0022-3565/96/2782-0808$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 278, No. 2

Printed in U.S.A.
JPET 278:808–816, 1996

Cloning and Functional Characterization of Human Heteromeric *N*-Methyl-D-Aspartate Receptors

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Accepted for publication April 25, 1996

ABSTRACT

Human cDNAs encoding *N*-methyl-D-aspartate receptor type (NMDAR)1A, NMDAR2A and NMDAR2B subunits were cloned and receptors encoded by these cDNAs were functionally expressed by injection of the respective mRNAs in *Xenopus* oocytes. The pharmacological properties of recombinant human *N*-methyl-D-aspartate (NMDA) receptors were characterized by profiling two agonists and four antagonists at both the NMDA and glycine sites in voltage-clamped oocytes. NMDA, glycine and D-serine were significantly more potent at human NMDAR (hNMDAR)1A/2B receptors than at hNMDAR1A/2A, whereas there was no detectable subtype-dependent difference in the potency of glutamate. Of the NMDA-site antagonists tested, CGP 43487 and 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonate exhibited 5.8- and 3.9-fold greater potency, respectively, at hNMDAR1A/2A receptors than at hNMDAR1A/2B. Of the four glycine-site competitive antagonists tested, L-689,560 displayed 5-fold greater potency at hNMDAR1A/2A, whereas 5,7-dichlorokynurenic acid, HA-966 and CGP 58411 did not discriminate between hNMDAR1A/2A and hNMDAR1A/2B. Receptors resulting from injection of hNMDAR1A, hNMDAR2A and hNMDAR2B transcripts in a 1:1:1 ratio were indistinguishable from hNMDAR1A/2B receptors in terms of their sensitivity to NMDA, glycine, D-serine, CGS 19755 and CGP 40116. Ifenprodil was approximately 350-fold more potent at hNMDAR1A/2B than at hNMDAR1A/2A receptors. Ifenprodil sensitivities of receptors formed in oocytes injected with a constant amount of hNMDAR1A mRNA but varying ratios of hNMDAR2A or hNMDAR2B mRNAs were compared. The receptors expressed at a 10:1 ratio of 2A:2B transcripts displayed an ifenprodil sensitivity that would be predicted for a population in which 51% was represented by hNMDAR$(1A)_2(2A)_3$ complexes. Our results underscore the need for subtype-selective compounds acting at novel sites to sufficiently probe the pharmacological differences between NMDA receptor subtypes formed by different subunit combinations.

Molecular cloning and subsequent localization studies have revealed a diversity of NMDA receptor subunits with distinct distribution patterns in the CNS. Subunits of NMDA receptors from rat (Moriyoshi *et al.*, 1991; Sugihara *et al.*, 1992; Nakanishi *et al.*, 1992; Monyer *et al.*, 1992; Ishii *et al.*, 1993) and mouse (Meguro *et al.*, 1992; Kutsuwada *et al.*, 1992) have been cloned and functionally expressed in *Xenopus* oocytes and mammalian cells. These studies have shown that, although oocytes injected with transcripts encoding NMDAR1 express functional receptors, the amplitudes of agonist-induced inward currents are dramatically increased when transcripts encoding NMDAR2A, -2B, -2C or -2D are coinjected with NMDAR1. The putative heteromeric recombinant channels display both the biophysical (Burnashev *et al.*, 1992; Stern *et al.*, 1992, 1994) and pharmacological (Kutsuwada *et al.*, 1992; Ishii *et al.*, 1993) properties observed with native receptors.

In situ hybridization studies in rat brain have revealed that NMDAR1 transcripts are expressed nearly uniformly throughout the CNS (Buller *et al.*, 1994), a finding consistent with the observation that mice carrying a null-mutant NMDAR1 allele did not express functional NMDA receptors (Forrest *et al.*, 1994). In contrast, the expression of the NMDAR2 transcripts is both developmentally and regionally regulated. Buller *et al.* (1994) used a combination of binding and *in situ* hybridization techniques to map the anatomical distribution of NMDAR1, -2A, -2B, -2C and -2D receptors in the adult rat CNS. That study showed that the distribution patterns of NMDAR2A and -2B mRNAs correlate well with

Received for publication September 27, 1995.

ABBREVIATIONS: CGP 40116, (2*R*)-(*E*)-2-amino-4-methyl-5-phosphono-3-pentenoic acid; CGP 43487, (2*R*)-(*E*)-2-amino-4-methyl-5-phosphono-3-pentenoic acid ethyl ester; CGP 58411, 7-chloro-4-hydroxy-3-phenyl-1*H*-quinolin-2-one; CGS 19755, *cis*-4-phosphonomethylpiperidine-2-carboxylic acid; CNS, central nervous system; CPP, 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonate; DCKA, 5,7-dichlorokynurenic acid; EC_{80}, concentration of agonist that produces 80% of the maximal response; GluR, glutamate receptor type; HA-966, (*RS*)-3-amino-1-hydroxypyrrolid-2-one; hNMDAR, human *N*-methyl-d-aspartate receptor type; L-689,560, (±)-4-*trans*-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline; NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptor type.

the localization of [^{3}H]CPP or L-[^{3}H]glutamate binding sites, which in turn correspond to the antagonist- or agonist-preferring NMDA receptors, respectively. Immunoprecipitation studies have demonstrated the existence of a population of NMDA receptors in the rat cortex consisting of NMDAR1A, NMDAR2A and NMDAR2B subunits (Sheng *et al.*, 1994). Together, these data show that the NMDAR2 subunits contribute to diversity in both the anatomical distribution and pharmacological properties of NMDA receptors.

NMDA receptors have been the focus of extensive drug discovery efforts. Consequently, a number of competitive antagonists are currently available for both the NMDA and glycine sites (reviews by Bigge *et al.*, 1993; Leeson, 1993, respectively), in addition to several noncompetitive antagonists and channel blockers. At present, the noncompetitive antagonist ifenprodil (Williams *et al.*, 1993; Williams, 1993) is the most subtype-selective compound active at NMDA receptors. The development of pharmacological tools that can distinguish NMDA receptor subtypes should aid in defining the possible differential roles of these receptors in normal and pathophysiological processes. The discovery of subtype-specific NMDA antagonists may lead to the development of therapeutic agents that could potentially maximize neuroprotection after ischemic and hypoxic events, with minimal side effects.

Recently, several groups have cloned and expressed human homologs of the NMDAR1 and NMDAR2A subunits (Karp *et al.*, 1993; Le Bourdellès *et al.*, 1994). In this work, we describe the cloning of cDNAs encoding hNMDAR1A, hNMDAR2A and hNMDAR2B receptor subunits and the heteromeric expression and pharmacological characterization of recombinant human NMDA receptors. Two agonists and four competitive antagonists for each of the glutamate and glycine sites were profiled to determine whether these compounds might be useful for discriminating between hNMDAR1A/2A, hNMDAR1A/2B and hNMDAR1A/2A/2B receptors. In addition, based on its significant subtype-selective profile, we used ifenprodil to probe the relative sensitivity of hNMDAR1A/2A/2B complexes formed in oocytes by injection of a constant amount of hNMDAR1A mRNA and varying amounts of hNMDAR2A or hNMDAR2B mRNAs. Some of the results have been previously reported in abstract form (Hess *et al.*, 1994).

Materials and Methods

cDNA libraries and construction of full-length NMDA receptor cDNAs. To isolate hNMDAR1A, 10^6 recombinants from a λgt10 human hippocampal cDNA library prepared as described by Ellis *et al.* (1988) were probed with an oligonucleotide probe based on the rat NMDAR1A sequence (nucleotides 2585–2628, according to the numbering system of Moriyoshi *et al.*, 1991). Two cDNAs, NMDA10 and NMDA11, were isolated. Clone NMDA10 is a 4238-nucleotide cDNA encoding a sequence homologous to the carboxyl-terminus of rat NMDAR1A. Clone NMDA11 is a 3851-nucleotide cDNA containing the entire NMDAR1 coding sequence but a different carboxyl-terminal sequence, compared with that of NMDA10. A full-length hNMDAR1A construct was obtained by ligating the 5′-end of NMDA11 to the 3′-end of NMDA10 (by using a common *Hind*III site) into the mammalian expression vector pcDNA I (Invitrogen, San Diego, CA).

To isolate hNMDAR2A cDNAs, degenerate oligonucleotides encoding the putative first and fourth transmembrane regions of rat NMDAR2A, NMDAR2B and NMDAR2C (Monyer *et al.*, 1992) were used to prime human cerebellar cDNA and amplify a 797-nucleotide hNMDAR2A fragment. This fragment was then used to probe 10^7 recombinants of a randomly primed, λgt10, cerebellar cDNA library and 6×10^5 recombinants from a specifically primed, λgt10, cerebellar cDNA library. Four overlapping partial hNMDAR2A cDNA clones, *i.e.*, NMDA54, NMDA47, NMDA50 and NMDA58, were isolated and ligated to construct the full-length hNMDAR2A cDNA. A cytomegalovirus promoter expression plasmid, pCMVβ (Clontech, Palo Alto, CA), was modified as described by Daggett *et al.* (1995). The mammalian expression plasmid pCMV-hNMDAR2A was constructed by ligating the following fragments: NMDA54 (−87-544) to NMDA47 (545-2105) to NMDA50 (2106–2902) to NMDA58 (2903–4440), with nucleotide numbers referring to the hNMDAR2A cDNA sequences and negative numbers representing 5′-untranslated sequence.

A hNMDAR2B cDNA was isolated by screening 10^6 recombinants from a human fetal brain λZAPII cDNA library (Stratagene, La Jolla, CA) with a cDNA encoding rat NMDAR2B (Sullivan *et al.*, 1994) as the probe. One 5467-nucleotide cDNA, NMDA81, was rescued by the *in vivo* excision procedure, and the resulting 8.5-kilobase plasmid, pBS-hNMDAR2B, contained the complete coding sequence of hNMDAR2B cDNA. The nucleotide sequences reported herein for hNMDAR2A and hNMDAR2B were determined by sequencing both strands of cDNA.

Recombinant hNMDAR1A, hNMDAR2A and hNMDAR2B *in vitro* transcripts were synthesized from linearized plasmids by using the mMessage mMachine RNA capping kit (Ambion Inc., Austin, TX).

Electrophysiology. Stage V oocytes were isolated from *Xenopus laevis* by using standard techniques (Goldin, 1992). Oocytes were typically injected with 50 nl containing 10 to 50 ng of one or more *in vitro* transcripts. Two to 6 days later, the cells were voltage-clamped (OC-725B Oocyte Clamp; Warner Instrument Corp., Hamden, CT) with two microelectrodes and held at −80 mV in a bath containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$ and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.3. Drugs were applied by using gravity feed and reached the recording chamber in approximately 600 msec, determined as the time necessary for application of 1 M KCl to elicit an inward current in oocytes held at −80 mV. Unless otherwise mentioned, glutamate or NMDA was coapplied with 30 μM glycine for the hNMDAR1A/2A combination and with 10 μM glycine for the hNMDAR1A/2B combination. For NMDA- and glycine-site antagonist studies, we used NMDA or glycine at a concentration corresponding to the EC_{80} for the receptor subtype. For NMDA-site antagonists, subsaturating concentrations of NMDA (100 μM for the hNMDAR1A/2A combination and 30 μM for the hNMDAR1A/2B and hNMDAR1A/2A/2B combinations) were used. The glycine-site antagonists, with the exception of DCKA, were tested in the presence of 300 μM NMDA and 10 μM D-serine for hNMDAR1A/2A or 100 μM NMDA and 3 μM D-serine for hNMDAR1A/2B. DCKA was tested with 100 μM NMDA and 10 μM glycine for the hNMDAR1A/2A receptor and 30 μM NMDA with 1.0 μM glycine for the hNMDAR1A/2B receptor. Repeated applications of agonists elicited reproducible responses, and recovery from antagonists was determined at the end of each experiment for each cell by measuring the response to application of the control dose of agonists. These recovery values were typically 70 to 95% for all compounds tested. Data were filtered at 100 Hz and digitized at 200 Hz. Axotape and Clampfit software (Axon Instruments, Foster City, CA) were used for data collection and analysis.

The NMDA-induced inward currents also included the endogenous Ca^{++}-activated Cl^- currents (Barish, 1983), because 1.8 mM Ca^{++} was present in the bath solution used in all recordings. To minimize the contribution of the Ca^{++}-activated Cl^- currents to NMDA-induced currents, we measured the steady-state currents at a minimum of 15 sec after the peak of the inward current, when the early transient inward Ca^{++}-activated Cl^- current had substantially decayed (Leonard and Kelso, 1990). The IC_{50} values estimated for

CGS 19755 from curves obtained by measuring the peak and steady-state portion of the current were not significantly different ($P > .05$).

Estimates of EC_{50} and IC_{50} values were obtained from curves fit to the equation for a single-site sigmoidal dose-response curve with a variable slope, $Y = Y_{max}/[1 + (EC_{50}/X)^n]$ or $Y = Y_{max} - Y_{max}/[1 + (IC_{50}/X)^n]$, where n is the Hill coefficient. For a two-equivalent site model, data were fit using $Y = \text{fraction} \cdot [Y_{max} - Y_{max}/(1 + (IC_{50}/X)^n)] + (1 - \text{fraction}) \cdot [Y_{max} - Y_{max}/(1 + (IC_{50}/X)^n)]$. The concentration data are presented as the antilogarithms of the geometric mean and the geometric mean ± S.D. (or the arithmetic mean for Hill coefficients) for 3 to 11 oocytes. For the antagonists, the IC_{50} values were converted to K_b values with the Leff-Dougall (Leff and Dougall, 1993) variant of the Cheng-Prusoff equation, $K_b = IC_{50}/[2 + ([A]/A_{50})^n]^{1/n} - 1$, where [A] is the agonist concentration used and A_{50} is the EC_{50} value for the agonist. The geometric means for the concentration or K_b data, or arithmetic means for Hill coefficients, were tested for significant differences between receptor subtypes by using either a two-tailed t test or analysis of variance with *post hoc* Student-Newman-Keuls or Mann-Whitney rank sum tests. The *post hoc* tests (SigmaStat, version 1.01; Jandel Corp., San Rafael, CA) examined whether the means were different at $P < .05$ but did not calculate an absolute P value; therefore, the differences may be larger than stated in the text and tables.

Reagents. Glutamate, NMDA, glycine and D-serine were obtained from Sigma Chemical Co. (St. Louis, MO). Racemic (±)-HA-966, DCKA, ifenprodil and CPP were from Research Biochemicals (Natick, MA). CGP 40116, CGS 19755, CGP 43487, L-689,560 (CGP 55391) (Leeson *et al.*, 1992) and CGP 58411 (Kulagowski *et al.*, 1994) were obtained from the Pharma Division of Ciba (Basel, Switzerland).

Results

Isolation and characterization of cDNAs encoding hNMDAR2A and hNMDAR2B. Full-length cDNAs encoding hNMDAR1A and hNMDAR2A were constructed from overlapping partial cDNAs isolated from either human adult hippocampal or cerebellar cDNA libraries, respectively. A single cDNA encoding the entire hNMDAR2B sequence was isolated from a human fetal brain cDNA library. Adopting the membrane topology proposed for rat GluR3 (Bennett and Dingledine, 1995) or for goldfish kainate receptors by Wo and Oswald (1994), all putative phosphorylation sites appear to be conserved in the deduced amino acid sequences of human and rat NMDA receptor subunits.

The hNMDAR1A cDNA encodes 938 amino acids. The sequence reported here is identical to the sequences previously reported for hNMDAR1A by Karp *et al.* (1993) and Planells-Cases *et al.* (1993) but varies by two amino acids from that reported by Le Bourdellès *et al.* (1994). The hNMDAR2A cDNA encodes 1464 amino acids, identical to those of rat and mouse NMDAR2A cDNAs. The deduced amino acid sequence of the hNMDAR2A we isolated differs from that reported by Le Bourdellès *et al.* (1994) at 12 amino acid positions, as follows (Le Bourdellès *et al.* sequence→our sequence): Gly^{61}→Ala, Val^{108}→Ala, Val^{248}→Leu, Ala^{376}→Gly, Lys^{379}→Glu, Asn^{381}→His, Ser^{386}→Arg, Ser^{760}→Gly, Val^{800}→Cys, Asn^{810}→Ser, Glu^{1265}→Gln and Gly^{1378}→Val. The underlined residues are identical to those reported for the rat sequence (Monyer *et al.*, 1992; Ishii *et al.*, 1993). The hNMDAR2B cDNA encodes 1484 amino acids, *i.e.*, 2 amino acids longer than the rat sequence, and yields a calculated molecular mass of 166.4 kDa. The deduced amino acid sequence of hNMDAR2B has an overall identity of 98.4% with the reported rat sequences (Monyer *et al.*, 1992; Ishii *et al.*, 1993) and 95.5% with the mouse sequence reported by Kutsuwada *et al.* (1992). The deduced amino acid sequence for hNMDAR2B reported by Adams *et al.* (1995) is 99.8% identical to that obtained for our clone. The only differences occur at Arg^{920}→Ser, Pro^{921}→Ala and Val^{1167}→Ile.

Functional expression of human recombinant NMDA receptors in *Xenopus* oocytes. Coinjection of *in vitro* transcripts encoding hNMDAR1A and hNMDAR2A or hNMDAR1A and hNMDAR2B into *Xenopus* oocytes resulted in the expression of receptors activated by either NMDA or glutamate in the presence of glycine and blocked by 1.0 mM external Mg^{++}. The currents recorded from oocytes injected with either combination were substantially larger than those recorded from oocytes injected with only the hNMDAR1A transcript. In oocytes injected with only hNMDAR1A, the peak inward currents in response to 100 μM glutamate and 30 μM glycine application ranged from 3 to 10 nA, whereas those in oocytes injected with hNMDAR1A and hNMDAR2A transcripts were in the range of 40 to 3400 nA. Oocytes injected with transcripts encoding only hNMDAR2A or hNMDAR2B did not produce detectable currents, whereas injection of the same batch of oocytes with hNMDAR1A and hNMDAR2A or hNMDAR1A and hNMDAR2B did result in functional receptor expression ($n = 1$).

Agonist profiles of hNMDAR1A/2A and hNMDAR1A/2B receptors. We examined the potencies of two agonists and two coagonists in activating hNMDAR1A/2A and hNMDAR1A/2B receptors. The data are summarized in table 1. Glutamate concentration-response curves obtained in the presence of 30 μM glycine for the hNMDAR1A/2A and hNMDAR1A/2B heteromeric receptors are shown in figure 1A. The EC_{50} values for glutamate were not significantly different between hNMDAR1A/2A and hNMDAR1A/2B ($P > .05$, two-tailed unpaired t test), and the Hill coefficients were approximately 1.0 for both receptors. Wafford *et al.* (1995) reported an EC_{50} of 3.26 μM for gluta-

TABLE 1

Summary of EC_{50} values and Hill coefficients for NMDA, glutamate, glycine and D-serine with human NMDA receptors expressed in *Xenopus* oocytes

Values represent the mean (−S.D., +S.D.) obtained from four to seven oocytes. n_H is the Hill coefficient (mean ± S.D.).

	Glutamate	NMDA	Glycine	D-Serine
hNMDAR1A/2A EC_{50} (μM)	1.6 (0.6, 4.2)	23.9 (13.2, 43.1)	1.9 (1.7, 2.2)	2.2 (1.5, 3.2)
n_H	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	1.0 ± 0.1
hNMDAR1A/2B EC_{50} (μM)	1.4 (0.7, 2.7)	9.9 (7.8, 12.5)*	0.2 (0.1, 0.3)*	0.6 (0.2, 2.1)*
n_H	1.3 ± 0.4	1.1 ± 0.1	0.9 ± 0.3	0.8 ± 0.2
hNMDAR1A/2A/2B EC_{50} (μM)		13.5 (6.6, 27.2)	0.4 (0.2, 0.7)*	0.6 (0.5, 0.9)*
n_H		1.3 ± 0.7	0.8 ± 0.3	0.9 ± 0.2

* Mean of hNMDAR1A/2B or hNMDAR1A/2A/2B is significantly different from the mean of hNMDAR1A/2A ($P < .05$, analysis of variance with *post hoc* Student-Neuman-Keuls test).

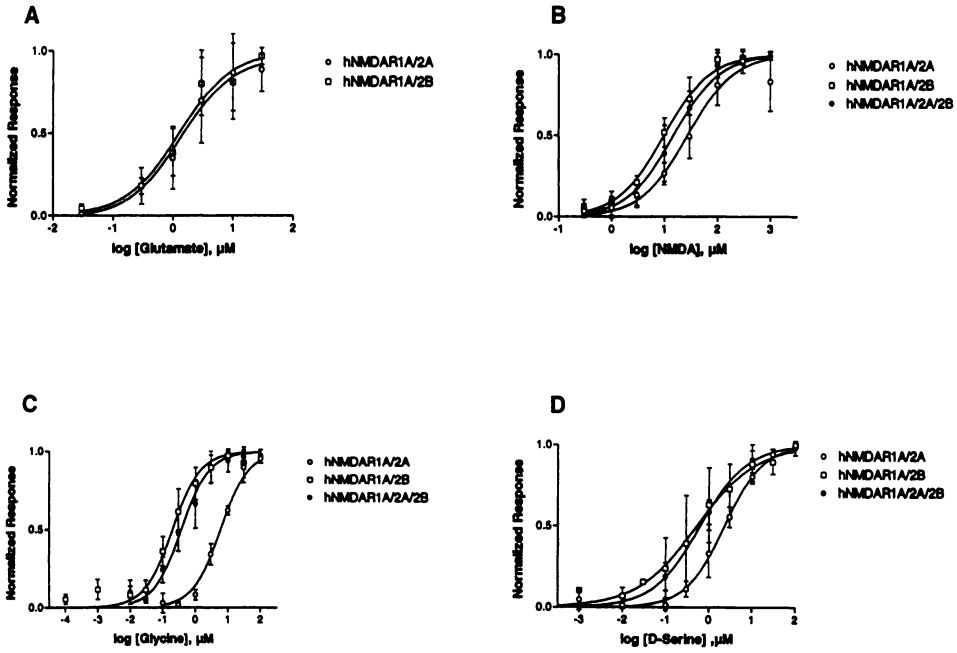

Fig. 1. Concentration-response curves for glutamate (A), NMDA (B), glycine (C) and serine (D) with human NMDA receptor combinations expressed in *Xenopus* oocytes. D-Serine sensitivity was determined in the presence of saturating levels of NMDA (300 μM for hNMDAR1A/2A and 100 μM for hNMDAR1A/2B and hNMDAR1A/2A/2B). The data points represent the mean ± S.D. for normalized responses from four to seven cells, and the solid lines are fits to the data points.

mate for hNMDAR1A/2A receptors expressed in oocytes, whereas Priestly *et al.* (1995) obtained values of 0.8 and 0.4 μM for hNMDAR1A/2A and hNMDAR1A/2B receptors expressed in L(tk$^-$) cells, respectively.

From concentration-response curves for NMDA (fig. 1B), we estimated EC_{50} values for hNMDAR1A/2A and hNMDAR1A/2B that were significantly different ($P < .05$), and the Hill coefficients were approximately 1.0 for both subunit combinations. For hNMDAR1A/2A and hNMDAR1A/2B receptors expressed in L(tk$^-$) cells, Priestly *et al.* (1995) obtained EC_{50} values of 8.9 and 6.8 μM, respectively.

The hNMDAR1A/2A and hNMDAR1A/2B combinations displayed markedly different sensitivities to glycine. Figure 1C shows glycine concentration-response curves obtained from five or six oocytes in the presence of an NMDA concentration corresponding approximately to its EC_{80}. The glycine EC_{50} values for the hNMDAR1A/2A and hNMDAR1A/2B receptors were significantly different from each other ($P < .05$). The Hill coefficients were approximately 1.0 for both subunit combinations. There was an approximately 10-fold difference in glycine sensitivity between the hNMDAR1A/2A and hNMDAR1A/2B receptors, markedly larger than the 2.4-fold and 1.1-fold differences obtained for NMDA and glutamate, respectively (table 1). The glycine EC_{50} value reported here for hNMDAR1A/2A receptors is in good agreement with an EC_{50} value of 2.3 μM for hNMDAR1A/2A receptors expressed in oocytes (Wafford *et al.*, 1995). Priestly *et al.* (1995) reported glycine EC_{50} values of 0.5 and 0.05 μM for hNMDAR1A/2A and hNMDAR1A/2B receptors, respectively, expressed in L(tk$^-$) cells. Kutsuwada *et al.* (1992) reported glycine EC_{50} values of 2.10 and 0.3 μM for mouse NMDAR1A/2A and NMDAR1A/2B receptors, respectively.

Next, we determined the potency of D-serine (Kleckner and Dingledine, 1988) on human NMDA receptors. D-Serine is a glycine-site agonist often used in brain-slice and whole-animal studies, where the concentration of glycine cannot be adequately controlled. From concentration-response curves for D-serine (fig. 1D), we determined that the EC_{50} value for hNMDAR1A/2A receptors was significantly different from that for hNMDAR1A/2B receptors ($P < .05$). The Hill coefficients were approximately 1.0 for both combinations. Thus, both glycine and D-serine were less potent at hNMDAR1A/2A than hNMDAR1A/2B receptors, by 9.5-fold and 3.7-fold, respectively.

Antagonist profiles of hNMDAR1A/2A and hNMDAR1A/2B receptors. Four NMDA-site competitive antagonists were tested for their ability to inhibit currents elicited by a concentration of NMDA corresponding to its EC_{80} for each receptor subtype, as determined in the studies shown in figure 1B. Table 2 summarizes the K_b values obtained with the Leff-Dougall variant of the Cheng-Prusoff equation (Leff and Dougall, 1993), which corrects for differences in agonist affinity. The K_b values estimated for CPP from the concentration-response curves shown in figure 2A

TABLE 2

Summary of Leff-Dougall K_b values and Hill coefficients for NMDA-site competitive antagonists

Values represent the mean (−S.D., +S.D.) obtained from three to five oocytes. n_H is the Hill coefficient (mean ± S.D.).

	CPP	CGP 43487	CGP 40116	CGS 19755
hNMDAR1A/2A K_b (μM)	0.15 (0.10, 0.24)**	0.28 (0.15, 0.49)**	0.04 (0.02, 0.08)	0.36 (0.30, 0.44)
n_H	1.1 ± 0.1***	1.4 ± 0.4	1.2 ± 0.5	1.1 ± 0.1
hNMDAR1A/2B K_b (μM)	0.59 (0.43, 0.81)	1.61 (0.98, 2.64)	0.03 (0.01, 0.09)	0.37 (0.26, 0.51)
n_H	1.6 ± 0.1	1.2 ± 0.3	0.9 ± 0.3	1.1 ± 0.2
hNMDAR1A/2A/2B K_b (μM)			0.12 (0.08, 0.17)	0.33 (0.25, 0.44)
n_H			1.1 ± 0.3	1.2 ± 0.2

** Significant differences between means of hNMDAR1A/2A and hNMDAR1A/2B, $P < .01$, two-tailed unpaired *t* test.
*** $P < .001$.

revealed that CPP is approximately 4-fold ($P < .01$) more potent at hNMDAR1A/2A than hNMDAR1A/2B. The Hill coefficient was also significantly larger for the hNMDAR1A/2B combination than for the hNMDAR1A/2A combination (table 2). For CGP 43487 (fig. 2B), the K_b values for hNMDAR1A/2A and hNMDAR1A/2B were also significantly different ($P < .01$); this compound was approximately 5.8-fold more potent at hNMDAR1A/2A, compared with hNMDAR1A/2B, and was the most subtype-selective NMDA-site antagonist examined. We observed no statistically significant differences in the sensitivity of hNMDAR1A/2A and hNMDAR1A/2B to CGP 40116 (Massieu *et al.*, 1993) or CGS 19755 (Hutchison *et al.*, 1989) (fig. 2, C and D).

We also examined the selectivity of several glycine-site competitive antagonists. The concentration-inhibition curves obtained in the presence of glycine added at its EC_{80} are shown in figure 3, and the K_b values are listed in table 3. The results indicated that DCKA could not detectably distinguish between hNMDAR1A/2A and hNMDAR1A/2B (fig. 3A). The K_b values were not significantly different, and the Hill coefficients were approximately 1.0 for both receptors (table 3). The potency of (±)-HA-966, L-689,560 and CGP 58411 were also determined in the presence of EC_{80} concentrations of glycine or D-serine. As determined from the curves shown in figure 3B, the K_b values for (±)-HA-966 for hNMDAR1A/2A and hNMDAR1A/2B were not significantly different. The Hill coefficients were 0.3 and 0.5, respectively (table 3), possibly reflecting multiple sites of action. The DCKA analog L-689,560 (fig. 3C) was 5-fold more potent at hNMDAR1A/2A than hNMDAR1A/2B ($P < .05$). The 7-chlorokynurenic acid derivative CGP 58411 did not discriminate between the two subunit combinations (fig. 3D; table 3). In summary, L-689,560 was the only glycine-site competitive antagonist tested that was significantly, albeit modestly, selective between the two subunit combinations.

Pharmacology of hNMDAR1A/2A/2B receptors. The results of immunoprecipitation studies (Sheng *et al.*, 1994) and oocyte expression studies (Wafford *et al.*, 1993) have suggested that NMDA receptors may be composed of more than one type of NMDAR2 subunit. Wafford *et al.* (1993) have shown that injection of oocytes with transcripts encoding hNMDAR1E, rat NMDAR2A and rat NMDAR2C subunits in a 1:3:3 ratio results in receptors that exhibit a glycine affinity intermediate between those of oocytes injected with NMDAR1E/2A or NMDAR1E/2C. We also examined the pharmacological properties of receptors formed by coinjecting oocytes with equal amounts of transcripts encoding hNMDAR1A, hNMDAR2A and hNMDAR2B (referred to as 1:1:1). From the concentration-response curves for NMDA (fig. 1B; table 1), we determined that the EC_{50} values were not significantly different from those for hNMDAR1A/2A or hNMDAR1A/2B ($P > .05$). In contrast, the EC_{50} values of glycine and serine for hNMDAR1A/2A/2B receptors (fig. 1C; table 1) were significantly different from those for hNMDAR1A/2A ($P < .05$) but were not distinguishable from those for hNMDAR1A/2B receptors. Likewise, the NMDA-site competitive antagonists CGS 19755 and CGP 40116 did not distinguish between hNMDAR1A/2A/2B and hNMDAR1A/2B. In summary, the 1:1:1 combination of hNMDAR1A/2A/2B subunits was indistinguishable from hNMDAR1A/2B receptors in terms of its sensitivity to NMDA, glycine, D-serine, CGS 19755 and CGP 40116 but differed from hNMDAR1A/2A receptors in sensitivity to glycine and D-serine.

It was apparent that we could not detect significant differences in the pharmacological properties of hNMDAR1A/2A/2B and hNMDAR1A/2B subunit combinations with compounds that were not highly selective between hNMDAR1A/2A and hNMDAR1A/2B. Therefore, to magnify the differences between the different combinations, we studied the sensitivities of different ratios of hNMDAR2A and hNMDAR2B subunits to ifenprodil, a noncompetitive antagonist that has been reported to discriminate between NMDAR1A/2A and NMDAR1A/2B by 430-fold (Williams,

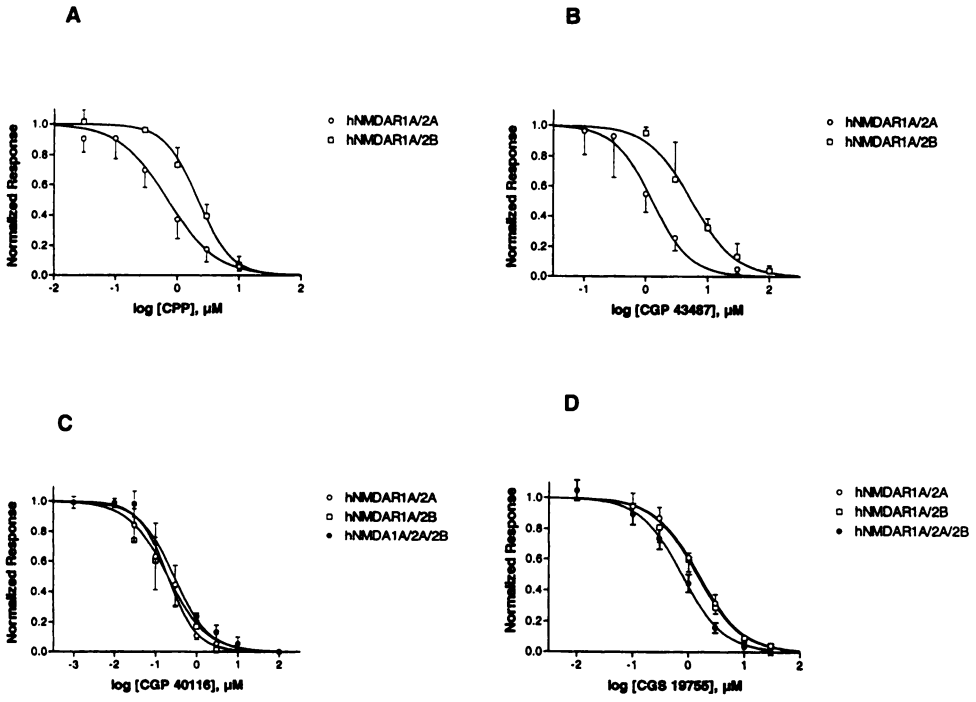

Fig. 2. Antagonist-response curves for CPP (A), CGP 43487 (B), CGP 40116 (C) and CGS 19755 (D) with human NMDA receptors. Subsaturating concentrations of NMDA (100 μM for the hNMDAR1A/2A combination and 30 μM for the hNMDAR1A/2B and hNMDAR1A/2A/2B combinations) were used. The data points represent the mean ± S.D. for normalized responses from three to five cells. The solid lines are fits to the data points.

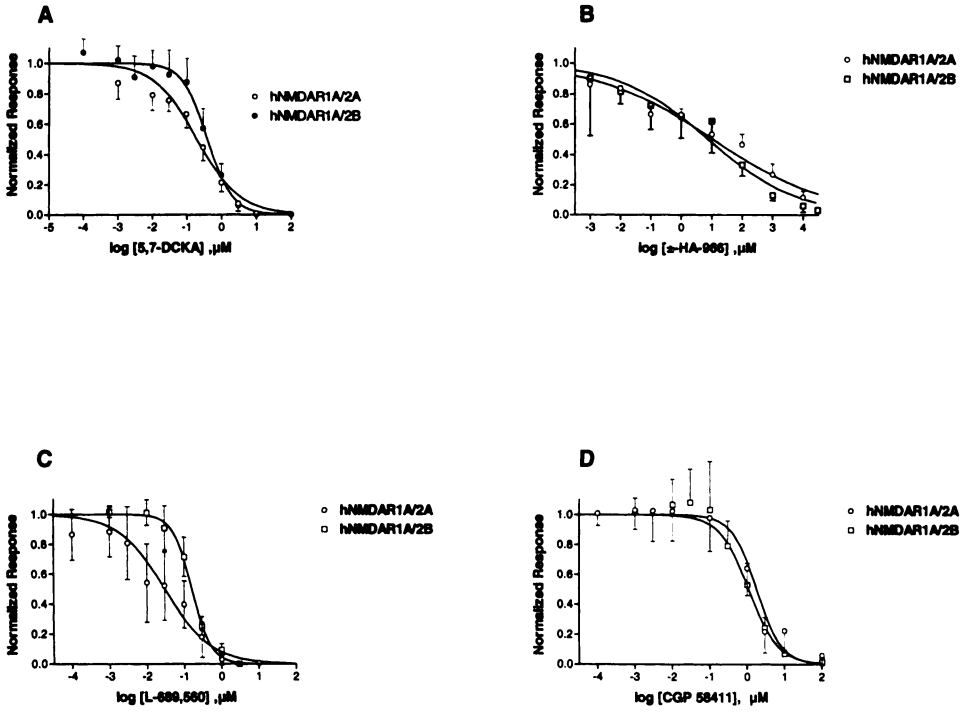

Fig. 3. Antagonist-response curves for glycine-site antagonists DCKA (A), (±)-HA-966 (B), L-689,560 (C) and CGP 58411 (D) with *Xenopus* oocytes expressing hNMDAR1A/2A and hNMDAR1A/2B. With the exception of DCKA, antagonists were tested in the presence of 300 μM NMDA and 10 μM D-serine for hNMDAR1A/2A or 100 μM NMDA and 3.0 μM D-serine for hNMDAR1A/2B. DCKA was tested with 100 μM NMDA and 10 μM glycine for the hNMDAR1A/2A receptor and with 30 μM NMDA and 1.0 μM glycine for the hNMDAR1A/2B receptor. The data points represent the mean ± S.D. for normalized responses from 3 to 11 cells. The solid lines are fits to the data points.

TABLE 3

Summary of Leff-Dougall K_b values and Hill coefficients for glycine-site competitive antagonists

Values represent the mean (−S.D., +S.D.) obtained from 3 to 11 oocytes. n_H is the Hill coefficient (mean ± S.D.).

	DCKA	HA 966	L-689,560	CGP 58411
hNMDAR1A/2A K_b (μM)	0.03 (0.02, 0.05)	11.6 (2.7, 50.5)	0.004 (0.001, 0.012)*	0.24 (0.09, 0.63)
n_H	0.7 ± 0.1	0.3 ± 0.03	0.7 ± 0.4	1.3 ± 1.0
hNMDAR1A/2B K_b (μM)	0.05 (0.03, 0.09)	0.7 (0.07, 7.30)	0.020 (0.016, 0.026)	0.13 (0.08, 0.22)
n_H	1.2 ± 0.5	0.5 ± 0.3	1.7 ± 0.8	1.3 ± 0.4

* Significant differences between means of hNMDAR1A/2A and hNMDAR1A/2B ($P < .05$, Mann-Whitney rank sum test).

1993) or 300-fold (Priestly *et al.*, 1995). Ifenprodil blocked currents obtained in response to 300 μM NMDA and 30 μM glycine in a dose-dependent manner, and we obtained IC_{50} values of 0.114 (0.04, 0.31) and 39.5 (17.5, 89.5) μM for hNMDAR1A/2B and hNMDAR1A/2A receptors, respectively (fig. 4). These values were significantly different ($P < .05$, two-tailed t test) and represent a 346-fold discrimination.

Ifenprodil reportedly blocks both recombinant (Williams, 1993) and native (Legendre and Westbrook, 1991) NMDA receptors with multiple mechanisms. Williams (1993) has reported that with NMDAR1A/2A receptors ifenprodil produces a voltage-dependent block at concentrations of 100 to 300 μM, whereas the inhibition of NMDAR1A/2B receptors by ifenprodil is not voltage dependent but is the result, at least in part, of noncompetitive antagonism at the glycine site. Therefore, the differences in ifenprodil sensitivity determined for recombinant receptors formed by injection of different ratios of 2A and 2B subunits may arise from a combination of subtype-dependent differences in potency and mechanism of action. More specifically, the relatively shallow Hill slopes of 0.6 to 0.8 estimated for the hNMDAR1A/2A and hNMDAR1A/2B receptors may reflect the multiple mechanisms of ifenprodil action.

Figure 4 shows that concentration-response curves for ifenprodil were progressively shifted to higher concentrations as the hNMDAR2A:hNMDAR2B transcript ratio was increased while the amount of hNMDAR1A transcript and the total amount of the three transcripts were kept constant. The data obtained from oocytes injected with hNMDAR2A and hNMDAR2B transcripts at a 10:1 ratio were best fit with a two-independent site model ($P < .001$ *vs.* a single-site model) in which 49% of the sites displayed high sensitivity to ifenprodil and 51% displayed low sensitivity. The mean IC_{50} values for the two sites were 0.246 and 94.8 μM, and the Hill slopes were 1.6 and 2.2. These IC_{50} values were not significantly different from those obtained for hNMDAR1A/2B and hNMDAR1A/2A receptors, respectively.

A binomial expansion predicts the relative proportions of subunit combinations resulting from injection of various ratios of 2A and 2B transcripts. We examined the efficiency of expression of hNMDAR1A/2A, hNMDAR1A/2B and hNMDAR1A/2A/2B subunits in oocytes from a single frog and found that the mean current amplitude in response to saturating concentrations of NMDA and glycine from five oocytes injected with transcripts encoding hNMDAR1A and -2A was 443 ± 248 nA (mean ± S.D.). This value was significantly ($P < .05$) less than that obtained from oocytes injected with transcripts encoding hNMDAR1A and -2B (871 ± 140 nA) or hNMDAR1A, -2A and -2B transcripts in a 1:1:1 ratio (828 ± 226 nA). Because the mean open time and single-channel conductances of rodent NMDAR1A/2A and NMDAR1A/2B receptors expressed in oocytes are not detect-

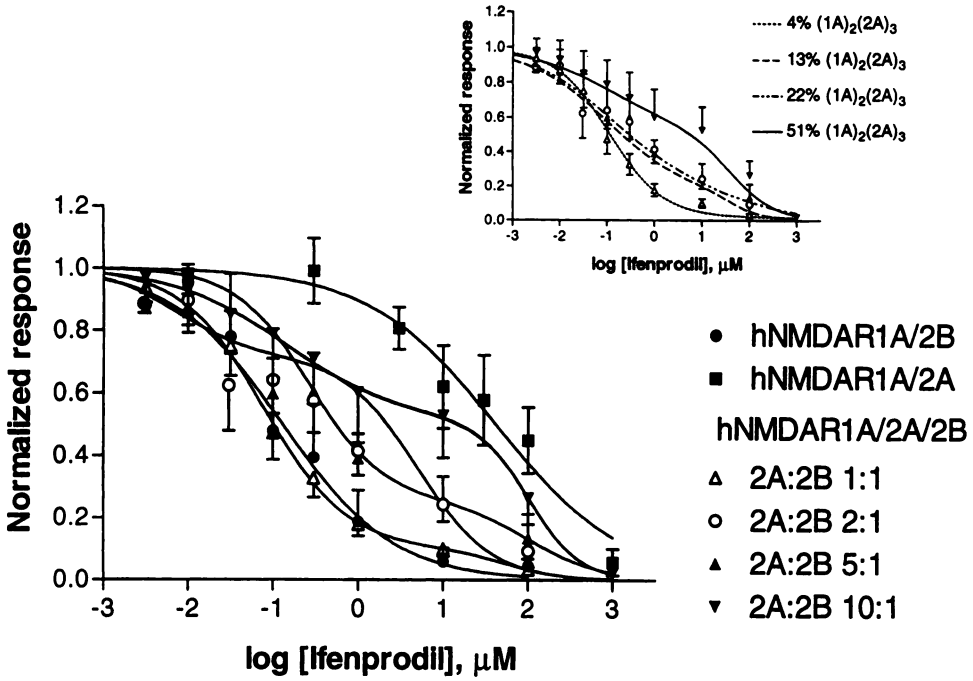

Fig. 4. Block of currents by ifenprodil in oocytes injected with either two or three different transcripts encoding NMDA receptor subunits. Ifenprodil was tested in the presence of 300 μM NMDA and 30 μM glycine. The data points represent the mean ± S.D. for normalized responses from four to eight cells. The solid lines are fits to the data. For the data obtained from oocytes injected with three subunits, the data were fit with a two-independent binding site model. Inset, data obtained for the four 2A:2B ratios tested and the binomial predictions for a two-independent binding site model assuming that 51%, 22%, 13% or 4% of the receptors contain three 2A subunits.

ably different (Stern *et al.*, 1992), these results suggest that hNMDAR2A may be expressed at approximately 2-fold lower levels than hNMDAR2B in oocytes. With correction for the lower expression of hNMDAR2A and with the assumption of a pentameric receptor consisting of two NMDAR1A subunits and three NMDAR2*X* subunits (Béhé *et al.* 1995), the binomial expansion predicts (table 4) that, if the 2A and 2B transcripts are injected at ratios of 1:1, 2:1, 5:1 and 10:1, then 4, 13, 22 and 51% of the receptors formed, respectively, should represent receptor complexes expressing only 2A subunits [*i.e.*, $(1A)_2(2A)_3$] (table 4). Because we could measure only the ifenprodil sensitivity of the $(1A)_2(2A)_3$ and $(1A)_2(2B)_3$ receptors, we fit the data with a two-independent site model in which the proportion of receptors with ifenprodil affinity equal to that of the 1A/2A combination was fixed at the fraction predicted by the binomial expansion and the IC_{50} values for the two sites were fixed at those measured for the hNMDAR1A/2A or hNMDAR1A/2B combinations, respectively. Figure 4, inset, shows that the data were well fit by the two-independent site model.

For each of the four injection ratios studied, we observed close agreement between the binomial prediction for the proportion of receptors containing only hNMDAR1A and hNMDAR2A subunits and the component measured that displayed low ifenprodil sensitivity. This suggests that the $(1A)_2(2A)_1(2B)_2$ and $(1A)_2(2A)_2(2B)_1$ receptors have an ifenprodil sensitivity similar to that of $(1A)_2(2B)_3$ receptors and, therefore, the presence of a single hNMDAR2B subunit in the receptor complex imparts high ifenprodil sensitivity to the receptor. Additional experiments are required to deduce the stoichiometry of the human NMDA receptor subunits in the various receptor complexes.

TABLE 4

Binomial expansion predictions of subunit compositions resulting from injection of transcripts encoding hNMDAR1A, hNMDAR2A and hNMDAR2B subunits in different 2A:2B ratios

Values were calculated assuming a pentameric receptor composed of two NMDAR1A subunits (Béhé *et al.*, 1995) and three 2X subunits and were corrected for the estimated relative expression levels of hNMDAR2A and hNMDAR2B subunits in oocytes.

2A:2B Injection Ratio	Proportion of Receptors Containing:			
	$(1A)_2(2A)_3$	$(1A)_2(2A)_2(2B)_1$	$(1A)_2(2A)_1(2B)_2$	$(1A)_2(2B)_3$
1:1	0.037	0.222	0.444	0.296
2:1	0.125	0.375	0.375	0.125
5:1	0.216	0.432	0.288	0.064
10:1	0.512	0.384	0.096	0.008

Discussion

We cloned cDNAs encoding hNMDAR1A, hNMDAR2A and hNMDAR2B and characterized the pharmacological properties of the recombinant receptors (hNMDAR1A/2A, hNMDAR1A/2B and hNMDAR1A/2A/2B) expressed in *Xenopus* oocytes with whole-cell current measurements. Comparison of the agonist potencies revealed that at the agonist site, NMDA was 2-fold more potent on hNMDAR1A/2B than hNMDAR1A/2A, but could not discriminate these subunit combinations from hNMDAR1A/2A/2B, and that glutamate was not selective between the different subunit combinations. At the coagonist site, glycine discriminated approximately 10-fold between hNMDAR1A/2A and hNMDAR1A/2B and approximately 5-fold between hNMDAR1A/2A and hNMDAR1A/2A/2B and was the most subtype-selective agonist tested. D-Serine was both less potent and less selective than glycine but, nevertheless, significantly discriminated between hNMDAR1A/2A and hNMDAR1A/2B and between hNMDAR1A/2A and hNMDAR1A/2A/2B.

We sought to determine whether NMDA-site or glycine-site antagonists could discriminate between the receptors formed from different subunit combinations. Of the NMDA-site antagonists, CPP displayed 4-fold and CGP 43487 displayed 6-fold selectivity for hNMDAR1A/2A over hNMDAR1A/2B. The structural basis for the subtype selectivity of CPP and CGP 43487 is not understood because both

of these compounds, as well as the nonselective CGS 19755 and CGP 40116, can adopt similar low-energy conformations consistent with the NMDA-site pharmacophore model of Whitten *et al.* (1992).

Of the glycine-site antagonists, L-689,560 was 5-fold more potent at hNMDAR1A/2A than hNMDAR1A/2B, whereas neither DCKA, (±)-HA-966 nor CGP 58411 was subtype-selective. The shallow Hill slopes obtained in our studies with HA-966 may reflect multiple sites of action of the compound. We obtained similar shallow Hill slopes for ifenprodil, a compound known to have multiple sites of action on NMDA receptors. Priestly *et al.* (1995) did not observe a significant difference in microscopic affinity constants for L-689,560 at hNMDAR1A/2A and hNMDAR1A/2B receptors. Recently, Woodward *et al.* (1995) reported that, when tested with 100 μM glutamate and 10 μM glycine at all four subtypes, the quinoxalinedione, glycine-site competitive antagonist ACEA-1021 was 10-fold more potent against rat NMDAR1A/2A receptors than against rat NMDAR1A/2B receptors and 50-fold less potent against rat NMDAR1A/2D receptors. However, there was only a 5.8-fold difference in the Leff-Dougall K_b values.

These data indicate that a new series of NMDA receptor antagonists, which do not compete at the NMDA or glycine sites, may be required to effectively discriminate between NMDA receptor subtypes. The noncompetitive antagonist ifenprodil has recently been shown to be 430-fold more potent in blocking rat NMDAR1A/2B receptors than rat NMDAR1A/2A receptors (Williams, 1993). Priestley *et al.* (1995) found a 300-fold separation of affinities for hNMDAR1A/2A and hNMDAR1A/2B receptors expressed in L(tk$^-$) cells; we likewise found that ifenprodil blocks hNMDAR1A/2B receptors with 346-fold greater potency than hNMDAR1A/2A receptors. Although incompletely understood, the antagonism by ifenprodil appears to result from a decrease in the duration of long single-channel openings of rat hippocampal cultured neurons (Legendre and Westbrook, 1991). Additionally, Williams (1993) observed that ifenprodil produced a weak open-channel block of rat NMDAR1A/2A receptors and a noncompetitive effect at the glycine site of rat NMDAR1A/2B receptors expressed in oocytes.

Recent evidence indicates that three-way combinations of NMDA receptor subunits may represent functional native NMDA receptor subunit combinations (Wafford *et al.*, 1993; Sheng *et al.*, 1994). Our results show that injection of oocytes with transcripts encoding hNMDAR1A, hNMDAR2A and hNMDAR2B in a 1:1:1 ratio results in the expression of receptors that are pharmacologically indistinguishable from hNMDAR1A/2B receptors, in terms of sensitivity to NMDA, glycine, D-serine, CGS 19755 and CGP 40116. Wafford *et al.* (1993) reported that oocytes injected with hNMDAR1E, rat NMDAR2A and rat NMDAR2C in a 1:3:3 ratio exhibit a glycine affinity intermediate between those of oocytes injected with NMDAR1E/2A or NMDAR1E/2C. We previously showed that oocytes injected with hNMDAR1A, hNMDAR2A and hNMDAR2C in a 1:1:1 ratio are significantly more sensitive to glycine than are hNMDAR1A/2A-injected oocytes (Daggett *et al.*, 1994). Thus, in oocyte expression studies, optimization of the ratio of transcripts injected is necessary for the functional characterization of three-way combinations of NMDA receptors.

Because ifenprodil can discriminate >300-fold between the hNMDAR1A/2A and hNMDAR1A/2B subtypes, we measured ifenprodil sensitivity to probe the pharmacological differences among hNMDAR1A/2A/2B receptors formed by increasing the 2A:2B transcript ratio in the hNMDAR1A/2A/2B injection mixture. We found that the NMDA receptors with low ifenprodil sensitivity formed by injection of hNMDAR2A and hNMDAR2B transcripts at 1:1, 2:1 or 5:1 ratios correlated with predictions of a mixture containing 4, 13 or 22% hNMDAR$(1A)_2(2A)_3$ receptors, respectively. This suggests that the $(1A)_2(2A)_1(2B)_2$ and $(1A)_2(2A)_2(2B)_1$ receptors have an ifenprodil sensitivity similar to that of $(1A)_2(2B)_3$ receptors and that the presence of a single hNMDAR2B subunit in the receptor complex imparts high ifenprodil sensitivity to the receptor. MacKinnon (1991) demonstrated a similar change in the pharmacological properties of Shaker K^+ channels, where the presence of a single wild-type subunit in the tetrameric receptor conferred high charybdotoxin sensitivity. Geiger *et al.* (1995) correlated the relative abundance of GluR2(R) mRNA, as determined by single-cell reverse transcription-polymerase chain reaction techniques, with the Ca^{++} permeability of nine different native neuronal and glial cell types expressing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. The data were well fit by a dominance model, suggesting that the incorporation of a single GluR2(R) subunit confers low Ca^{++} permeability to heteromeric α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. Together, these data suggest that the incorporation of a single subunit can be sufficient to change the pharmacological or biophysical properties of heteromeric receptors.

Our data suggest that development of subtype-selective antagonists necessitates testing against NMDA receptors formed from NMDAR1A together with two or possibly three different NMDAR2 subunits. These studies must examine the subunit stoichiometry necessary to produce pharmacologically distinct receptors by using both pharmacological and biochemical approaches.

Acknowledgments

We wish to thank CIBA-GEIGY Pharma Research for providing the CIBA compounds and J. Sullivan and S. Heinemann of the Salk Institute for providing the rat NMDAR2B probe. We also thank M. Harpold, K. Lloyd, M. Varney and J. Winkelman for critically reading the manuscript, M. Hans for helping with curve fitting and M. Washburn for providing some of the results.

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