A novel family of negative and positive allosteric modulators of NMDA receptors

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Abbreviations: AMPA, 2-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; NMDA, N-methyl-D-aspartate; NR2, NMDA receptor subunit 2; NSC339614, 6-, 7-, 8-, and 9-nitro isomers of naphth[1,2-c][1,2,5]oxadiazole-5-sulfonic acid; UBP512, 9-iodophenanthrene-3-carboxylic acid; UBP551, 3,5-dihydroxynaphthalene-2-carboxylic acid; UBP608, 6-bromo-2-oxo-2H-chromene-3-carboxylic acid;
UBP618, 1-bromo-2-hydroxy-6-phenynaphthalene-3-carboxylic acid; UBP710, 9-cyclopropylphenanthrene-3-carboxylic acid; UBP646, 9-(4-methylpent-1-yl) phenanthrene-3-carboxylic acid.

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Abstract

The N-methyl-D-aspartate (NMDA) receptor family regulates various CNS functions such as synaptic plasticity, however hypo- or hyper-activation of NMDA receptors is critically involved in many neurological and psychiatric conditions such as pain, stroke, epilepsy, neurodegeneration, schizophrenia, and depression. Consequently, subtype-selective positive and negative modulators of NMDA receptor function have many potential therapeutic applications not addressed by currently available compounds. We have identified allosteric modulators with several novel patterns of NMDA receptor subtype selectivity and having a novel mechanism of action. In a series of carboxylated naphthalene and phenanthrene derivatives, compounds were identified that selectively potentiate responses at GluN1/GluN2A (e.g. UBP512), GluN1/GluN2A and GluN1/GluN2B (UBP710), GluN1/GluN2D (UBP551), or GluN1/GluN2C and GluN1/GluN2D receptors (NSC339614) while having no effect or inhibiting responses at the other NMDA receptors. Selective inhibition was also observed; UBP512 inhibits only GluN1/GluN2C and GluN1/GluN2D receptors whereas UBP608 inhibits GluN1/GluN2A receptors with a 23-fold selectivity compared to GluN1/GluN2D receptors. The actions of these compounds were not competitive with the agonists L-glutamate or glycine and were not voltage-dependent. While the N-terminal regulatory domain (NTD) was not necessary for activity of either potentiators or inhibitors, segment 2 (S2) of the agonist ligand-binding domain was important for potentiating activity while subtype-specific inhibitory activity was dependent upon the segment 1 (S1). In terms of chemical structure, activity profile, and mechanism of action, these modulators represent a new class of pharmacological agents for the study of NMDA receptor subtype function and provide novel lead compounds for a variety of neurological disorders.
Introduction

The primary excitatory neurotransmitter in the vertebrate CNS, L-glutamate, activates three distinct families of ligand-gated ion channels that are named for agonists by which they are selectively activated, NMDA, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and kainate (Watkins et al., 1981; Monaghan et al., 1989; Dingledine et al., 1999). While AMPA and kainate receptors underlie fast excitatory synaptic transmission in the CNS, NMDA receptor activation triggers diverse calcium-dependent intracellular responses that regulate distinct forms of synaptic plasticity such as long-term potentiation, long-term depression and experience-dependent synaptic refinement (Monaghan et al., 1989; Dingledine et al., 1999). Such NMDA receptor-mediated mechanisms are thought to play key roles in learning and memory, but also contribute to the expression of epilepsy, schizophrenia, drug addiction, mood disorders, post-traumatic stress disorder and neuropathic pain (Kalia et al., 2008; Sanacora et al., 2008). Excessive NMDA receptor activation may also be a common mechanism causing neuronal cell death in stroke, traumatic brain injury and various neurodegenerative diseases such as Alzheimer’s, Parkinson’s, Huntington’s, amyotrophic lateral sclerosis (ALS), and Creutzfeldt-Jakob disease (Villmann and Becker, 2007; Kalia et al., 2008). These findings have led to high expectations for clinical studies of NMDA receptor-based therapeutic agents. Unfortunately, the results from these studies have been largely disappointing due to adverse effects and limited therapeutic efficacy (O’Collins et al., 2006; Kalia et al., 2008). To date, most NMDA receptor pharmacological agents tested in the clinic have been non-selective agents that cannot distinguish among NMDA receptor subtypes. This lack of subtype-selectivity has probably contributed to the inability to optimize the therapeutic effect of NMDA receptor pharmacological agents while minimizing their adverse effects (see discussion).
NMDA receptor complexes are composed of subunits from seven genes - GluN1, GluN2A-GluN2D, and GluN3A-GluN3B (Dingledine et al., 1999). The majority of NMDA receptors are thought to be composed of two GluN1 subunits and two GluN2 subunits (Laube et al., 1998). L-Glutamate and a necessary co-agonist (either glycine or D-serine), bind to homologous binding sites on GluN2 and GluN1 subunits, respectively, to cause the opening of the receptor’s Na⁺/K⁺/Ca²⁺ -permeable ion channel (Dingledine et al., 1999). Importantly, the GluN2 subunits have varied developmental profiles and anatomical distributions and confer distinct physiological, biochemical, and pharmacological properties to the NMDA receptor complex (Buller et al., 1994; Monyer et al., 1994; Cull-Candy et al., 2001). Evidence suggests that specific NMDA receptor subunits have distinct, and sometimes opposing, roles in various physiological and pathophysiological actions (Hrabetova et al., 2000; DeRidder et al., 2006; Chen et al., 2008). However, their specific roles have been difficult to study in the absence of highly-selective antagonists.

Currently, there are four functionally-distinct classes of compounds that are therapeutic candidates for the inhibition of NMDA receptor function, those that inhibit glutamate or glycine binding, block the ion channel, or compounds that inhibit the receptor by binding to an N-terminal regulatory domain (NTD) (Jane et al., 2000). Of these four drug targets, the first three drug binding sites are highly conserved in different NMDA receptor subtypes and it is only the NTD drug binding site for which there are compounds that fully distinguish GluN2 subunits. Presently, these latter compounds are limited to ones that are selective for GluN2B-containing receptors. Hence, the only subtype-selective agents that have been tested in the clinic are antagonists that selectively block GluN1/GluN2B receptors. While there are several potential therapeutic applications for positive modulators of NMDA receptor function, there are no such compounds available for clinical studies.
In this study, we have identified a series of naphthalene and phenantherene derivatives that display inhibitory and/or potentiating activity with remarkably different patterns of selectivity at NMDA receptors containing different GluN2 subunits. These agents, and their future derivatives, represent a novel class of NMDA receptor allosteric modulator drugs that do not act at either the glutamate or glycine binding sites, the ion channel, or the NTD. They may be acting at the dimer interface between individual subunit ligand-binding domains. This group of compounds should be valuable tools for identifying the physiological roles of distinct NMDA receptor subtypes and serve as lead compounds for a variety of therapeutic applications.
Methods

Compounds:

Structures of compounds synthesized and tested for this report are presented in Fig. 1. UBP512, UBP618, UBP646 and UBP710 were synthesized and purified by methods to be reported elsewhere. Following synthesis and purification, compound structure was verified by $^1$H 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. The other compounds were obtained from Alfa Aesar (UBP551), Sigma Aldrich (UBP608) and the National Cancer Institute’s Developmental Therapeutics Program Open Repository [http://dtp.cancer.gov](http://dtp.cancer.gov) (NSC339614).

NMDA receptor constructs

cDNA encoding the NMDAR1a subunit (GluN1a) was a generous gift of Dr. Shigetada Nakanishi (Kyoto, Japan). cDNA encoding the GluN2A, GluN2C and GluN2D were kindly provided by Dr. Peter Seeburg (Heidelberg, Germany) and the GluN2B [5 UTR] cDNA was the generous gift of Drs. Dolan Pritchett and David Lynch (Philadelphia, USA). GluN2A chimeras containing either the S1 (GluN2A$^{2CS1}$) or the S2 domain (GluN2A$^{2CS2}$) of GluN2C were constructed by overlap-extension PCR. In GluN2A$^{2CS1}$, the GluN2C S1 domain, amino acids 352-535, replaced the corresponding sequence in GluN2A (Monyer et al., 1992). In GluN2A$^{2CS2}$, the region between M3 and M4, GluN2C amino acids 634-795, replaced the corresponding sequence in GluN2A (Monyer et al., 1992). Constructs were verified by sequencing by the University of Nebraska Medical Center Sequencing Facility. The NTD-deleted NR1 (NR1$^{NTD}$) and the NTD-deleted NR2 constructs (NR2A$^{NTD}$ and NR2D$^{NTD}$) were kindly provided by Dr. Bodo Laube (Madry et al., 2008) and Dr. Pierre Paoletti (Rachline et al., 2005), respectively. Plasmids were linearized with Not I (GluN1a, GluN2C, GluN2D, and NR1$^{NTD}$), EcoRI (GluN2A, GluN2A$^{2CS1}$, and GluN2A$^{2CS2}$) or Sal I (GluN2B, NR2A$^{NTD}$ and NR2D$^{NTD}$) and
transcribed \textit{in vitro} with T7 (GluN1a, GluN2A, GluN2C, GluN2D, GluN2A\textsuperscript{2CS1}, and GluN2A\textsuperscript{2CS2}), SP6 (NR1\textsuperscript{ANTD}, NR2A\textsuperscript{ANTD}, NR2D\textsuperscript{ANTD} and GluN2B) RNA polymerase using the mMMessage mMMachine transcription kits (Ambion, Austin, TX, USA).

\textbf{NR subunit expression and electrophysiology in Xenopus oocytes}

Oocytes from mature female \textit{Xenopus laevis} (Xenopus One, Ann Arbor, MI, USA) were removed and isolated. GluN1a and GluN2 RNAs were dissolved in sterile distilled H\textsubscript{2}O and mixed in a molar ratio of 1:1-3. 50 nl of the final RNA mixture was microinjected (15-30 ng total) into the oocyte cytoplasm. Oocytes were incubated in ND-96 solution at 17°C prior to electrophysiological assay (1-5 days). Electrophysiological responses were measured using a standard two-microelectrode voltage clamp (model OC-725B, Warner Instruments, Hamden, Connecticut). The recording buffer contained 116 mM NaCl, 2 mM KCl, 0.3 mM BaCl\textsubscript{2} and 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4. Agonist-evoked responses were clamped at \(-60\) mV, unless stated otherwise. Response amplitudes for the four heteromeric complexes were generally between 0.1 to 3 \(\mu\)A. After obtaining a steady-state response to agonist application, test compounds were bath applied (Automate Scientific 16-channel perfusion system, Berkeley, CA, USA) and the responses were digitized for quantification (Digidata 1440A and pClamp-10, Molecular Devices, Sunnyvale, CA, USA). Dose-response relationships were fit to a single-site with variable slope (GraphPad Prism, ISI Software, San Diego, CA, USA), using a nonlinear regression to calculate IC\textsubscript{50} or EC\textsubscript{50} and % maximal inhibition. All experiments were performed a minimum of 4 times.
Results:

A variety of structures containing either two or three fused aromatic rings were evaluated for their ability to modulate NMDA receptor responses evoked by 10 µM L-glutamate and 10 µM glycine. GluN1/GluN2A, GluN1/GluN2B, GluN1/GluN2C, and GluN1/GluN2D receptors were expressed in *Xenopus* oocytes and receptor activity was determined by two-electrode voltage clamp. Of the compounds screened, 7 compounds represent the different activities that were observed. Four of these compounds were novel and were synthesized. UBP512 (9-iodophenanthrene-3-carboxylic acid) inhibited GluN1/GluN2C, and GluN1/GluN2D receptors, had minimal effect on GluN2B-containing receptors, and caused a small potentiation of GluN1/GluN2A receptors (Fig. 1A). At 3-10 µM, UBP512 weakly inhibited GluN1/GluN2A and GluN1/GluN2B receptor responses (~10-15%). At higher doses, UBP512 potentiated GluN1/GluN2A receptor-mediated responses and inhibited responses at GluN1/GluN2C (IC$_{50}$ = 51 ± 11 µM, Hill coefficient = 1.3 ± 0.3) and GluN1/GluN2D receptors (IC$_{50}$ = 46 ± 6 µM, Hill coefficient = 1.35 ± 0.1). Under these conditions, UBP512 maximally inhibited 69 ± 6 % and 72 ± 2 % of the total GluN1/GluN2C and GluN1/GluN2D receptor responses, respectively.

In contrast to UBP512, UBP551 (3,5-dihydroxynaphthalene-2-carboxylic acid) inhibited responses at receptors containing GluN2A, GluN2B, or GluN2C subunits and potentiated activity at GluN1/GluN2D receptors (Fig. 1B). UBP551 displayed IC$_{50}$s of 9.7 ± 0.2 µM, 9.4 ± 0.6, and 15 ± 6 µM for receptors containing GluN2A-C subunits, respectively, and Hill coefficients of 1.4 ± 0.1, 1.8 ± 0.2, 1.2 ± 0.3, with maximal inhibition of 91 ± 1.3%, 83.9 ± 7.1%, and 85.0 ± 2.3%. Maximal potentiation of GluN1/GluN2D responses was found at a concentration of 30 µM; higher concentrations resulted in reduced potentiating activity.
UBP608 (6-bromo-2-oxo-2H-chromene-3-carboxylic acid) and UBP618 (1-bromo-2-hydroxy-6-phenylnaphthalene-3-carboxylic acid) displayed only inhibitory activity when tested against receptor responses evoked by 10 µM L-glutamate plus 10 µM glycine (Figs. 1C and 1D). UBP608 fully inhibits (maximal inhibition = 104 ± 0.6 %) GluN1/GluN2A responses with an IC₅₀ of 18.6 ± 1.4 µM and a Hill coefficient = 1.08 ± 0.02. Several-fold higher concentrations of UBP608 were required to inhibit GluN1/GluN2B (IC₅₀ = 90 ± 4 µM, Hill coefficient = 1.25 ± 0.06) and GluN1/GluN2C responses (IC₅₀ = 68 ± 9 µM, Hill coefficient = 1.22 ± 0.07). GluN2D-containing receptors were least affected with an extrapolated IC₅₀ of 426 ± 40 µM and a Hill coefficient = 1.16 ± 0.1. UBP618 was a relatively potent, non-selective inhibitor at NMDA receptors (Fig. 1C) with IC₅₀s of GluN1/GluN2A: 1.8 ± 0.2 µM, GluN1/GluN2B: 2.4 ± 0.1 µM, GluN1/GluN2C: 2.0 ± 0.08 µM, and GluN1/GluN2D: 2.4 ± 0.3 µM. Corresponding Hill coefficients were 0.98 ± 0.07, 0.94 ± 0.04, 0.98 ± 0.05, and 1.48 ± 0.15 and maximal inhibitions were 83 ± 4%, 88 ± 2.0%, 87 ± 2% and 87 ± 5%, respectively.

In contrast to UBP512, UBP710 (9-cyclopropylphenanthrene-3-carboxylic acid) displayed greater activity in potentiating GluN2B-containing receptors than those containing GluN2A (Fig. 1E). UBP710 potentiated responses at receptors containing GluN2A and GluN2B subunits (approximately 50 - 150%) and, at 100 µM, usually caused a weak potentiation of responses at GluN2C and GluN2D-containing receptors (GluN2C: 8 out of 12 cells; GluN2D: 8 out of 12 cells). A more universal potentiator was UBP646 (9-(4-methylpent-1-yl) phenanthrene-3-carboxylic acid). This agent most effectively potentiated GluN1/GluN2D receptors and also consistently potentiated the other 3 subtypes (Figs. 1F).

A different pattern of potentiation activity was observed for the compound NSC339614. This compound potentiated GluN1/GluN2C and GluN1/GluN2D receptor responses while inhibiting GluN1/GluN2A and GluN1/GluN2B receptor activity (Supplemental Fig. 1). Chemical
characterization revealed that this compound is a mixture of the 6-, 7-, 8-, and 9-nitro isomers of naphth[1,2-c][1,2,5]oxadiazole-5-sulfonic acid potassium salt (Supplemental Fig. 2).

The structural features of these compounds do not conform to any known group of NMDA receptor antagonists or modulators. Thus, further studies were directed at defining the site of action. UBP512 does not appear to act as an NMDA receptor ion channel blocker. The ability of UBP512 to inhibit GluN1/GluN2D responses was not voltage-dependent (Fig. 2B), suggesting that UBP512 does not block by binding within the ion channel pore that is exposed to the transmembrane electric field. Voltage-dependency of inhibition was also evaluated for UBP618; 100 µM UBP618 inhibited GluN1/GluN2A responses 76 ± 4% at +40 mV and by 66 ± 3% at -60 mV.

UBP512 is not a competitive antagonist at either the L-glutamate or glycine binding sites. The blockade of GluN2C or GluN2D by 100 µM UBP512 could not be overcome by increasing concentrations of glycine (Fig. 2C) or L-glutamate (Fig. 2D). At the highest doses of L-glutamate in the presence of UBP512, there was a small reduction in both GluN1/GluN2C and GluN1/GluN2D receptor activation (Fig. 2D). In converse experiments, UBP512 activity was tested with a range of concentrations in the presence of low (10 µM) or high (300 µM) concentrations of L-glutamate and glycine (Fig. 2E). High agonist concentrations did not significantly alter UBP512 potency for inhibition (GluN1/GluN2C and GluN1/GluN2D) or potentiation (GluN1/GluN2A). GluN1/GluN2C and GluN1/GluN2D receptor responses to high agonist concentrations (300 µM L-glutamate / 300 µM glycine) were inhibited by UBP512 with IC₅₀s of 108 ± 12 µM and 53 ± 6 µM, respectively. However, in the presence of high agonist concentrations, UBP512 blockade became more effective. Under these conditions, UBP512 fully blocked GluN2C- and GluN2D-containing receptor responses (104 ± 8% and 97 ± 7%, respectively), and displayed Hill coefficients near 2 (1.8 ± 0.3 and 2.1 ± 0.5, respectively). The greater blockade by UBP512 in the presence of high agonist concentrations can...
account for the decrease in response size that occurs in the presence of 100 µM UBP512 when increasing L-glutamate concentration (Fig. 2D). Increasing agonist concentrations also increased the magnitude of UBP512 potentiation of GluN1/GluN2A receptors (Fig. 2E).

In a similar manner, the potency of UBP618 for inhibiting GluN1/GluN2A and GluN1/GluN2D responses were mostly unaffected by increasing agonist concentration (Fig. 2F; GluN1/GluN2A IC50 = 3.2 ± 0.4 µM, GluN1/GluN2D IC50 = 1.8 ± 0.1 µM) while the maximal % inhibition of GluN1/GluN2A responses was decreased from 83 ± 4% to 72 ± 1% and the maximal % inhibition of GluN1/GluN2D responses were increased from 92.0 ± 0.7% to 99.9 ± 0.4%. These results indicate that the inhibitory actions of UBP512 and UBP618 are not due to a competitive interaction at either the L-glutamate or glycine binding sites. However, agonist binding does alter the ability of UBP512 and UBP618 to inhibit channel function.

Zn++ is a high affinity negative modulator of GluN1/GluN2A receptors that binds to the N-terminal regulatory domain (NTD) of GluN2A (Paoletti et al., 2000). Thus, the selective potentiation of GluN2A-containing receptors by UBP512 could potentially be due to the reversal of Zn++-inhibition by Zn++ chelation. However, UBP512 potentiation was not affected by the presence of a potent Zn++ chelator, nor by the addition of 100 nM Zn++ (Supplemental Fig. 3A). Conversely, UBP512 addition did not alter the EC50s for either the high affinity or the low affinity components of Zn++ inhibition at GluN1/GluN2A receptors (Supplemental Fig. 3B).

UBP512, UBP608, and UBP710 do not require the NTD for their modulatory activity. Removal of the NTD region of both GluN1 and GluN2 subunits enhances, rather than blocks, UBP512 potentiation of GluN1/GluN2A receptors (Fig. 3A). Likewise, NTD-deletion of GluN1/GluN2D receptors does not eliminate UBP512 inhibitory activity, but does reduce UBP512 inhibitory potency a
few-fold (Fig. 3A). NTD deletion also did not block UBP608’s inhibitory activity (Fig. 3C) nor the ability of UBP710 to potentiate responses at GluN1/GluN2A receptors (Fig. 3E).

Since the NTD region is not necessary for modulator activity, these compounds are most likely binding on the remaining extracellular region that comprises the ligand-binding domain – either segment 1 (S1) between the NTD and the first intra-membrane domain (M1) and/or on segment 2 (S2) the extracellular loop between the third (M3) and fourth (M4) intra-membrane domain (see Fig 2A). The GluN1/GluN2A potentiators UBP512 and UBP710 and the inhibitor UBP608 were tested on GluN2A chimeras containing either the S1 or the S2 domain of GluN2C (Fig. 3). GluN2A-containing NMDA receptors with the S1 domain of GluN2C were still potentiated by UBP512 and UBP710, however GluN2A-containing receptors with the S2 domain of GluN2C were inhibited, instead of potentiated, by UBP512 and UBP710. In contrast, the inhibitory actions of UBP608 (which has higher potency at GluN2A- than GluN2C-containing receptors) were reduced in receptors having the GluN2A subunit with the GluN2C S1 domain. The S2 domain of GluN2C in GluN2A had a negligible affect on UBP608 inhibitory activity. Thus, the S2 domain is important for the binding and/or the downstream potentiating actions of UBP512 and UBP710 while the S1 domain appears to be more important for the inhibitory actions of UBP608.

All compounds were also evaluated for agonist or partial agonist activity by testing for excitatory activity alone or when paired with glycine or L-glutamate at each of the 4 GluN1/GluN2 receptors (Supplemental Table 2). Compounds were found to be devoid of agonist or partial agonist activity. Since the inhibitory modulators generally have maximal inhibitions of 70 – 90%, the absence of partial agonist activity confirms that these compounds are not acting at either of the agonist (L-glutamate or glycine) binding sites.
Discussion:

Over the past 30 years, there have been more than 30,000 publications characterizing NMDA receptor function. This work has established that NMDA receptors are critically involved in many physiological and pathophysiological activities and has led to many preclinical and clinical studies attempting to develop NMDA receptor therapeutic agents for the treatment of epilepsy, schizophrenia, depression, pain, drug addition, alcoholism, Alzheimer’s, Huntington’s, Parkinson’s, ALS, traumatic brain injury, stroke and other conditions (Koutsilieri and Riederer, 2007; Kalia et al., 2008; Sanacora et al., 2008). Unfortunately, results from most clinical trials have been disappointing due to adverse effects and limited therapeutic efficacy (Villmann and Becker, 2007; Kalia et al., 2008). Optimal therapeutic effectiveness of NMDA receptor pharmacological agents may require targeting the most appropriate subtypes of NMDA receptors. Thus, it is significant that most NMDA receptor agents that have been evaluated in the clinic, other than GluN2B-selective agents, are not subtype-selective. Due to the highly conserved nature of their respective binding sites, compounds that inhibit glutamate or glycine binding, or that block the ion channel, have very low subtype-selectivity.

In the present study, we have identified a class of allosteric modulators with a novel mechanism of action which imparts greater subtype-selectivity than the other classes of NMDA receptor agents. For UBP512 and UBP710 (and NSC339614, Supplemental Fig. 1), there is a general separation of activities at GluN2A/GluN2B vs GluN2C/GluN2D-containing receptors consistent with their relative degree of sequence homology (Dingledine et al., 1999). Some of the compounds described here can also distinguish between GluN2A and GluN2B (e.g. UBP512 and UBP608) and between GluN2C and GluN2D (e.g. UBP551 and UBP608). Thus, the corresponding pharmacophores for these agents appear to vary between the GluN2 subunits, especially between GluN2A/B and GluN2C/D subunits. The degree of selectivity by these compounds is already greater than that displayed by glutamate and
glycine binding site antagonists and channel blockers. Thus, this class of agents should be a valuable approach for further development of subtype-selective agents.

Since NMDA receptors are involved in a wide variety of psychiatric and neurological conditions, there are many potential applications of subtype-selective positive and negative NMDA receptor modulators. Most clinical interest has focused on the use of NMDA receptor blockers as neuroprotective agents. Over-activation of NMDA receptors can lead to neuronal cell death in stroke, head injury, and probably, neurodegenerative diseases. Importantly, several studies have indicated that NMDA receptor subtypes differ in their ability to cause cell death. GluN2B-containing NMDA receptors initiate cell death whereas GluN2A-containing receptors have been reported to contribute to neuroprotection signaling in traumatic mechanical injury and ischemia models (DeRidder et al., 2006; Chen et al., 2008; Terasaki et al., 2010). This may correspond to an enrichment of GluN2A and GluN2B subunits in synaptic and extrasynaptic compartments, respectively (Tovar and Westbrook, 1999; Lozovaya et al., 2004), and the ability of synaptic NMDA receptors to promote neuroprotection while extrasynaptic NMDA receptor activation signals to neuronal cell death (Hardingham and Bading, 2003; Papadia et al., 2008) (but see Thomas et al., 2006; von Engelhardt et al., 2007). Thus, the neuroprotective properties of GluN2B-selective antagonists have been actively studied.

Multiple lines of evidence also suggest that GluN2D may have a special role in initiating cell death in various conditions. As mentioned above, extrasynaptic NMDA receptors may preferentially contribute to cell death (Hardingham and Bading, 2003). Thus, it is noteworthy that GluN2D is found exclusively in the extrasynaptic compartment at some CNS synapses (Momiyama, 2000; Brickley et al., 2003; Lozovaya et al., 2004). Consistent with an excitotoxic role, we find that GluN2D knockout mice display reduced cerebral cortical damage, but unchanged hippocampal damage in the middle cerebral artery occlusion stroke model (D. T. Monaghan, H. Zhao, V. Gautam, H. Sun, and W. Mayhan,
Society for Neuroscience abstract 873.2, 2010). Related to this observation, tissue plasminogen activator (TPA) – enhanced stroke damage in the cerebral cortex appears to be dependent specifically upon GluN2D subunits (Baron et al., 2010). GluN2C and/or GluN2D may also play a specific role in white matter injury (Salter and Fern, 2005) and (specifically GluN2D) a role in Creutzfeldt-Jakob disease (Khosravani et al., 2008) and Alzheimer’s disease (Khosravani et al., 2008; Lauren et al., 2009). Hence, compounds with partial GluN2D-selectivity, such as UBP512, may have neuroprotective actions in some brain regions without affecting the larger populations of GluN2A- and GluN2B-containing receptors. GluN2-selective inhibitors of NMDA receptor signaling may also be useful for treating pain. GluN2D subunits are essential for the expression of pain in the sciatic nerve ligation neuropathic pain model and in the prostaglandin PGF2-α-induced pain model while GluN2A is important in the expression of tonic inflammatory pain (Hizue et al., 2005).

The compound class identified here has several additional therapeutic applications due to their ability to potentiate NMDA receptor activity. One intriguing possibility is that the potentiation of synaptic NMDA receptors containing the GluN2A subunit may stimulate neuroprotective-signaling pathways (Chen et al., 2008; Terasaki et al., 2010). In an in vivo context, direct agonist activation would activate inappropriate receptors while a potentiator should specifically increase the response of endogenously activated receptors, thus enhancing an appropriate biological response. Consequently, compounds that selectively potentiate GluN2A subunits, while inhibiting GluN2D-containing receptors (e.g. UBP512), may have combined pro-survival and neuroprotective properties. Such an activity may also have cognitive enhancement properties by promoting synaptic plasticity.

NMDA receptor potentiators might also be useful in treating post-traumatic stress disorder and schizophrenia. The reversal of post-traumatic stress disorder has been reported to be enhanced by increasing NMDA receptor function (Davis et al., 2006). In the case of schizophrenia, this disease is
thought to be associated with NMDA receptor hypofunction (Lindsley et al., 2006). Thus the ability to selectively potentiate the most appropriate subpopulations of NMDA receptors may be useful in these conditions.

The structure-activity relationships for these compounds are unusual and thus they represent a novel class (or classes) of compounds. The structural features corresponding to the activities described here do not conform to any known group of NMDA receptor antagonists or modulators, they do not contain an amino group alpha to a carboxylic acid group as is common for either glutamate or glycine binding site ligands (Jane et al., 2000). These compounds also do not have a T-shaped hydrophobic multi-ring system with a positive charge center commonly found in NMDA receptor channel blockers. They also do not have an extended structure with an aromatic ring containing a proton donor linked via a basic nitrogen to another aromatic ring – a structure that is typical of ifenprodil-like agents that act at the NTD (Jane et al., 2000).

Consistent with these structure-activity considerations, UBP512 and related compounds, do not act as competitive ligands at either the L-glutamate or glycine binding sites. Interestingly, however, high agonist concentrations differentially affect modulator activity at GluN1/GluN2A and GluN1/GluN2D receptors, by enhancing modulator potentiation (or reducing inhibition) at GluN1/GluN2A and enhancing modulator inhibition at GluN1/GluN2D receptors. This dichotomy parallels the differential actions of the NTD on NMDA receptors – removing the NTD domains of GluN2A and GluN2D have opposite actions on both channel open probability and glutamate affinity (Gielen et al., 2009; Yuan et al., 2009). The NTD also has an influence on modulator activity, but it is not required for either the inhibitory or potentiating actions (Fig. 3). Thus the modulator binding site is not at the L-glutamate or glycine binding sites or on the NTD, although these sites interact allosterically with the modulator site.
UBP512 and UBP710’s potentiating actions become inhibitory at GluN1/GluN2A receptors that have the GluN2A S2 domain replaced by GluN2C’s S2 domain (Fig. 3). Thus, these modulators might be binding to this domain or this domain contributes to transducing the effect of modulator binding to its effect on receptor function. In contrast, the subunit-specific inhibitory actions of UBP608 are influenced by the GluN2 subunit’s S1 domain and not the S2 domain. These findings suggest that the potentiating and inhibiting activities are at different binding sites. This would be consistent with the biphasic effect that some compounds display upon adding or removing the modulator (Fig. 1A,E,F).

The dimer interface between the agonist ligand-binding domains may be the site of action for UBP512 and related compounds. In the AMPA receptor family, several positive and negative modulators have been identified. Site-directed mutagenesis and crystallography studies indicate that the inhibitory 2,3-benzodiazepines (e.g. GYKI-52466) bind at the dimer interface formed by the ligand-binding domains (Balannik et al., 2005; Ahmed and Oswald, 2010). Also in the ligand-binding dimer interface is a binding site for the allosteric potentiator cyclothiazide (Ahmed and Oswald, 2010). Consistent with this possible location, we find that GluN2 identity of the S2 domain influences UBP512 and UBP710 potentiation while the S1 domain is important for the subunit-selective inhibitory actions of UBP608.

The compounds described here represent several novel lead compounds for a variety of activities at NMDA receptors. We have found that small structural modifications of these compounds lead to significant changes in potency and selectivity. Hence, these compounds should be useful tools for determining the function of discrete NMDA receptor subtypes, but also serve as a unique starting point for developing highly-specific NMDA receptor modulator agents for a variety of neuropsychiatric and neurological conditions.
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References


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Figure Legends

Figure 1

A series of two-ring and three-ring aromatic structures display varied activities on the responses of NMDA receptor subtypes. Representative voltage-clamped (-60 mV) current responses are shown for GluN1/GluN2A (2A) and GluN1/GluN2D (2D) receptors evoked by 10 µM L-glutamate and 10 µM glycine (black bar) plus the addition of 100 µM of the indicated compound (gray bar). Scale bar: x-axis = 17 seconds, y-axis = 300 nA (mean values, see Supplemental Table 1 for individual values). In the bottom of each panel is a dose-response of compound potentiation (values > 1) or inhibition (values < 1) of agonist responses by GluN1/GluN2A (filled squares), GluN1/GluN2B (filled circles), GluN1/GluN2C (open squares), and GluN1/GluN2D (open circles) receptors. Values represent means ± s.e.m. with N = 4 or more.

Figure 2

Compound inhibition of NMDA receptor responses is not voltage dependent and does not compete with L-glutamate or glycine binding to NMDA receptors. (A) A schematic illustrating a GluN1/GluN2 dimer and the domain structure and binding sites for L-glutamate (hexagon), glycine (star), NTD ligands (oval), and channel blockers (square). (B) UBP512 (100 µM) inhibition of GluN1/GluN2D receptor responses at different membrane potentials. Inserts: current traces showing agonist (black bar) and UBP512 application (grey bar), scale bars (X = seconds, Y = µA): -60 mV (180, 1.1); +40 mV (72, 2.0). (C, D) GluN1/GluN2C (2C) or GluN1/GluN2D (2D) receptors were activated by increasing concentrations of glycine (C) or L-glutamate (D) and 10 µM of the other agonist in the absence (filled symbols) or presence (open symbols) of 100 µM UBP512. (E, F) UBP512 (E) and UBP618 (F) modulation of NMDA receptor responses evoked by low (10 µM L-glutamate and 10 µM glycine, open
symbols with dashed lines) or high agonist concentrations (300 µM L-glutamate / 300 µM glycine, closed symbols with solid lines). UBP512 more effectively inhibited GluN1/GluN2C (inverted triangles) and GluN1/GluN2D (circles) receptor responses and more effectively potentiated GluN1/GluN2A (squares) receptor responses evoked by high agonist concentrations than by low concentrations. (F) UPB618 displays greater maximal inhibition of GluN1/GluN2D receptor responses and decreased maximal inhibition of GluN1/GluN2A receptor responses in the presence of high agonist concentrations.

**Figure 3**

(A, C, E) Compound activity was tested on responses evoked by 10 µM L-glutamate / 10 µM glycine of wildtype GluN1/GluN2A (2A) and GluN1/GluN2D (2D) receptors (dashed lines) or receptors without NTDs of both GluN1 and GluN2 subunits (solid lines) - GluN1/GluN2A (2A^NTD) and GluN1/GluN2D (2D^NTD). (B, D, E) Compounds were tested on responses by wildtype GluN1/GluN2A (2A) and GluN1/GluN2C (2C) receptors (dashed lines) and by chimeric receptors (solid lines) where the GluN2A subunit has the GluN2C S1 (2A^2CS1) or the GluN2C S2 (2A^2CS2) domain.
Figure 1
Figure 3