at native NMDA receptors, we evaluated the ability of 5 μ M UBP141 to block the NMDA receptor-mediated EPSC in the hippocampal CA3-CA1 synapse after burst stimulation. The concentration of UBP141 used (5 μ M) is approximately twice the K_i value for NR1/NR2D receptors but only 25% of the K_i value for NR1/NR2B receptors. Thus, at this concentration, UBP141 would be expected to block more of NR2D-mediated responses than those from NR2B-containing receptors. UBP141 was clearly able to block a significant portion (~40%) of the slow-decaying NMDA current while having very little effect (~5%; Fig. 5) on the peak response that appears to represent NR2A- and NR2B-containing NMDA synaptic current (Lozovaya et al., 2004). In contrast to UBP141 (5 μ M), ifenprodil inhibited the fast early peak response in addition to inhibiting the very slow-decaying NMDA receptor response. For example, in the representative experiments in Figs. 4 and 5 (Lozovaya et al., 2004), ifenprodil inhibited an ~30% after-burst peak response and ~40% slow-decaying current. The greater selectivity of UBP141 for inhibition of the slow component than that displayed by ifenprodil (or PPDA) supports our previous conclusion that the NR2D subunit may be contributing to this slow-decaying current in the hippocampal CA3-CA1 synapse (Lozovaya et al., 2004).

The fast early peak NMDA receptor-mediated synaptic response appears to be largely mediated by NR2A-containing NMDA receptors because it is fast decaying, preferentially inhibited by (R)-CPP, and, relative to the slow component, more weakly inhibited by ifenprodil and PPDA (Lozovaya et al., 2004). A fast-decaying peak response mediated in part by NR2A and a very slow-decaying current mediated in part by NR2D are consistent with the known deactivation kinetics of recombinant NR2A-, NR2B-, and NR2D-containing receptors (Monyer et al., 1994; Vicini et al., 1998). The presence of a functionally active NR2D subunit in the CA3-CA1 synapse supports our previous suggestion that NR2D-containing NMDA receptors participate in generating LTD (Hrabetova et al., 2000). For the interpretation of physiological experiments in native preparations, it will be important to determine the effects of subtype-selective antagonists on triheteromeric (e.g., NR1/NR2A/NR2B or NR1/NR2B/NR2D) NMDA receptors.

The observation that NR2D subunits might primarily contribute to just the slow-decaying NMDA receptor response may be important to the development of neuroprotective agents. The slow response is thought to be due to the activation of extrasynaptic NMDA receptors that are activated by L-glutamate spillover following a train of stimuli (Lozovaya et al., 2004). Various studies have shown that the synaptic and extrasynaptic populations of NMDA receptors activate different intracellular signaling pathways and that the extrasynaptic population plays a key role in causing cell death while the synaptic population may be neuroprotective (Hardingham and Bading, 2003). Thus, it is possible that an NR2D-selective antagonist would provide a more selective neuroprotective action than would an NR2A- or NR2B-selective antagonist or a nonselective NMDA receptor antagonist.

The selectivity of UBP141 (or UBP145) for extrasynaptic currents may be enhanced due to the low micromolar affinity

of UBP141. A low affinity antagonist is likely to display rapid dissociation. Such a competitive antagonist may more readily inhibit extrasynaptic NMDA receptors in the presence of low L-glutamate concentrations than inhibit synaptic NMDA receptors with higher concentrations of L-glutamate (Diamond, 2001). Hence, the low affinity of UBP141 may contribute to the apparent selectivity of this compound for extrasynaptic NMDA receptors in addition to its low selectivity for NR2D-containing NMDA receptors.

Acknowledgments

We thank Drs. Shigetada Nakanishi, David Lynch, Dolan Pritchett, and Peter Seeburg for providing NMDA receptor cDNA constructs.

References

- Armstrong N, Sun Y, Chen GQ, and Gouaux E (1998) Structure of a glutamate-receptor ligand-binding core in complex with kainate. *Nature* **395**:913–917.
- Auberson YP, Allgeier H, Bischoff S, Lingenhoeck K, Moretti R, and Schmutz M (2002) 5-Phosphonomethylquinolinediones as competitive NMDA receptor antagonists with a preference for the human 1A/2A, rather than 1A/2B receptor composition. *Bioorg Med Chem Lett* **12**:1099–1102.
- Buller AL, Larson HC, Schneider BE, Beaton JA, Morrisett RA, and Monaghan DT (1994) The molecular basis of NMDA receptor subtypes: native receptor diversity is predicted by subunit composition. *J Neurosci* **14**:5471–5484.
- Buller AL and Monaghan DT (1997) Pharmacological heterogeneity of NMDA receptors: characterization of NR1a/NR2D heteromers expressed in *Xenopus* oocytes. *Eur J Pharmacol* **320**:87–94.
- Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099–3108.
- Childers WE Jr and Baudy RB (2007) N-Methyl-D-aspartate antagonists and neuropathic pain: the search for relief. *J Med Chem* **50**:2557–2562.
- Choi D (1998) Antagonizing excitotoxicity: a therapeutic strategy for stroke? *Mt Sinai J Med* **65**:133–138.
- Clausen RP, Christensen C, Hansen KB, Greenwood JR, Jørgensen L, Micale N, Madsen JC, Nielsen B, Egebjerg J, Bräuner-Osborne H, et al. (2008) N-Hydroxy-pyrazolyl glycine derivatives as selective N-methyl-D-aspartic acid receptor ligands. *J Med Chem* **51**:4179–4187.
- Colquhoun D (2007) Why the Schild method is better than Schild realised. *Trends Pharmacol Sci* **28**:608–614.
- Diamond JS (2001) Neuronal glutamate transporters limit activation of NMDA receptors by neurotransmitter spillover on CA1 pyramidal cells. *J Neurosci* **21**:8328–8338.
- Durand GM, Bennett MV, and Zukin RS (1993) Splice variants of the N-methyl-D-aspartate receptor NR1 identify domains involved in regulation by polyamines and protein kinase C. *Proc Natl Acad Sci U S A* **90**:6731–6735.
- Erreger K, Geballe MT, Kristensen A, Chen PE, Hansen KB, Lee CJ, Yuan H, Le P, Lyuboslavsky PN, Micale N, et al. (2007) Subunit-specific agonist activity at NR2A-, NR2B-, NR2C-, and NR2D-containing N-methyl-D-aspartate glutamate receptors. *Mol Pharmacol* **72**:907–920.
- Feng B, Morley RM, Jane DE, and Monaghan DT (2005) The effect of competitive antagonist chain length on NMDA receptor subunit selectivity. *Neuropharmacology* **48**:354–359.
- Feng B, Tse HW, Skifter DA, Morley R, Jane DE, and Monaghan DT (2004) Structure-activity analysis of a novel NR2C/NR2D-preferring NMDA receptor antagonist: 1-(phenanthrene-2-carbonyl) piperazine-2,3-dicarboxylic acid. *Br J Pharmacol* **141**:508–516.
- Frizelle PA, Chen PE, and Wyllie DJ (2006) Equilibrium constants for [(R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-2,3-dioxo-1,2,3,4-tetrahydroquinolizin-5-yl)-methyl]-phosphonic acid (NVP-AA0077) acting at recombinant NR1/NR2A and NR1/NR2B N-methyl-D-aspartate receptors: implications for studies of synaptic transmission. *Mol Pharmacol* **70**:1022–1032.
- Hansen KB, Bräuner-Osborne H, and Egebjerg J (2008) Pharmacological characterization of ligands at recombinant NMDA receptor subtypes by electrophysiological recordings and intracellular calcium measurements. *Comb Chem High Throughput Screen* **11**:304–315.
- Hardingham GE and Bading H (2003) The Yin and Yang of NMDA receptor signaling. *Trends Neurosci* **26**:81–89.
- Hollmann M, Boulter J, Maron C, Beasley L, Sullivan J, Pecht G, and Heinemann S (1993) Zinc potentiates agonist-induced currents at certain splice variants of the NMDA receptor. *Neuron* **10**:943–954.
- Hrabetova S, Serrano P, Blace N, Tse HW, Skifter DA, Jane DE, Monaghan DT, and Sacktor TC (2000) Distinct NMDA receptor subpopulations contribute to long-term potentiation and long-term depression induction. *J Neurosci* **20**:RC81.
- Ikeda K, Nagasawa M, Mori H, Araki K, Sakimura K, Watanabe M, Inoue Y, and Mishina M (1992) Cloning and expression of the epsilon 4 subunit of the NMDA receptor channel. *FEBS Lett* **313**:34–38.
- Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M, Akazawa C, Shigemoto R, Mizuno N, Masu M, et al. (1993) Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. *J Biol Chem* **268**:2836–2843.
- Jane DE, Tse H-W, Skifter DA, Christie JM, and Monaghan DT (2000) Glutamate receptor ion channels: activators and inhibitors, in *Handbook of Experimental Pharmacology: Pharmacology of Ionic Channel Function: Activators and Inhibitors* (Endo M, Kurachi Y, and Mishina M eds) pp 415–478, Springer, Berlin.
- Kárádóttir R, Cavellier P, Bergersen LH, and Attwell D (2005) NMDA receptors are expressed in oligodendrocytes and activated in ischaemia. *Nature* **438**:1162–1166.
- Kinarsky L, Feng B, Skifter DA, Morley RM, Sherman S, Jane DE, and Monaghan DT (2005) Identification of subunit- and antagonist-specific amino acid residues in the N-methyl-D-aspartate receptor glutamate-binding pocket. *J Pharmacol Exp Ther* **313**:1066–1074.
- Laube B, Hirai H, Sturgess M, Betz H, and Kuhse J (1997) Molecular determinants of agonist discrimination by NMDA receptor subunits: analysis of the glutamate binding site on the NR2B subunit. *Neuron* **18**:493–503.
- Laurie DJ and Seeburg PH (1994) Ligand affinities at recombinant N-methyl-D-aspartate receptors depend on subunit composition. *Eur J Pharmacol* **268**:335–345.
- Lozovaya NA, Grebenyuk SE, Tsintsadze T, Feng B, Monaghan DT, and Krishtal OA (2004) Extrasynaptic NR2B and NR2D subunits of NMDA receptors shape 'super-slow' afterburst EPSC in rat hippocampus. *J Physiol* **558**:451–463.
- Meldrum BS (2002) Implications for neuroprotective treatments. *Prog Brain Res* **135**:487–495.
- Minami T, Matsumura S, Okuda-Ashitaka E, Shimamoto K, Sakimura K, Mishina M, Mori H, and Ito S (2001) Characterization of the glutamatergic system for induction and maintenance of allodynia. *Brain Res* **895**:178–185.
- Monaghan DT (1993) Autoradiographic analysis of excitatory amino acid receptors, in *Receptor Autoradiography Principles and Practice* (Wharton J and Polak JM eds) pp 171–193, Oxford University Press, Oxford.
- Monyer H, Burnashev N, Laurie DJ, Sakmann B, and Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* **12**:529–540.
- Mori H and Mishina M (1995) Structure and function of the NMDA receptor channel. *Neuropharmacology* **34**:1219–1237.
- Morley RM, Tse HW, Feng B, Miller JC, Monaghan DT, and Jane DE (2005) Synthesis and pharmacology of N1-substituted piperazine-2,3-dicarboxylic acid derivatives acting as NMDA receptor antagonists. *J Med Chem* **48**:2627–2637.
- Nakanishi S (1992) Molecular diversity of glutamate receptors and implications for brain function. *Science* **258**:597–603.
- Neyton J and Paoletti P (2006) Relating NMDA receptor function to receptor subunit composition: limitations of the pharmacological approach. *J Neurosci* **26**:1331–1333.
- Sugihara H, Moriyoshi K, Ishii T, Masu M, and Nakanishi S (1992) Structures and properties of seven isoforms of the NMDA receptor generated by alternative splicing. *Biochem Biophys Res Commun* **185**:826–832.
- Tong G, Takahashi H, Tu S, Shin Y, Talantova M, Zago W, Xia P, Nie Z, Goetz T, Zhang D, et al. (2008) Modulation of NMDA receptor properties and synaptic transmission by the NR3A subunit in mouse hippocampal and cerebrocortical neurons. *J Neurophysiol* **99**:122–132.
- Urwiler S, Campbell E, Fricker G, Jenner P, Lemaire M, McAllister KH, Neijt HC, Park CK, Perkins M, Rudin M, et al. (1996) Biphenyl-derivatives of 2-amino-7-phosphono-heptanoic acid, a novel class of potent competitive N-methyl-D-aspartate receptor antagonists—II. Pharmacological characterization in vivo. *Neuropharmacology* **35**:655–669.
- Vicini S, Wang JF, Li JH, Zhu WJ, Wang YH, Luo JH, Wolfe BB, and Grayson DR (1998) Functional and pharmacological differences between recombinant N-methyl-D-aspartate receptors. *J Neurophysiol* **79**:555–566.

Address correspondence to: Dr. Blaise Mathias Costa, Department of Pharmacology, 985800 Nebraska Medical Center, Omaha, NE 68198-5800. E-mail: bmathiascosta@unmc.edu