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Pharmacological Properties of Recombinant Human *N*-Methyl-D-Aspartate Receptors Comprising NR1a/NR2A and NR1a/NR2B Subunit Assemblies Expressed in Permanently Transfected Mouse Fibroblast Cells

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SUMMARY

The pharmacological properties of two recombinant human *N*-methyl-D-aspartate (NMDA) receptor subtypes, comprising either NR1a/NR2A or NR1a/NR2B subunits permanently transfected into mouse L(tk−) cells, have been compared using whole-cell voltage-clamp electrophysiology. Glutamate was a full agonist at both receptors, having a modestly but statistically significant ($p < 0.002$) higher affinity for the NR2B- than the NR2A-containing receptor (microscopic K_d [mK_d] = 0.76 and 0.43 μM, respectively). In comparison to glutamate, NMDA, quinolinic acid, and *cis*-2,3-piperidinedicarboxylic acid were partial agonists at both receptor subtypes. Maximal amplitude currents resulted when glutamate-site agonists were combined with either glycine or D-serine; both of these amino acids were, therefore, defined as full agonists at the glycine site. Glycine had an ~10-fold higher affinity ($p < 0.0001$) for NR2B- than for NR2A-containing receptors (mK_d = 0.057 and 0.53 μM, respectively). D-Cycloserine, (+)-(3*R*)-3-amino-1-hydroxypyrrolidin-2-one, (+)-*cis*-(4*R*)-methyl-(3*R*)-amino-1-hydroxypyrrolidin-2-one, and 1-aminocyclobutanecarboxylic acid also had higher affinities for the NR2B-containing receptor but were partial agonists, at both receptor subtypes, unlike glycine. Agonist-evoked whole-cell currents were antagonized by D-(−)-2-amino-5-phosphonopentanoic acid, *cis*-4-(phosphonomethyl)piperidine-2-carboxylic acid, and 3-((*R*)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid, all of which had slightly, but statistically significant, higher affinities (2.2-, 2.8-, and 5.5-fold, respectively) for the NR2A-containing receptor. Responses were also antagonized by the glycine-site antagonists 7-chlorokynurenic acid, 7-chloro-4-hydroxy-3-(3-phenoxy)phenylquinolin-2-(1*H*)-one, and (±)-4-(*trans*)-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline. The atypical NMDA antagonist ifenprodil showed the largest separation in functional affinity (IC_{50} values, 0.6 and 175 μM at NR2B- and NR2A-containing receptors, respectively). These experiments demonstrate the usefulness of permanently transfected L(tk−) cells for electrophysiological studies of recombinant NMDA receptor function and provide the first detailed functional pharmacological analysis of human NMDA receptor subtypes.

The NMDA receptor is a cationic channel that is gated by the co-agonists glutamate and glycine (1, 2). The molecular cloning of the cDNAs of the individual rodent NMDA receptor subunits in several laboratories (3–6) has suggested a subunit composition for the receptor and has provided a molecular basis for native NMDA receptor heterogeneity (7–10). The family of rat NMDA receptor subunits is believed to comprise an NR1 subunit (ζ in mouse), a single gene product generating at least eight different isoforms (11, 12), and four classes of highly homologous NR2 subunits, referred to as A through D in the rat and ε1 through 4 in mouse (4–6, 13), each of which is the product of distinct genes.

The subunit stoichiometry of a functional NMDA receptor is currently unknown. The NR1 (or ζ1) subunit is a fundamental requirement; it possesses binding sites for glycine (14–16).[1] and glutamate (15). Expression of NR1 alone, but not NR2 alone, in *Xenopus* oocytes is capable of forming a functional channel with many of the features expected of a native NMDA receptor (3). However, more robust currents

[1] S. Grimwood, B. Le Bourdellès, J. R. Atack, C. Barton, W. Cockett, S. M. Cook, E. Gilbert, P. H. Hutson, R. M. McKernan, J. Myers, C. I. Ragan, P. B. Wingrove, and P. J. Whiting. Characterization of recombinant human *N*-methyl-D-aspartate receptor subtypes in stably transfected cells: homomeric and heteromeric receptors can coexist within the same cell. Submitted for publication.

ABBREVIATIONS: ACBC, 1-aminocyclobutanecarboxylic acid; CGS 19755, *cis*-4-(phosphonomethyl)piperidine-2-carboxylic acid; R-CPP, 3-((*R*)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; D-AP5, D-(−)-2-amino-5-phosphonopentanoic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; L-687,414, (+)-*cis*-(4*R*)-methyl-(3*R*)-amino-1-hydroxypyrrolidin-2-one; L-701,324, 7-chloro-4-hydroxy-3-(3-phenoxy)phenylquinolin-2-(1*H*)-one; L-689,560, (±)-4-(*trans*)-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline; NMDA, *N*-methyl-D-aspartic acid; *cis*-2,3-PDA, *cis*-2,3-piperidinedicarboxylic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; mK_d, microscopic K_d; D-AP7, (−)-2-amino-7-phosphonoheptanoic acid; ifenprodil, α-(4-hydroxyphenyl)-β-(4-benzylpiperidin-1-yl)-β-methylmethanol tartrate; 7-Cl-KYNA, 7-chlorokynurenic acid.

result from the coexpression of NR1 with one or more of the NR2 family (5, 13). This, together with other evidence, suggests that the majority of native NMDA receptors are hetero-oligomeric assemblies of NR1 and NR2 subunits. The pharmacological profile of such receptors is influenced by the type of NR2 subunit coexpressed (15, 18, 19).

Although the human homologues of NR1 have been cloned (20–22), as has the human NR2A (22, 23) and NR2B,[1] much of the currently available information on recombinant NMDA receptors has been derived from the expression of rodent subunit cDNAs in *Xenopus* oocytes or transiently transfected cells. Although NR1 exhibits a high degree of amino acid conservation between rat and human (only 8 substitutions) (22), the NR2B subunit shows more diversity (21 substitutions and 2 extra residues in the human sequence)[1] and the NR2A subunit shows even greater diversity (81 substitutions) (22). We wanted to characterize in detail the pharmacology of recombinant NMDA receptor subtypes and to investigate whether the amino acid differences between rat and human NR2 conferred pharmacological novelty. Accordingly, receptor assemblies composing NR1a/NR2A or NR1a/NR2B subunit combinations were permanently transfected into a mouse cell line [L(tk−)]. The functional affinities of a number of ligands for each of the co-agonist binding sites and that of the atypical, noncompetitive NMDA receptor antagonist ifenprodil were determined with the use of whole-cell voltage-clamp electrophysiology.

Materials and Methods

Generation of stably transfected cell lines expressing human NR1a/NR2A and NR1a/NR2B recombinant receptors. The cDNAs encoding human NR1a and NR2A (22) and human NR2B[1] were subcloned into the dexamethasone-inducible eukaryotic expression vector pMSGneo and mouse L(tk−) cells (American Type Culture Collection CCL1.3) transfected with NR1a/NR2A (clone BA7) or NR1a/NR2B (clone J4) assemblies. Precise details are described elsewhere (24).[1] The transfections were performed with 10 μg total DNA in a ratio of 1:3 for NR1a/NR2A or with 32 μg total DNA in a ratio of 1:7 for NR1a/NR2B. Geneticin-resistant colonies were isolated using cloning cylinders, and cell clones expressing recombinant heteromeric NMDA receptors were identified by measuring [^{3}H]dizocilpine binding.[1] The cell clones expressing the highest levels of [^{3}H]dizocilpine binding were recloned by limiting dilution.

Induction of expression of recombinant receptors. Cell cultures were maintained in Dulbecco's modified Eagle's medium supplemented initially with 10% fetal calf serum or later with 10% Fetal-Clone-II, 2 mM L-glutamine, 2 mg/ml Geneticin, 100 μg/ml penicillin, and 100 U/ml streptomycin at 37° in a 5% CO_2 atmosphere. For electrophysiological studies, cells were seeded at low density onto poly-D-lysine-coated glass coverslips. After a period of 24 hr, the culture medium was changed to an induction medium with the same composition as described but without Geneticin and supplemented with 1 μM dexamethasone (this was reduced to 0.1 μM during later experiments without any apparent effects on the levels of receptor expression) and 0.5 mM ketamine.

Immunocytochemistry. The induction of receptor expression was visualized by immunofluorescence using a polyclonal rabbit antiserum generated to the cDNA sequence corresponding Ser-14/Leu-76 of rat NR1. Briefly, this amino acid sequence was incorporated into pRSET 5a (a T7 polymerase expression vector) and expressed in *Escherichia coli* BL21 (DE3) Lys S, as detailed previously (25). The recombinant protein was purified by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroelution and antisera raised in rabbits as described by McKernan *et al.* (26). The antiserum was affinity purified using the recombinant polypeptide coupled to Sepharose CL4B. Cells used in the immunofluorescence assay were cultured on Nunc glass chamber slides. After 24 hr in the presence or absence of induction medium, the cells were washed with PBS and then fixed for 15 min in 4% paraformaldehyde in PBS. After permeabilization in PBS containing 0.4% Triton-X-100 for 15 min, the slides were washed twice in PBS and incubated in PBS containing BSA (3% PBS-BSA) and normal goat serum (3%) for 15 min. Affinity purified rabbit anti-NR1 antibodies were added at a concentration of 20 μg ml^{-1} to each well. After a 2-hr incubation, the slides were washed three times for 10 min each in PBS before being overlaid with affinity fluorescein isothiocyanate-labeled goat anti-rabbit IgG at a dilution of 1:40 in PBS-BSA. After an additional 1-hr incubation, the slides were washed three times in PBS and mounted with the use of Immu-mount. Photomicrographs were acquired with Kodak TMAX 3200 film with a standard exposure time of 10 sec.

Electrophysiological recordings. Cells were used for electrophysiological recordings 24–72 hr after the induction of receptor expression with dexamethasone. The cultures were observed with the use of phase-contrast optics and continuously perfused with a salt solution of the following composition (in mM): NaCl 124, KCl 3.25, $CaCl_2$ 2, $MgCl_2$ 2, HEPES buffer 10, and D-glucose 11. pH of the perfusate was adjusted to 7.4 using NaOH, and osmolality was adjusted to 350 mOsm using sucrose. Individual cells were voltage-clamped at a holding potential of −60 mV using an Axoclamp-2A amplifier in the whole-cell clamp mode. Patch pipettes were fabricated from thin-wall glass (1.2 mm o.d., 0.9 mm i.d., Clark Electromedical) with tip diameters in the order of 2–3 μm and were filled with a solution of the following composition (in mM): CsF 120, CsCl 10, HEPES 10, EGTA 10, and $CaCl_2$ 0.5. pH of the pipette solution was adjusted to 7.25 using CsOH, and osmolality was adjusted to 320 mOsm using sucrose.

Glutamate receptor agonists and antagonists were diluted from concentrated aqueous stock solutions into a modified salt solution, which other than the omission of $MgCl_2$ had the same composition as that described for the external perfusate. Experiments involving glycine-site agonists and antagonists required additional modifications to the salt solution to minimize the effects of contaminating glycine. For these latter experiments, all solutions were prepared in high performance liquid chromatography-grade water and in glassware that had been extensively rinsed in high performance liquid chromatograph-grade water. Drugs were diluted into a modified salt solution that lacked $MgCl_2$, D-glucose, and sucrose. In all cases, drugs were applied to localized regions of the culture by fast perfusion from a double-barrelled pipette assembly.

Data analysis. Concentration-response curves to NMDA or glycine receptor agonists were generated by measuring the inward current response produced by increasing concentrations of the agonist in the presence of a saturating concentration of the co-agonist, glycine or glutamate, respectively. Data from individual cells were analyzed by computing best-fit lines using a two-equivalent binding site model (9, 27), which predicts the affinity of the ligand at individual binding sites (i.e., mK_d, the concentration of agonist effecting 50% receptor occupancy, rather than EC_{50} or macroscopic K_d) obtained with a single-site model. These data were converted to $\log_{10}$ values (i.e., pmK_d) to facilitate statistical analyses. The resulting geometric mean pmK_d values, determined from a minimum of four experiments, are quoted as their corresponding antilogarithms together with their associated asymmetric standard error values (i.e., −standard error, +standard error). Efficacy values are quoted relative to glutamate or glycine response amplitudes determined on the same cell. For each agonist, the maximum response predicted from the curve fit was used. In the case of experiments involving glycine or glycine-site agonists, the residual contaminating level of glycine was estimated by obtaining responses to glutamate in nominally glycine-free salt solution on all cells. The "basal" response obtained under such conditions was never >10% of the response obtained with a saturating concentration of glycine; the basal response was subtracted from all subsequent agonist responses.

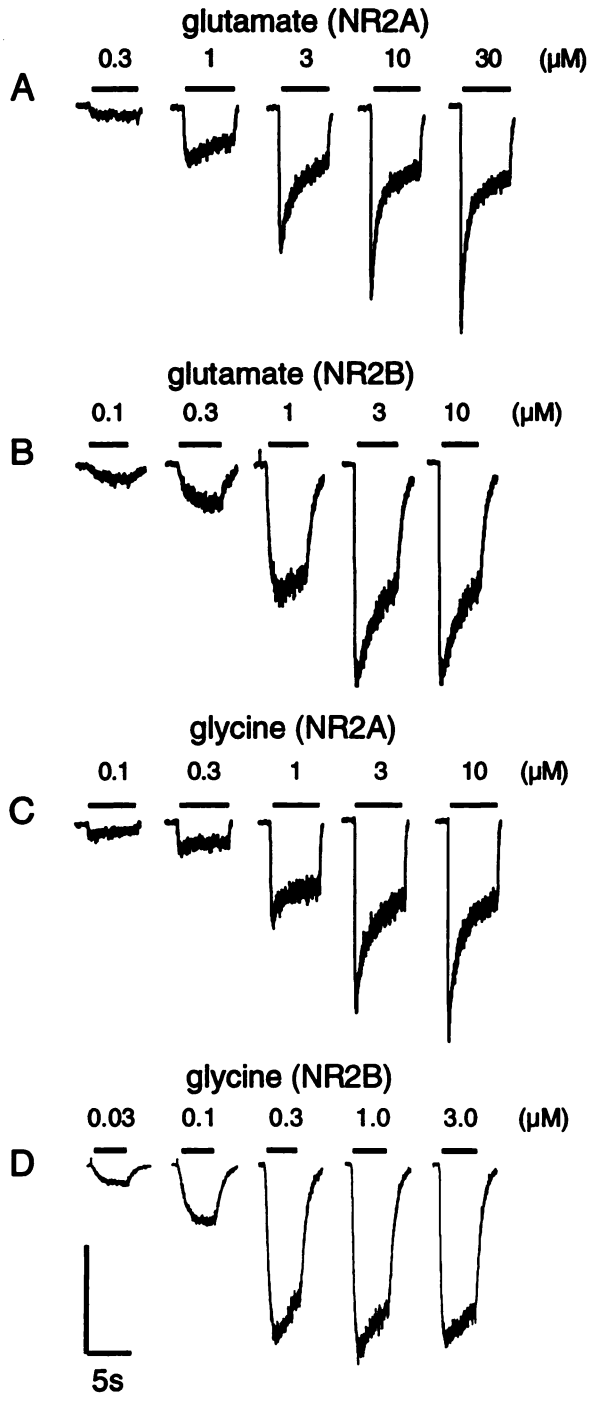

Fig. 1. Whole-cell currents evoked by the application of increasing concentrations of L-glutamate (A and B) or glycine (C and D) to L(tk−) cells expressing either NR1a/NR2A (A and C) or NR1a/NR2B (B and D) human recombinant NMDA receptors. Each cell was voltage-clamped at a holding potential of −60 mV, and discrete 5-sec glutamate applications (*horizontal bars* above each response; concentrations as indicated) were applied by fast perfusion at 30-sec intervals (intervening sections of trace omitted for clarity). Concentration-response relationships for glutamate were performed in the continuous presence of glycine (3 μM for NR2A- or 1 μM for NR2B-containing subunit assemblies, whereas concentration-response relationships for glycine were performed in the continuous presence of glutamate (10 μM). Note that the responses to either co-agonist were slower in rise time and decay with the NR1a/NR2B subunit combination, but responses to either co-agonist showed faster rates of fading for the NR1a/NR2A receptor. *Vertical calibration bar*, current amplitude of 680 pA (A), 540 pA (B), 470 pA (C), and 440 pA (D).

Concentration-response relationships for several NMDA and glycine-site antagonists were determined by applying increasing concentrations of antagonist in the presence of fixed concentrations of the co-agonists glutamate and glycine. Experiments involving glutamate-site antagonists were performed with 10 μM glutamate and 10 μM glycine. Glutamate concentration was also fixed at 10 μM when glycine-site antagonists were studied; however, in these experiments, the glycine concentration was adjusted to reflect the difference in glycine affinity for the two subunit assemblies (i.e., NR1a/NR2A or NR1a/NR2B). Thus, 3 μM glycine was used for experiments involving the NR1a/NR2A assemblies, whereas 300 nM was used for NR1a/NR2B. To enable more rigorous comparisons of antagonist affinities, the concentration effecting 50% receptor occupancy (K_o) (9) was converted to an antagonist microscopic inhibition constant (mK_i) using the Cheng-Prusoff relationship (28). As with agonists, statistical analyses were performed on $\log_{10}$-transformed data (i.e., pmK_i), and tabulated values represent antilogarithms of geometric mean values (−standard error, +standard error) of data from several experiments.

Drugs and bulk chemicals. The sources of bulk chemicals (all were analytical grade) and drugs are as follows: NaCl, KCl, D-glucose, and sucrose (Fisons, Loughborough, UK); glycine, HEPES, and $CaCl_2$ (BDH, Pool, UK); D-serine, D-cycloserine, L-glutamate, quinolinate, EGTA, poly-D-lysine, and dexamethasone (Sigma Chemical Co., Pool, UK); ACBC, D-AP5, D-AP7, R-CPP, NMDA, ifenprodil, and *cis*-2,3-PDA (Tocris Cookson, Bristol, UK); CsCl, CsF, and CsOH (Aldrich, Gillingham, UK); dizocilpine and CGS 19755 (Research Biochemicals International, Massachusetts); Geneticin, Dulbecco's modified Eagle's medium, penicillin, streptomycin, L-glutamine, and fetal calf serum (GIBCO-BRL, Paisley, UK); FetalClone-II (Hyclone Laboratories, Utah); ketamine HCl (Parke-Davis); and R-HA-966, 7-Cl-KYNA, L-687,414, L-689,560, and L-701,324 (synthesized by Medicinal Chemistry Department, Merck Sharp & Dohme Research Laboratories, Terlings Park, Pontypool, UK).

Results

Electrophysiological characterization of recombinant human NR1a/NR2A and NR1a/NR2B receptors expressed in permanently transfected cells. Voltage-clamped, mouse L(tk−) cells that had been stably transfected with recombinant human NMDA receptor assemblies consisting of either NR1a/NR2A or NR1a/NR2B subunits responded by generating inward currents after the application of glutamate receptor agonists (Fig. 1). In contrast, cells permanently transfected with NR1a alone (40 experiments, data not shown) or with NR1a/NR2A but in which expression was not induced (by the omission of dexamethasone from the culture medium; 11 experiments, data not shown) failed to generate inward currents to agonist application. The induction of receptor expression in NR1a/NR2A transfected cells was also confirmed by immunofluorescence (Fig. 2). In common with reports from other laboratories (29, 30), we found it necessary to include an NMDA receptor antagonist in the culture medium to prevent cell loss due to excitotoxicity after the induction of NMDA subunit expression. In our study, optimal protection was afforded by the use of 0.5 mM ketamine. Preliminary experiments (data not shown) revealed that this low affinity, voltage-dependent NMDA ion-channel blocker was rapidly eliminated by washing the cells in ketamine-free salt solutions, after which whole-cell currents were not increased by clamping the cell membrane at +60 mV while repeatedly applying glutamate.

Maximal current amplitudes required the coadministration of both a glutamate- and a glycine-site ligand confirming the presence of a glycine co-agonist site (1, 2). Responses to a

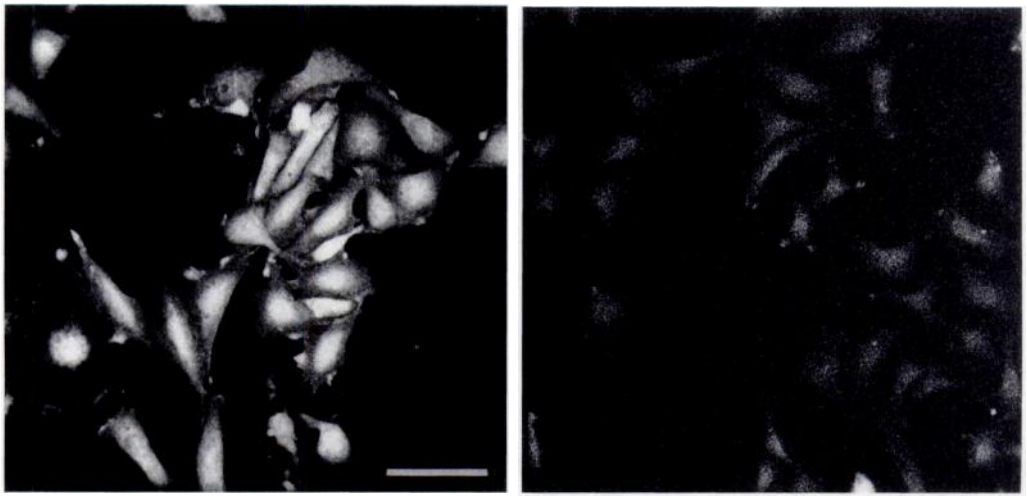

Fig. 2. Immunofluorescence visualization of NMDA receptors expressed in L(tk−) cells permanently transfected with NR1a/NR2A subunits using an NR1 polyclonal antiserum. *NR1a/NR2A induced*, cells cultured for 24 hr in medium containing 1 μM dexamethasone. *NR1a/NR2A uninduced*, cells cultured in medium without dexamethasone. *Calibration bar*, 50 μm.

saturating concentration of glutamate in nominally glycine-free solutions evoked responses that were 4.5 ± 1.4% (16 experiments) and 9.1 ± 1.9% (16 experiments) of maximal with NR1a/NR2A- and NR1a/NR2B-transfected cells, respectively. The number of successfully transfected cells has remained constant with passage, so essentially every cell responded to agonist application (passage numbers: 34, clone J4 and 26, clone BA7); this was true for both stably transfected cell lines. The profile of the inward current response to glutamate was different for the two subunit assemblies. Currents evoked by glutamate on recombinant receptors comprising NR1a/NR2A subunits peaked and desensitized more rapidly than those recorded from the NR1a/NR2B combination (Fig. 1). To quantify this phenomenon, we measured the ratio of pseudo-steady state (I_{ss} = 5 sec after commencing agonist application) to peak (I_{pk}) inward current (10 μM glutamate + 1 μM glycine for NR2B or 10 μM glycine for NR2A) for the two receptor assemblies. These analyses confirmed that NR1a/NR2A currents consistently desensitized to a greater extent (I_{ss}/I_{pk} = 0.55 ± 0.05; 11 experiments) than those recorded from NR1a/NR2B (I_{ss}/I_{pk} = 0.75 ± 0.03, 8 experiments).

Pharmacology of the glutamate-binding site. Both NR1a/NR2A and NR1a/NR2B assemblies responded to several established NMDA receptor ligands. Thus, glutamate, NMDA, quinolinate, and *cis*-2,3-PDA evoked inward current responses. Concentration-response curves to each of these agonists were constructed by measuring whole-cell currents in the continuous presence of the co-agonist, glycine, for each of the subunit assemblies. To directly compare agonist efficacies, a response to a saturating concentration (10 μM) of glutamate was obtained on the same cell. Glutamate consistently evoked responses that were larger in amplitude than those seen with the other agonists and was, therefore, defined as a full agonist (Table 1). In comparison, NMDA, quinolinate, and *cis*-2,3-PDA were partial agonists. The intrinsic efficacies of NMDA and quinolinate varied depending on the subunit assembly (Table 1). *cis*-2,3-PDA behaved as a low efficacy (<6% glutamate) partial agonist on both assemblies. The level of efficacy was so low with this analogue that it was considered more appropriate to calculate its affinity from inhibition curves describing the antagonism of responses evoked by a fixed concentration of glutamate and glycine.

The affinities of four competitive NMDA receptor antagonists (D-AP5 and D-AP7 and their conformationally constrained analogues, CGS19755 and R-CPP, respectively) were determined from inhibition curves for each subunit assembly. These experiments revealed all except D-AP7 to have a significantly higher affinity for the NR1a/NR2A subunit assembly compared with NR1a/NR2B. In most cases, the difference in affinity was modest, except for R-CPP, which showed a slightly more marked (>5-fold) separation (Table 1).

Pharmacology of the glycine site. Similar experiments were performed to determine the functional affinity of several compounds acting at the glycine co-agonist site on the NMDA receptor complex. Bell-shaped curves were particularly common with glycine, D-serine, and D-cycloserine; in such instances, curve fits described the experimental data more accurately when high agonist concentrations (i.e., >EC_{90}) were excluded from the data set (Fig. 3). Thus, in the case of glycine, agonist concentrations up to 10 μM or 1 μM were subjected to curve-fitting routines with NR1a/NR2A and NR1a/NR2B assemblies, respectively. Truncated concentration ranges (details are indicated in legend to Fig. 3) were also used for curve fits with the glycine-site agonist D-serine and the partial agonists D-cycloserine, ACBC, R-HA-966, and L-687,414. Comparisons of agonist efficacy were complicated by the bell-shaped nature of the concentration-response curves. Relative agonist efficacies were always determined with respect to 3 μM (NR1a/NR2A) or 1 μM (NR1a/NR2B) glycine applications on the same cell; these concentrations were deliberately chosen to be submaximal (~80% and 86% of maximum, as estimated from the fitted lines describing the mean data generated from eight NR1a/NR2A- and five NR1a/NR2B-tranfected cells, respectively). Thus, when comparing currents evoked by agonists, the reference glycine current amplitudes were scaled by a factor of 1/0.8 and 1/0.86 for

TABLE 1

Affinities and intrinsic efficacies of glutamate-site ligands

Microscopic affinity constants (μM) are quoted as geometric mean values (−standard error, +standard error), mK_d for agonists, mK_i[a] for antagonists/low efficacy partial agonists. 2B/2A, ratio of affinities at NR1a/NR2B:NR1a/NR2A. Statistically significant (p value) differences in affinity at the two subunit assemblies were determined using a nonpaired Student's t test on log-transformed data. Intrinsic efficacy quoted by comparison to glutamate (=100%) for heteromeric assemblies comprising NR1a/NR2A (NR2A) or NR1a/NR2B (NR2B).

	Affinity					Intrinsic efficacy		
	NR1 + NR2A	n	NR1 + NR2B	n	2B/2A	p	NR2A	NR2B
	mK_d or mK_i[a]					% glutamate		
Agonist								
Glutamate	0.76 (0.70, 0.82)	10	0.43 (0.37, 0.50)	6	0.6	< .002	100	100
NMDA	8.9 (7.6, 10.5)	7	6.8 (6.2, 7.4)	5	0.8	NS	83 ± 3	65 ± 6
Quinolinate	400 (300, 500)	6	502 (448, 561)	7	1.3	NS	64 ± 8	89 ± 3
cis-2,3-PDA	21 (16, 28)	4	38[a] (35, 41)	6	1.8	< .02	3 ± 1	7 ± 0.5
Antagonist[a]								
D-AP5	1.9 (1.4, 2.0)	5	4.2 (4.0, 4.3)	6	2.2	< .001	0	0
D-AP7	3.7 (3.4, 4.1)	5	4.2 (4.0, 4.4)	4	1.1	NS	0	0
CGS 19755	0.46 (0.39, 0.54)	4	1.1 (1.0, 1.2)	4	2.4	< .002	0	0
R-CPP	0.13 (0.07, 0.23)	8	0.71 (0.63, 0.91)	5	5.5	< .002	0	0

n, Number of experiments; NS = $p > 0.05$.

NR1a/NR2A and NR1a/NR2B, respectively. These experiments revealed glycine and D-serine to be high affinity, full agonists at both receptor assemblies. D-Serine may be slightly more efficacious than glycine at NR1a/NR2B receptors, but complications due to bell-shaped curves make interpretations of small differences in efficacy equivocal, and additional experiments would be required to substantiate this observation. Interestingly, glycine had a ~10-fold higher affinity for the NR2B-containing receptor than for the NR2A-containing receptor, but the difference in affinity for the two receptor subtypes was much more modest (~1.5-fold) for the glycine analogue D-serine. All of the remaining glycine-site ligands tested, i.e., D-cycloserine, ACBC, R-HA-966, and L-687,414, were found to be partial agonists with varying levels of intrinsic efficacy. In all cases, the partial agonists had a higher level of efficacy at the NR1a/NR2A assembly but higher affinity for the NR1a/NR2B receptor, although the difference in affinity was always less than 10-fold (Table 2).

The affinities of the glycine-site antagonists L-701,324, L-689,560, and 7-Cl-KYNA were determined from inhibition curves. Of these three antagonists, only the low affinity 7-Cl-KYNA appeared to discriminate between the two subunit assemblies, having approximately 2- to 3-fold higher affinity for the NR1a/NR2B receptor (Table 2).

Antagonism by ifenprodil. The noncompetitive NMDA antagonist ifenprodil showed the largest separation in affinity at the two receptor assemblies, with ~300-fold higher affinity for the NR1a/NR2B assembly (IC_{50} = 0.6 ± 0.08 μM; four experiments) compared with NR1a/NR2A (IC_{50} = 175 ± 14 μM, five experiments).

Discussion

These experiments describe the use of permanently transfected cell lines expressing recombinant human NMDA receptor subtypes as tools to allow detailed functional and pharmacological characterization.

Whole-cell current responses elicited by NMDA receptor agonists in cells expressing heteromeric NR1a/NR2A or NR1a/NR2B assemblies were extremely robust, with amplitudes that were comparable to those obtained from primary cultured neurons (10). In contrast, the lack of a detectable response in cells transfected with NR1a alone suggests, as has been shown previously with rat recombinant NMDA receptors expressed in Chinese hamster ovary cells (31), that this subunit does not assemble into functional receptor ion-channel complexes, despite apparently possessing both glutamate- (15) and glycine- (14–16, 32) binding sites. We can assume, therefore, that the pharmacological profiles presented here are from hetero-oligomeric subunit assemblies.

As is the case with native NMDA receptors, responses to saturating concentrations of glutamate in L(tk−) cells expressing either NR1a/NR2A or NR1a/NR2B assemblies were small in the absence of added glycine. This confirms the presence of a functional modulatory binding site for the co-agonist glycine (1, 2) on these recombinant human receptors.

Glutamate-site ligands. Glutamate was a full agonist at both NR1a/NR2A and NR1a/NR2B receptor subtypes. It had the highest affinity of those agonists tested and had a modestly, yet statistically significant, higher affinity for the NR1a/NR2A receptor compared with NR1a/NR2B. In comparison to glutamate, NMDA, quinolinate, and *cis*-2,3-PDA were partial agonists, as has been found to be the case with native NMDA receptors (33). Neither NMDA nor quinolinate discriminated between NR1a/NR2A and NR1a/NR2B, whereas *cis*-2,3-PDA showed a selectivity that was the reverse of that seen with glutamate, i.e., higher affinity for NR1a/NR2B. In this regard, *cis*-2,3-PDA compared more fa-

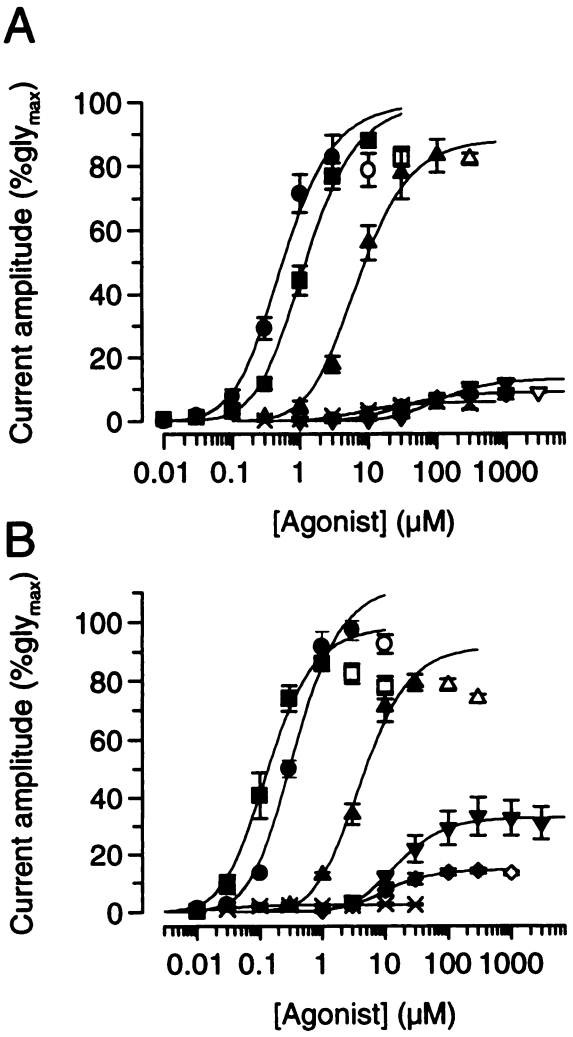

Fig. 3. Concentration-response curves for glycine site ligands on L(tk−) cells permanently transfected with (A) NR1a/NR2A or (B) NR1a/NR2B NMDA receptor subunits. Peak inward currents were measured in response to 5-sec fast applications of D-serine (●), glycine (■), D-cycloserine (▲) R-HA-966 (▼), ACBC (♦), or L-687,414 (×) at 30-sec intervals. In each experiment, agonist responses were normalized with respect to the predicted maximal response to glycine obtained on the same cell (details given in Data Analysis). *Curves*, best-fit lines computed through filled-symbol data points (error bars show ± standard error unless obscured by the symbol), which represent mean values obtained from a number of cells (values given in Table 2). *Open symbols*, data points that were omitted from the curve-fitting routine and show the bell-shaped nature of the concentration-response curves. Glycine and D-serine were full agonists on both subunit assemblies, whereas D-cycloserine, R-HA-966, ACBC, and L-687,414 were partial agonists with varying levels of efficacy.

vorably to several of the glutamate antagonists (see below), and this may reflect the low level of intrinsic activity of this compound. The functional affinities of glutamate-recognition site agonists at NR1a/NR2A were in good agreement with the corresponding values determined with radioligand binding .[1] The rank order of agonist affinities remained the same at NR1a/NR2A and NR1a/NR2B assemblies (i.e., glutamate > NMDA > *cis*-2,3-PDA > quinolinic acid; Table 1) and was similar to that seen previously with native rat cortical (33) and cerebellar granule cell NMDA receptors.[2] However, there was no corresponding relationship with agonist efficacy. NMDA had a greater efficacy at NR1a/NR2A compared with NR1a/NR2B, whereas the reverse was true for quinolinic acid.

The archetypal NMDA receptor antagonist D-AP5 and its partially conformationally constrained analogue, CGS 19755, were both found to have a ~2.5-fold higher affinity for the NR1a/NR2A subunit assembly compared with NR1a/NR2B. A similar NR1a/NR2A selectivity has been reported previously for D-AP5 using rat NMDA receptor cDNAs expressed in *Xenopus* oocytes (18). Likewise, the microscopic affinity constants (mK_i) found in the present study for CGS 19755 at human NR1a/NR2A and NR1a/NR2B receptors are in good agreement with previous estimates of affinity determined from the displacement of [^{3}H]glutamate from rat homologues using a ligand binding assay (15). R-CPP, a conformationally constrained analogue of D-AP7, was also found to have a >5-fold higher affinity for the NR1a/NR2A assembly, whereas D-AP7 failed to discriminate between the two receptor subtypes. The ~5-fold difference in affinity seen with R-CPP is somewhat greater than reported previously with the unsaturated analogue D-CPPene for the antagonism of NMDA-evoked currents generated in *Xenopus* oocytes injected with rat recombinant receptors (~1.6-fold; Ref. 18) and for the displacement of [^{3}H]CGP 39653 binding from HEK 293 cells transiently transfected with rat NMDA receptor subunit combinations (~2-fold; Ref. 15). In summary, the present data confirm and extend the results of other laboratories (15, 18) and suggest that the competitive phosphonate antagonists possess a modestly, but statistically significant, higher affinity for the NR1a/NR2A receptor and, therefore, support the concept of an antagonist-preferring NMDA receptor subtype (18).

Glycine-site ligands. Glycine and D-serine were both found to be high affinity, full agonists, but only glycine showed a substantial discrimination between subunit assemblies, having, in agreement with Kutsuwada *et al.* (3), a ~10-fold higher affinity for NR1a/NR2B. Laurie and Seeburg (15) found a considerably more modest (~1.8-fold) difference in glycine affinity for the rat recombinant NR1/NR2A and NR1/NR2B homologues, as determined from the displacement of [^{3}H]5,7-dichlorokynurenate binding. The reasons for the discrepancy between binding and functional data are unlikely to lie in a species difference, as we have found a similar difference in glycine affinity for subtypes of native rat NMDA receptors (discussed in greater detail below). Conceivably, the inconsistency may be due to the fact that a radiolabeled antagonist ([^{3}H]5,7-dichlorokynurenic acid), as opposed to an agonist, was used in the binding assay described by Laurie and Seeburg (15). Consistent with this notion, the present antagonist inhibition curves for the monohalogenated kynurenic acid derivative 7-Cl-KYNA revealed a much more modest selectivity (~2.5-fold) for the human NR1a/NR2B receptor compared with glycine. This, with the fact that other glycine-site antagonists, i.e., L-689,560 (34) and L-701,324 (35), do not discriminate between NR2A- and NR2B-containing receptors, is perhaps not surprising when considered in the context of recent mutagenesis experiments. A number of amino acid substitutions in either the amino terminal region of NR1 (14, 16) or in the region between

[2] T. Priestley, T. Laughton, unpublished observations.

TABLE 2

Affinities and intrinsic efficacies of glycine-site ligands

Microscopic affinity constants (μM) are quoted as geometric mean values (−standard error, +standard error), mK_d for agonists, mK_i[a] for antagonists/low efficacy partial agonists. 2B/2A, ratio of affinities at NR1a/NR2B:NR1a/NR2A. Statistically significant (p value) differences in affinity at the two subunit assemblies were determined using a non-paired Student's t test on log-transformed data; Intrinsic efficacy quoted by comparison to glycine (=100%) for heteromeric assemblies comprising NR1a/NR2A (NR2A) or NR1a/NR2B (NR2B).

	Affinity					Intrinsic efficacy		
	NR1 + NR2A	n	NR1 + NR2B	n	2B/2A	p	NR2A	NR2B
	mK_d or mK_i[a]						% glycine	
Agonist								
Glycine	0.53 (0.43, 0.64)	8	0.057 (0.046, 0.072)	5	0.11	0.0001	100	100
D-Serine	0.22 (0.19, 0.26)	4	0.15 (0.14, 0.16)	5	0.68	0.003	99 ± 9	113 ± 4
D-Cycloserine	3.2 (2.7, 3.9)	6	1.32 (1.26, 1.38)	4	0.41	0.002	93 ± 6	88 ± 4
R-HA-966	12 (11, 13)	4	4.6 (3.4, 6.2)	5	0.38	0.02	12 ± 1	14 ± 1
L-687 414	4.0[a] (3.4, 4.8)	5	1.1[a] (1.0, 1.2)	5	0.28	0.0002	5 ± 2	3 ± 1
ACBC	45 (43, 47)	4	6.6 (6.1, 7.1)	6	0.15	0.0001	13 ± 2	33 ± 7
Antagonist[a]								
7-Cl-KYNA	0.63 (0.59, 0.68)	4	0.20 (0.17, 0.22)	4	0.32	0.0001	0	0
L-701, 324	0.0050 (0.0037, 0.0049)	5	0.0048 (0.0043, 0.0053)	5	0.96	NS	0	0
L-689, 560	0.041 (0.039, 0.043)	4	0.038 (0.030, 0.049)	5	0.93	NS	0	0

n, Number of experiments; NS = $p > 0.05$.

TM3 and TM4 (14) were found to produce marked changes in glycine affinity but more modest effects on glycine-site antagonist affinities. These experiments suggest that there are fundamental differences in the binding domains of agonists and antagonists. The additional sites of interaction between the generally more bulky antagonist molecules (particularly so with L-689,560 and L-701,324) and the receptor may negate the presumably steric influences of NR2 subunits on agonist affinity. This does not explain why D-serine, a close structural analogue of glycine, had essentially the same affinity at both subunit assemblies. Furthermore, although all of the remaining glycine-site ligands studied, i.e., D-cycloserine, ACBC, R-HA-966, and L-687,414, were found to have a higher affinity for the NR1a/NR2B assembly, the ratio of affinities at the two subunit assemblies showed considerable variation. Taken together, these observations suggest a complex structure-activity relationship for ligand affinity at the glycine site on the NMDA receptor.

When D-cycloserine, ACBC, R-HA-966, and L-687,414 were compared with glycine, they behaved as glycine-site partial agonists. These glycine-site ligands showed varying levels of efficacy at NR1a/NR2A and NR1a/NR2B human recombinant receptors. Efficacy comparisons were complicated by the apparent bell-shaped nature of the glycine agonist concentration-response curves. Significantly, however, compounds with less intrinsic efficacy than glycine at recombinant receptors also behave as partial agonists at native rat NMDA receptors where, with the exception of D-cycloserine, bell-shaped concentration-response curves do not occur (10). We have not studied possible underlying reasons for the apparently premature curtailing of inward current, but the fact that we have not observed similar phenomena in our work with native NMDA receptors suggests that this may be a property peculiar to non-neuronal cells or may reflect the fact that recombinant NMDA receptors differ in their desensitization characteristics compared with native receptors expressed in neurons.

Comparison of recombinant and native NMDA receptor profiles. We previously suggested that the NMDA receptors expressed by cortical neurons have a predominantly NR1/NR2B composition (9, 10), whereas rat cerebellar granule cells, specifically those prepared from 5–7-day-old rat pups, have a predominantly NR1/NR2A subunit composition (10, 36). The ~1.8- and ~10-fold higher affinities of glutamate and glycine, respectively, for the NR1a/NR2B receptor compared with NR1a/NR2A agrees very closely with the observed differences in agonist affinity for the native receptors (i.e., ~2- and ~10-fold higher affinity for glutamate and glycine at cortical neurons compared with granule cells; Ref. 9). There also are clear parallels between the high and low affinity components of ifenprodil antagonism of NMDA responses on native receptors (10, 37) and the ~300-fold difference in affinity for human recombinant NR2A- and NR2B-containing receptors. The higher affinity of ifenprodil for NR2B-containing receptors has been reported previously for rat recombinant receptors (38).

In conclusion, these experiments demonstrate the usefulness of the L(tk−) cell line for the stable transfection and inducible expression of recombinant NMDA receptors comprising different subunit assemblies. Stable transfection offers the advantage of a potentially inexhaustible and homogeneous supply of recombinant receptors. This approach has enabled an extensive pharmacological profile to be compiled for human NR1a/NR2A and NR1a/NR2B subunit combinations and has revealed an overall similarity to rat recombinant and native NMDA receptors.

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