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Pharmacological heterogeneity of NMDA receptors: characterization of NR1a/NR2D heteromers expressed in *Xenopus* oocytes

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Abstract

The pharmacology of recombinant NR1a/NR2D NMDA receptors expressed in *Xenopus* oocytes was examined and compared to the pharmacology of NR1a/NR2A, NR1a/NR2B and NR1a/NR2C heteromers. The NR1/NR2D heteromer showed a pharmacological profile distinct from each of the other NR1/NR2 heteromers. This unique pharmacological profile was characterized by a relatively lower affinity for the agonist homoquinolinate and the antagonists 2-amino-5-phosphonopentanoate (D-AP5) and (*R,E*)-4-(3-phosphonoprop-2-enyl)piperazine-2-carboxylic acid (D-CPPene) but not for the antagonists (±)-4-(4-phenylbenzoyl) piperazine-2,3-dicarboxylic acid (PBPD) and α-amino-5-(phosphonomethyl)[1,1′-biphenyl]-3-propanoic acid (EAB515). NR2D-containing receptors displayed a pharmacological profile most similar to that observed for receptors containing the genetically related NR2C subunit. These findings parallel observations obtained for native NMDA receptors in the medial thalamus (presumed to contain NR2D subunits) and forebrain (presumed to contain NR2A and NR2B subunits). Thus, only compounds that discriminate between either NR2A- or NR2B-containing heteromers and NR2D-containing hetermers also discriminate between forebrain and medial thalamic NMDA receptors. While the pharmacology of the NR1a/NR2D receptor shows many parallels to the medial thalamic NMDA receptor, some differences were observed. Certain compounds which discriminate between medial thalamic and cerebellar (presumed to contain NR2C subunits) receptors (e.g., homoquinolinate, D-CPPene) did not show a similar selectivity for NR1a/NR2D receptors relative to NR1/NR2C receptors. Co-expression of NR1a, NR2B and NR2D subunits in *Xenopus* oocytes resulted in the formation of heteromeric complexes with unique pharmacological properties, suggesting the co-existence of these two distinct NR2 subunits in the same receptor complex.

Keywords: NMDA receptor; Glutamate receptor; *Xenopus* oocyte; Ligand-gated ion channel

1. Introduction

NMDA receptors mediate synaptic transmission and neural plasticity at many sites in the mammalian central nervous system (CNS; Bear and Malenka, 1994; Monaghan et al., 1989). NMDA receptors are also involved in epileptiform activity and neuronal cell death in a number of experimental and pathological conditions (Lipton and Rosenberg, 1994). Radioligand binding and electrophysiological studies have identified four pharmacologically distinct populations of NMDA receptors. Two of these populations are typified by receptors in the cerebellum (Perkins and Stone, 1983; Sekiguchi et al., 1990; Ebert et al., 1991; Monaghan and Beaton, 1991; O'Shea et al., 1991; Beaton et al., 1992; Buller et al., 1994) and the medial thalamic nuclei (Buller et al., 1994; Beaton et al., 1992) which display pharmacological heterogeneity at both the glutamate and channel blocker binding sites. Two additional populations of NMDA-selective glutamate binding sites have been identified based on their relative affinities for agonists and antagonist; these binding sites have been termed agonist-preferring and antagonist-preferring NMDA receptors (Sakurai et al., 1993; Monaghan, 1991; Monaghan et al., 1988).

Two NMDA receptor subunit families (NR1a–h and NR2A–D) have been identified in rat (Ishii et al., 1993; Monyer et al., 1992, 1994; Moriyoshi et al., 1991) and mouse (Sugihara et al., 1992; Yamazaki et al., 1992; Meguro et al., 1992; Kutsuwada et al., 1992). Alternative splicing of a single NR1 gene generates eight isoforms with distinct functional properties (Hollmann et al., 1993; Nakanishi et al., 1992; Sugihara et al., 1992; Durand et al., 1992, 1993; Anantharam et al., 1992). Heterogeneity within the NR2 subunit family results from expression of four closely related genes (Sugihara et al., 1992; Yamazaki et

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al., 1992; Meguro et al., 1992; Kutsuwada et al., 1992; Ishii et al., 1993; Monyer et al., 1992, 1994). The contribution of the individual subunits to the mature receptor complex has not been determined. Native NMDA receptors are believed to be oligomeric complexes formed from combinations of NR1 and NR2 subunits, with the NR2 subunit imparting distinct functional and pharmacological properties (Buller et al., 1994; Laurie and Seeburg, 1994a,b; Meguro et al., 1992; Kutsuwada et al., 1992; Monyer et al., 1992).

The distribution of the NMDA receptor subunit mRNA parallels the distribution of NMDA receptor subtypes in native brain. NR2A and NR2C subunit mRNAs are found in regions expressing antagonist-preferring and cerebellar NMDA receptor subtypes, respectively (Buller et al., 1994; Laurie and Seeburg, 1994a,b; Watanabe et al., 1993). Agonist-preferring forebrain NMDA receptors are found only in regions expressing both NR2B and NR1 subunits lacking cassette 1 (Buller et al., 1994). The NR2D subunit mRNA is expressed exclusively in brain regions that display a distinct NMDA receptor subtype pharmacology (Buller et al., 1994; Monyer et al., 1994; Watanabe et al., 1992; Monaghan and Beaton, 1992; Beaton et al., 1992). Thus, NR2D mRNA is found in specific midline thalamic nuclei (paratenial nucleus, paraventricular nucleus, intermediodorsal nucleus, medial dorsal lateral nucleus and rhomboid nucleus) and other select nuclei, including the anteroventral nucleus, medial geniculate, periaqueductal gray, interpeduncular nucleus, the glomerular layer of the olfactory bulb and the nucleus of the diagonal band.

Previous studies have demonstrated that native NMDA receptor subtype pharmacology can be reproduced in *Xenopus* oocytes expressing specific NR1a/NR2 combinations (Buller et al., 1994; Williams et al., 1993). The parallel distribution of the NR2D subunit mRNA and the medial thalamic NMDA receptor suggests that the NR2D subunit confers the unique pharmacological properties upon the medial thalamic NMDA receptor subtype. In the present report, we have characterized the pharmacology of the NR1a/NR2D heteromer expressed in *Xenopus* oocytes. In addition, the pharmacology of heteromers formed in oocytes co-expressing NR1a, NR2B and NR2D subunits was investigated to evaluate potential heterotrimeric complexes.

2. Materials and methods

2.1. Materials

2.1.1. Compounds

Structures of compounds used in this report are presented in Fig. 1. *N*-Methyl-D-aspartate (NMDA) and 2-amino-5-phosphonopentanoate (D-AP5; Davies and Watkins, 1982) were from Sigma (St. Louis, MO, USA). Homoquinolinate (Stone, 1984) and (±)-4-(4-phenylbenzoyl) piperazine-2,3-dicarboxylic acid (PBPD) were provided by Drs. David Jane and Jeff Watkins. (*R,E*)-4-(3-phosphonoprop-2-enyl)piperazine-2-carboxylic acid (D-CPPene; Aebischer et al., 1989) was provided by Dr. Paul Herrling (Sandoz Pharmaceuticals, Basle, Switzerland). (±)-6-(1*H*-Tetrazol-5-ylmethyl)decahydroisoquinoline-3-carboxylic acid (LY233536; Ornstein et al., 1991) was from Eli Lilly (Indianapolis, IN, USA). α-Amino-5-(phosphonomethyl)[1,1′-biphenyl]-3-propanoic acid (EAB515; Müller et al., 1992) was provided by Drs. Aebischer and Mueller (Sandoz Pharmaceuticals).

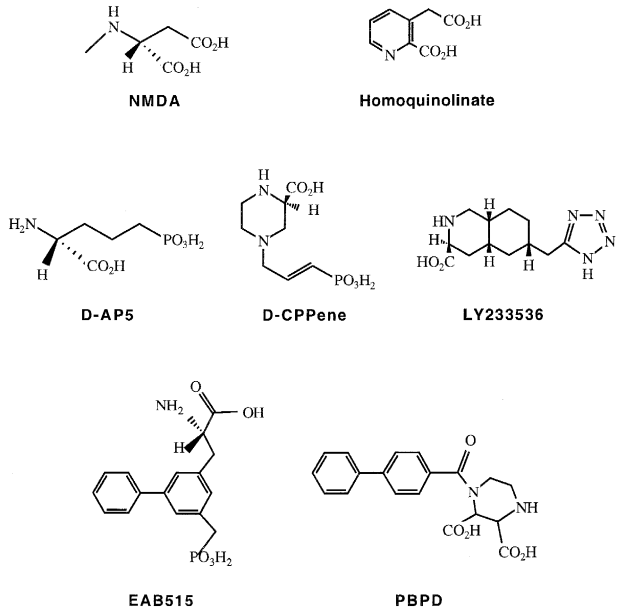

Fig. 1. Structures of NMDA receptor agonists and antagonists.

2.1.2. cDNAs

pN60 containing the 4.2 kb NR1 subunit cDNA (Moriyoshi et al., 1991) inserted into pBluescript (Stratagene, La Jolla, CA, USA) was provided by Dr. Shigetada Nakanishi (Kyoto University Faculty of Medicine, Kyoto, Japan). This cDNA corresponds to the predominant splice variant found in the CNS, NR1a (10,53). cDNAs encoding the NR2A, NR2C (Monyer et al., 1992) and NR2D (Monyer et al., 1994) were the generous gift of Dr. Peter Seeburg (University of Heidelberg, Heidelberg, Germany). The NR2D subunit cDNA was subcloned into pcDNA1/Amp (Invitrogen, San Diego, CA, USA), for use in the studies described below. The NR2B[5′UTR] cDNA was the generous gift of Drs. Dolan Pritchett and David Lynch (University of Pennsylvania, Philadelphia, PA, USA) and contains the NR2B subunit cDNA from which most of the upstream 5′ untranslated sequences have been removed to allow more efficient translation in *Xenopus* oocytes (Williams, 1994).

2.2. In vitro transcription

Plasmids were linearized with *Not*I (NR1a), *Eco*RI (NR2A, NR2C and NR2D) or *Sal*I (NR2B) and tran-

scribed in vitro with T3 (NR2A, NR2C), SP6 (NR2B) or T7 (NR1a, NR2D) RNA polymerase using the mMessage mMachine transcription kit (Ambion, Austin, TX, USA).

2.3. Translation in Xenopus oocytes

Oocytes were removed from mature female *Xenopus laevis* (Xenopus One, Ann Arbor, MI, USA) as previously described (Buller et al., 1994). NMDA receptor subunit RNAs were dissolved in sterile distilled H_2O. NR1a and NR2 RNAs were mixed in a molar ratio of either 1:1 or 1:3 to minimize the formation of NR1a homomers. 50 nl of the final RNA mixture was microinjected (15–30 ng total) into the oocyte cytoplasm (Buller et al., 1994, 1995). Oocytes were incubated in ND-96 solution at 17°C prior to electrophysiological assay (2–8 days).

2.4. Electrophysiology

Electrophysiological responses were measured using a standard two-microelectrode voltage clamp (model OC-725A oocyte clamp, Warner Instruments, Hamden, CT, USA). Electrodes were filled with 3 M KCl and had resistances of 0.6–3 MΩ. The recording chamber was continuously perfused with Ba^{2+}-Ringers solution (116 mM NaCl, 2 mM KCl, 2 mM $BaCl_2$, 5 mM HEPES, pH 7.4) and all drugs were dissolved in the same solution. Electrophysiological recordings were performed in Ba^{2+}-Ringers solution to minimize the contribution of the Ca^{2+}-activated Cl^- current to the whole-cell current response to NMDA (Leonard and Kelso, 1990). Response magnitude was determined by the steady-state plateau response elicited by bath application of 100 μM NMDA and 10 μM glycine, unless otherwise indicated, at a holding potential of −60 mV. The presence of the plateau response was taken as an indication of a lack of significant activation of the endogenous Cl^- current by Ba^{2+} in these cells. Because the different heteromers show variability in the level of NMDA receptor expression, coupled with the inherent frog-to-frog variability in expression, electrophysiological recordings were conducted on different days (1–5 days post-injection) to allow for similar response magnitudes. Response amplitudes for the four heteromers were generally between 35–200 nA. Attempts were made to keep response magnitudes within this range to minimize activation of the endogenous Cl^- current. Dose-response curves for agonists were fit (GraphPad Prism, ISI Software, San Diego, CA, USA) according to the equation $I = I_{max}/[1 + EC_{50}/A)^n]$ where I is the current response, I_{max} is the maximal current response, n is the Hill coefficient, A is the agonist concentration and EC_{50} is the agonist concentration producing a half-maximal response. Antagonist inhibition curves were determined in the presence of 100 μM NMDA and 10 μM glycine and were fit according to the equation $I = I_{max} - I_{max}/[1 + (IC_{50}/A)^n]$ where I_{max} is the current response in the absence of antagonist, A is the antagonist concentration and IC_{50} is the antagonist concentration producing half-maximal inhibition. IC_{50} values were corrected for agonist affinity according to the equation $IC_{50} = IC_{50}(obs)/1 + ([agonist]/EC_{50})$ as described (Durand et al., 1992). Data were analyzed by analysis of variance followed by the Scheffe F test or Fisher probable least-squares difference (PLSD) test post hoc (Statview, Abacus Concepts, Berkeley, CA, USA). NMDA, homoquinolinate, D-AP5 and D-CPPene dose-response curves at NR1/NR2A and NR1/NR2C heteromers have been previously published (Buller et al., 1994) and are included in the present report for comparison.

3. Results

Fig. 2 shows dose-response curves for agonist activation of recombinant heteromeric NMDA receptors expressed in *Xenopus* oocytes. Relative to the other heteromers, the NR1a/NR2D receptor showed a higher affinity for NMDA, with EC_{50} values of 35.9 ± 3.3 μM, 20.7 ± 2.7 μM, 22.0 ± 2.4 μM and 9.0 ± 0.3 μM for NR1a/NR2A, NR1a/NR2B, NR1a/NR2C and NR1a/NR2D, respectively ($P < 0.05$, ANOVA with Scheffe F test post hoc NR1a/NR2D against NR1a/NR2A and NR1a/NR2C; $P < 0.05$, ANOVA with Fisher PLSD post hoc, NR1a/NR2D against NR1a/NR2B).

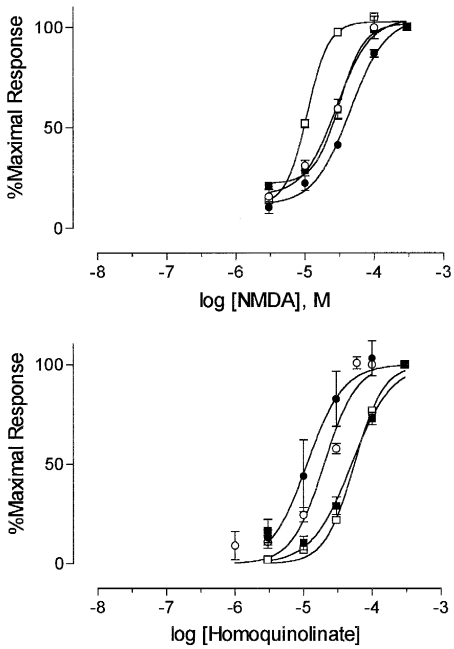

Fig. 2. Agonist activation of NR1a/NR2 receptor complexes expressed in *Xenopus* oocytes. Dose-response curves for activation of NR1a/NR2A (●), NR1a/NR2B (○), NR1a/NR2C (■) and NR1a/NR2D (□) heteromeric NMDA receptors expressed in oocytes by NMDA (top panel) or homoquinolinate (bottom panel) were performed in the presence of 10 μM glycine and were fit according to the equation $I = I_{max}/[1 + (EC_{50}/A)^n]$ as described in Section 2. Each point represents the mean ± S.E.M. of 3–6 oocytes taken from a single frog. Dose-response curves for NR1a/NR2A and NR1a/NR2C are taken from Buller et al. (1994) and are presented here for purposes of comparison.

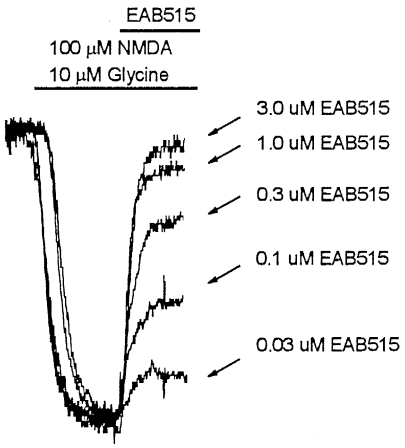

Fig. 3. Inhibition of NR1a/NR2A NMDA receptors by EAB515. Shown are normalized current traces taken sequentially from a single oocyte microinjected with RNA encoding the NR1a and NR2A subunits. Currents in the absence of antagonist were 40–50 nA.

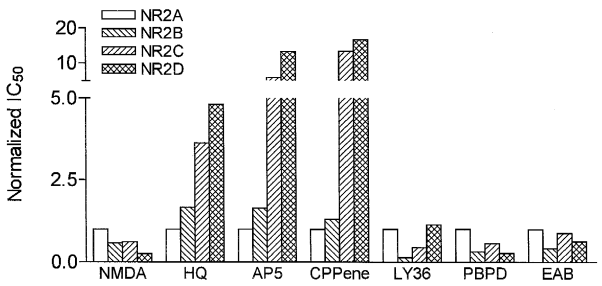

Fig. 4. Comparison of normalized agonist and antagonist affinities for heteromeric NMDA receptors expressed in *Xenopus* oocytes. EC_{50} and corrected IC_{50} values were normalized by dividing by the corresponding EC_{50} (agonists) or IC_{50} (antagonists) values at the NR1a/NR2A heteromer. Values less than 1.0 indicate a higher affinity relative to the NR1a/NR2A heteromer while values greater than 1 indicate a lower affinity relative to the NR1a/NR2A heteromer.

In contrast to NMDA (Fig. 2), glutamate (Buller et al., 1995; Kutsuwada et al., 1992) and glycine (Ikeda et al., 1992), the agonist homoquinolinate (Stone, 1984) displayed a significantly lower affinity for the NR1a/NR2D heteromer relative to the other heteromers (Fig. 2). The EC_{50} values for homoquinolinate activation of NMDA receptors were 15.5 ± 5.1 μM, 25.8 ± 3.3 μM, 56.4 ± 5.4 μM and 74.6 ± 3.6 μM for NR1a/NR2A, NR1a/NR2B, NR1a/NR2C and NR1a/NR2D, respectively ($P < 0.05$, ANOVA with Scheffe F test post hoc NR1a/NR2D against NR1a/NR2A and NR1a/NR2B; $P < 0.05$, ANOVA with Fisher PLSD post hoc, NR1a/NR2D against NR1a/NR2C).

Dose-response curves for antagonist inhibition of heteromeric NMDA receptors expressed in oocytes were generated. Fig. 3 shows representative current traces from an individual oocyte expressing NR1a/NR2A receptors in the absence and presence of the antagonist EAB515 (Müller et al., 1992). Antagonist IC_{50} values were derived from inhibition curves and corrected for agonist affinity (Durand et al., 1992). The corrected IC_{50} values are shown in Table 1. Relative to either NR2A- or NR2B-containing heteromers, the NR1a/NR2D receptor displayed a lower affinity for the antagonists D-AP5 and D-CPPene (Table 1). In addition, the NR1a/NR2D receptor also showed a significantly lower affinity for D-AP5 than the NR1a/NR2C receptor. In comparison to the NR1a/NR2B and NR1a/NR2C heteromers, but not to the NR1/NR2A heteromer, the NR1a/NR2D receptor had a significantly lower affinity for the antagonist LY233536 (Ornstein et al., 1991). In contrast to the generally lower affinities of the NR1/NR2D receptor for D-AP5, D-CPPene and LY233536, the structurally related antagonists (see Fig. 1), 4-(*p*-phenyl)benzoyl piperazine dicarboxylic acid (PBPD; Jane, Pook and Watkins, personal communication) and EAB515 (Müller et al., 1992) displayed affinities at NR1/NR2D receptors similar to, or even higher than, that observed at either NR1/NR2A or NR1/NR2C receptors (Table 1). In addition, both compounds also showed a relatively higher affinity for NR1a/NR2B receptors suggesting that these compounds preferentially interact with NR2B- and NR2D-containing receptors while the antagonists D-AP5 and D-CPPene have a higher affinity for NR2A- and NR2B-containing receptors (see Table 1).

Table 1
Agonist and antagonist EC_{50} and corrected IC_{50} values for recombinant NMDA receptors expressed in *Xenopus* oocytes

	NR1/NR2A	NR1/NR2B	NR1/NR2C	NR1/NR2D
D-AP5	0.28 ± 0.02	0.46 ± 0.14	1.64 ± 0.14	3.71 ± 0.67 [a,b,c]
D-CPPene	0.11 ± 0.03	0.14 ± 0.04	1.46 ± 0.08	1.84 ± 0.74 [A,B]
LY233536	1.75 ± 0.29	0.25 ± 0.02 [a]	0.79 ± 0.07 [A]	2.01 ± 0.06 [b,c]
PBPD	15.79 ± 0.43	5.01 ± 0.25 [a,c]	8.98 ± 0.18 [a]	4.29 ± 0.11 [a,c]
EAB515	0.04 ± 0.002	0.017 ± 0.002	0.035 ± 0.006	0.025 ± 0.007

Xenopus oocytes were microinjected with RNA coding for the NR1 subunit and either the NR2A, NR2B, NR2C or NR2D subunit. Agonist dose-response curves were determined in the presence of 10 μM glycine and antagonist dose-response curves were generated in the presence of 100 μM NMDA and 10 μM glycine as described in Section 2. IC_{50} values were corrected for agonist affinity according to the equation $IC_{50} = IC_{50}(obs)/1 + ([agonist]/EC_{50})$. EC_{50} values for NMDA, homoquinolinate, D-AP5 and D-CPPene at the NR1/NR2A and NR1/NR2C receptors have been previously presented (Buller et al., 1994) and are included in this table for comparison. The mean EC_{50} or corrected IC_{50} (μM) ± S.E.M. for individual dose-response curves taken from 4–6 separate oocytes are shown. Statistically significant comparisons ($P < 0.05$), performed by analysis of variance followed by Scheffe F-test post hoc (lower case) or by Fisher PLSD post hoc (upper case), are indicated as: [a] compared to NR1/NR2A; [b] compared to NR1/NR2B; [c] compared to NR1/NR2C.

Fig. 4 shows a comparison of normalized agonist and antagonist affinities for heteromeric NMDA receptors expressed in oocytes. The EC_{50} or apparent affinity was normalized by dividing by the corresponding EC_{50} (agonists) or apparent affinity (antagonists) at the NR1a/NR2A heteromer. Values less than 1.0 indicate a higher affinity relative to the NR1a/NR2A heteromer while values greater than 1 indicate a lower affinity relative to the NR1a/NR2A heteromer. As shown in Fig. 4, relative to the other heteromers, the NR1a/NR2D receptor showed the highest relative affinity for NMDA and the lowest relative affinity for homoquinolinate. Relative to the other heteromers, the NR1a/NR2D receptor displayed a lower relative affinity for the antagonists D-AP5 and D-CPPene and a relatively higher affinity for the antagonists PBPD and EAB515.

To determine whether the NR2D subunit could assemble into a complex containing two distinct NR2 subunits in combination with an NR1a subunit, NR2D subunits were co-expressed with NR1a and NR2B in *Xenopus* oocytes. The NR2B subunit was chosen because it shows overlapping developmental and anatomical patterns of expression with the NR2D subunit (Watanabe et al., 1992; Buller et al., 1994; Monyer et al., 1994). We examined whether the NR1a/NR2B/NR2D heteromer displayed unique pharmacological properties that might contribute to the native pharmacology of the medial thalamic receptors.

With the exception of homoquinolinate, heterotrimeric NMDA receptors generally showed an intermediate affinity for agonists (Table 2, Fig. 5). Thus, the EC_{50} for glycine and NMDA activation of recombinant NR1a/NR2B/NR2D receptors generally fell between that observed for either NR1a/NR2B or NR1a/NR2D receptors. Surprisingly, the NR1a/NR2B/NR2D heteromer displayed a significantly lower affinity for homoquinolinate than either NR1a/NR2 heteromer (Table 2, Fig. 5). The Hill coefficients for the NR1a/NR2B/NR2D heteromer did not significantly differ from that observed for the NR1a/NR2 heteromers (Table 2), indicating that a single receptor population, presumably containing three distinct species of subunits, was formed.

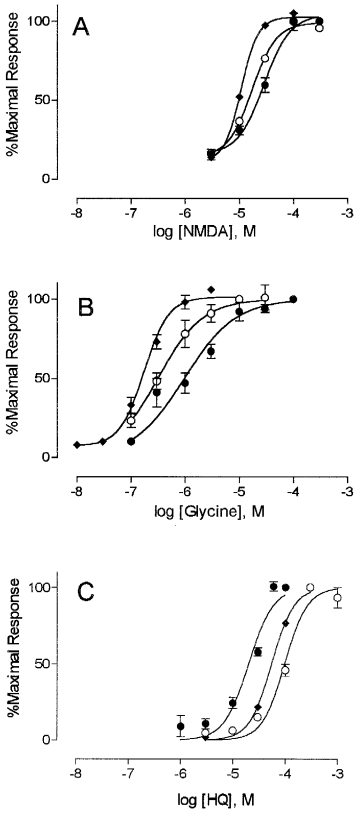

Fig. 5. Comparison of agonist pharmacology at NR1a/NR2B/NR2D receptor complexes expressed in *Xenopus* oocytes. Dose-response curves for NMDA (A), glycine (B) and homoquinolinate (C) activation of heterodimeric NR1a/NR2B (●), NR1a/NR2D (♦) receptors and NR1a/NR2B/NR2D (○) heterotrimeric receptors expressed in *Xenopus* oocytes. Dose-response curves were performed in the presence of 10 μM glycine and were fit according to the equation $I = I_{max}/[1+(EC_{50}/A)^n]$ as described in Section 2. Each point represents the mean ± S.E.M. of 3–6 oocytes taken from a single frog.

Table 2
EC_{50} and corrected IC_{50} values and Hill slopes for NR1/NR2B, NR1/NR2D and NR1/NR2B/NR2D NMDA receptors expressed in *Xenopus* oocytes

	NR1/NR2B		NR1/NR2D		NR1/NR2B/NR2D	
	EC_{50}	Hill slope	EC_{50}	Hill slope	EC_{50}	Hill slope
Agonists						
NMDA	20.7 ± 2.5	0.99 ± 0.19	9.0 ± 0.3 [b]	1.67 ± 0.08 [b]	14.0 ± 0.7 [b]	1.10 ± 0.70
Glycine	1.21 ± 0.25	0.92 ± 0.07	0.16 ± 0.02 [b]	1.25 ± 0.25	0.4 ± 0.1 [b]	1.07 ± 0.05
HQ	25.8 ± 3.3	1.06 ± 0.09	90.0 ± 15.5 [B]	1.25 ± 0.01	141 ± 14.4 [b,d]	1.10 ± 0.12
Antagonists						
D-AP5	0.46 ± 0.14	−0.73 ± 0.09	3.71 ± 0.67 [b]	−1.46 ± 0.25 [b]	3.35 ± 1.36 [b]	−0.69 ± 0.11 [d]
D-CPPene	0.14 ± 0.04	−1.01 ± 0.25	1.84 ± 0.74	−1.06 ± 0.26	0.06 ± 0.01 [D]	−0.78 ± 0.04
LY233536	0.26 ± 0.02	−1.24 ± 0.16	2.01 ± 0.06 [b]	−1.36 ± 0.13	0.59 ± 0.05 [D]	−1.39 ± 0.10

Xenopus oocytes were microinjected with RNAs coding for the NR1 subunit and either the NR2B, NR2D or both NR2B and NR2D subunits. Agonist dose-response curves were determined in the presence of 10 μM glycine (for the NMDA and homoquinolinate (HQ) curves) or 100 μM NMDA (for the glycine curve). Antagonist inhibition curves were determined in the presence of 100 μM NMDA and 10 μM glycine. Agonist and antagonist dose-response curves were fit as described in Section 2. Shown are the mean EC_{50} or IC_{50} ± S.E.M. for individual dose-response curves taken from 4–6 separate oocytes. Statistically significant comparisons ($P < 0.05$), performed by analysis of variance followed by Scheffe *F*-test post hoc (lower case) or by Fisher PLSD post hoc (upper case), are indicated as: [b] compared to NR1/NR2B; [d] compared to NR1/NR2D.

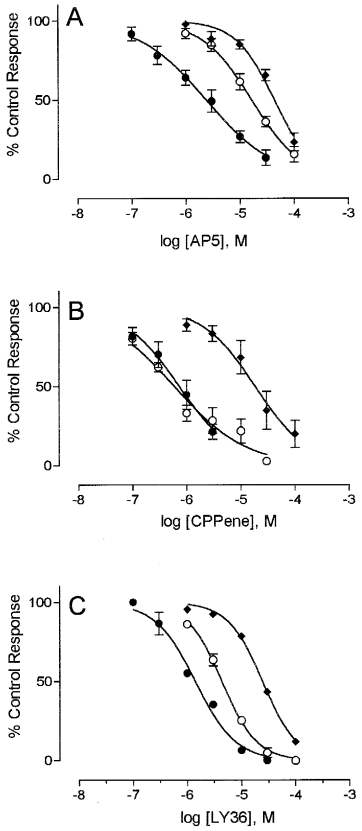

Fig. 6. Comparison of antagonist inhibition of NR1a/NR2B, NR1a/NR2D and NR1a/NR2B/NR2D receptor complexes expressed in *Xenopus* oocytes. Inhibition curves for D-AP5 (A), D-CPPene (B), and LY233536 (C) inhibition of currents elicited by 100 μM NMDA in the presence of 10 μM glycine in oocytes expressing NR1a/NR2B (●), NR1a/NR2D (◆), and NR1a/NR2B/NR2D (○) heterotrimeric receptors. Inhibition curves were fit according to the equation $I = I_{max}/[1 + (EC_{50}/A)^n]$ as described in Section 2. Each point represents the mean ± S.E.M. of 3–6 oocytes taken from a single frog.

Similar to that observed for agonists, heterotrimeric NMDA receptors expressed in oocytes generally showed antagonist affinities intermediate to those observed for either heterodimeric receptor (Fig. 6). Interestingly, the affinity of the NR1a/NR2B/NR2D receptor for D-CPPene was lower than that observed for either NR1a/NR2B or NR1a/NR2D, although this did not reach statistical significance. The affinity of the trimer for LY233536 was increased relative to the NR1a/NR2D heteromer and was also higher than that observed for NR1a/NR2C receptors (Table 1), paralleling observations in native cerebellum versus midbrain (see Section 4).

4. Discussion

In this report, we find that the NR2D subunit confers pharmacological properties to the NR1/NR2 heteromer, that are distinct from the other NR1/NR2 heteromeric receptors. This pharmacological profile is characterized by a significantly lower affinity for homoquinolinate and D-AP5. In contrast, the antagonist PBPD had a higher affinity at NR1a/NR2D receptors than at either NR1a/NR2A or NR1a/NR2D receptors. As shown in Fig. 4, while the pharmacological profile of the NR1/NR2D receptor was unique, it was most similar to that of NR2C-containing receptors. This finding is consistent with the higher degree of homology observed between NR2D and NR2C subunits than between NR2D and either NR2A or NR2B subunits (Ishii et al., 1993; Monyer et al., 1994).

The NR2 subunit contributes to the pharmacological heterogeneity of native NMDA receptors (Laurie and Seeburg, 1994b; Buller et al., 1994). The co-localization of at least three distinct NMDA receptor radioligand binding sites (antagonist preferring, agonist preferring and cerebellar) with NR2A, NR2B (and NR1 subunits lacking insert 1) and NR2C subunits, coupled with the observations that the pharmacology of distinct NR1a/NR2 heteromers can reproduce native pharmacology, provides strong support for the hypothesis that discrete populations of native NMDA receptors have differing subunit compositions. The distribution of the NR2D subunit is developmentally regulated, with expression in adults limited to several midbrain thalamic nuclei, the medial geniculate, the nucleus of the diagonal band, the interpeduncular nucleus and the periaqueductal gray (Buller et al., 1994; Monyer et al., 1994; Watanabe et al., 1992). It is intriguing that in autoradiographic studies, these same nuclei show a unique pharmacological profile, characterized by a relatively low affinity for most antagonists except LY233536, PBPD and EAB515 (Andaloro et al., 1996; Buller et al., 1994; Beaton et al., 1992; Monaghan and Beaton, 1992). These data strongly suggest that the NR2D subunit contributes to the observed pharmacology of the medial thalamic NMDA receptors.

Several similarities between the pharmacology of native medial thalamic NMDA receptors and NR1a/NR2D heteromers expressed in *Xenopus* oocytes were observed in the present report. Both the NR1a/NR2C and NR1a/NR2D receptors have a low affinity for homoquinolinate, an agonist which binds weakly to both cerebellar and medial thalamic NMDA receptors (Buller et al., 1994; Monaghan and Beaton, 1992). Unlike that observed for NMDA (Fig. 2), glycine (Buller et al., 1995) or glutamate (Ikeda et al., 1992), homoquinolinate readily discriminates between NR2C- or NR2D-containing heteromers and NR2A- or NR2B-containing heteromers. However, in contrast to native brain where homoquinolinate preferentially binds to medial thalamic receptors relative to cerebellar receptors (Monaghan and Beaton, 1992), homoquinolinate does not show a higher affinity for NR1a/NR2D heteromers relative to NR1a/NR2C heteromers.

The antagonists D-AP5 and D-CPPene selectively inhibit both NR1a/NR2A and NR1a/NR2B heteromers (relative to NR1a/NR2C and NR1a/NR2D heteromers), a pattern similar to that seen for homoquinolinate (see Fig. 4). This

is due to the significantly lower affinity of D-AP5 and D-CPPene for NR1a/NR2C and NR1a/NR2D heteromers. In contrast, PBPD and EAB515 had either a similar or higher affinity for NR1a/NR2D receptors relative to NR1a/NR2A or NR1a/NR2B receptors. This antagonist profile is consistent with observations regarding native NMDA receptors in the medial thalamus; D-AP5 and D-CPPene have lower affinities at medial thalamic NMDA receptors, than at forebrain NMDA receptors, whereas PBPD and EAB515 each inhibit medial thalamic and other forebrain NMDA receptors with similar affinities (Andaloro and Monaghan, unpublished observations). However, while D-CPPene and D-AP5 show significantly higher affinities for medial thalamic NMDA receptors compared to cerebellar NMDA receptors, in the present report, NR1a/NR2C receptors showed a higher affinity for these compounds relative to NR1a/NR2D heteromers.

Taken together with previous pharmacological studies of native and recombinant NMDA receptors, the present data demonstrate that those compounds that discriminate between NR2D-containing heteromers and NR2A- or NR2B-containing heteromers also discriminate between medial thalamic (predominantly NR2D-containing) and forebrain (predominantly NR2A- and NR2B-containing) NMDA receptors. However, certain compounds which are selective for medial thalamic receptors relative to cerebellar NMDA receptors (e.g., homoquinolinate, D-CPPene), do not show a similar selectivity for NR1a/NR2D receptors relative to NR1a/NR2C receptors. Thus, while the NR2D subunit most likely contributes to the unique pharmacology of medial thalamic NMDA receptors, the relative relationship between NR2C- and NR2D-containing receptors and cerebellar and medial thalamic NMDA receptors requires further investigation. It remains to be determined whether additional subunits (NR1 or NR2) modify the pharmacology of native NMDA receptor in the medial thalamus and cerebellum.

Co-expression of NR1a, NR2B and NR2D subunits in *Xenopus* oocytes resulted in the formation of heteromeric complexes with distinct pharmacological properties, including a unique low affinity for homoquinolinate, high affinity for D-CPPene and intermediate affinities for the other compounds examined (see Table 2). Previous studies have demonstrated that functional heteromers can be formed from the coexpression of NR1a, NR2A and NR2C subunits (Chazot et al., 1994; Wafford et al., 1993). In agreement with these results, the present data suggest that two different NR2 subunits (in this case, NR2B and NR2D) may exist in the same complex. Furthermore, the combination of two NR2 subunits, in the presence of NR1, results in the formation of an oligomeric complex with unique pharmacology. However, although the NR1a/NR2B/NR2D heteromer shows a novel pharmacology, it cannot account for the observed differences between recombinant (NR2C versus NR2D) and native (cerebellar versus medial thalamic) receptors.

While considerable evidence suggests that the NR1 subunit, minimally, contains binding sites for glutamate and glycine, it is clear that NR2 influences the pharmacology of the NR1/NR2 heteromer (Laurie and Seeburg, 1994b; Buller et al., 1994). Whether the NR2 subunit contains a binding site for glutamate in addition to that present on the NR1 subunit or whether it influences the formation of a binding site on the NR1 subunit is not known. However, it is becoming apparent that the pharmacological diversity observed in native brain is dependent upon the specific subunit composition of the native receptor.

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