Functional and Pharmacological Differences Between Recombinant 
N-Methyl-d-Aspartate Receptors

STEFANO VICINI, 1 JIAN FENG WANG, 1 JIN HONG LI, 1 WEI JIAN ZHU, 1 YUE HUA WANG 2, JIAN HONG LUO 2, BARRY B. WOLFE, 2 AND DENNIS R. GRAYSON 3
1 Department of Physiology and Biophysics and 2 Department of Pharmacology, Georgetown University School of Medicine, Washington, DC 20007; and 3 Departments of Psychiatry and Anatomy and Neurobiology, Neurosciences Research Center, Allegheny Singer Research Institute, Allegheny University of the Health Sciences, Pittsburgh, Pennsylvania 15212

Vicini, Stefano, Jian Feng Wang, Jin Hong Li, Wei Jian Zhu, Yue Hua Wang, Jian Hong Luo, Barry B. Wolfe, and Dennis R. Grayson. Functional and pharmacological differences between recombinant N-methyl-d-aspartate receptors. J. Neurophysiol. 79: 555–566, 1998. N-methyl-d-aspartic acid (NMDA) receptors transiently transfected into mammalian HEK-293 cells were characterized with subunit-specific antibodies and electrophysiological recordings. Deactivation time course in response to fast L-glutamate pulses were studied in isolated and lifted cells, as well as in outside-out membrane patches excised from cells expressing recombinant NR1 subunits in combination with the NR2A, NR2B, NR2C, or NR2D NMDA receptor subunits. Transfected cells were preidentified by the fluorescence emitted from the coexpressed Aequorea victoria jellyfish Green Lantern protein. Currents generated by NR1/NR2A channels displayed double exponential deactivation time course being faster than that in NR1/NR2B or NR1/NR2C channels. However, a large decay variability was observed within each cotransfection, suggesting that mechanisms additional to subunit composition may also regulate deactivation time course. NR1/NR2D channels displayed slowly deactivating currents. Channel deactivation was fast and comparable among receptors obtained by cotransfecting five distinct spliced variants of the NR1 subunit, each with the NR2A subunit. Additionally, recovery from desensitization was slower for NR1/NR2B than for NR1/NR2A channels. The average deactivation time course of responses to brief L-glutamate applications in cells where NR1/NR2A/NR2B cDNAs were cotransfected at variable ratio was intermediate between those of the NR1/NR2A and NR1/NR2B channels. Although immunocytochemical evidence indicates that the majority of cells are cotransfected by all plasmids in triple transfection, our experimental condition did not allow for a tight control of the expression of NMDA receptor subunits. This produced the result that many cells were characterized by deactivation time course and haloperidol sensitivities of separate NR1/NR2A and NR1/NR2B subunit heteromers. We also speculate on the possible formation of channels resulting from the coassembly in the same receptor of NR1/NR2A/NR2B subunits from a minority of cells that gave responses to brief application of L-glutamate characterized by slow deactivation time course and decreased haloperidol sensitivity.

INTRODUCTION

Five genes encoding subunits of the N-methyl-d-aspartate (NMDA) receptor have been cloned from rat brain. These include several spliced forms of the NR1 subunit and the NR2A, NR2B, NR2C, and NR2D subunits (McBain and Mayer 1994; Zukin and Bennett 1995, for review). Although various forms of the NR1 subunit mRNAs are expressed in a wide spectrum of neuronal populations (Laurie and Seeburg 1994b; Laurie et al. 1995), the NR2 subunit mRNAs are more restricted in their distribution and show differential patterns of expression with respect to temporal events occurring during development (Monyer et al. 1994) and neuronal maturation (Vallano et al. 1996). The NMDA receptor plays an important role in synaptic plasticity during development (Sheetz and Constantine-Paton 1994). It is therefore possible that changes in subunit composition of native NMDA receptor may underlie changes in synaptic plasticity. Among the properties characterizing NMDA receptors, the deactivation time course of currents produced by brief L-glutamate transients is crucial to synaptic transmission because it underlies the kinetics of NMDA–excitatory postsynaptic currents (EPSCs) (Lester et al. 1990). Furthermore, it has been proposed that desensitization and recovery from desensitization may also affect the deactivation time course and thereby the duration of NMDA-EPSCs (Lester and Jahr 1992). Native NMDA receptors at excitatory synapses have been shown to have a faster time course with development (Carmignoto and Vicini 1992; Flint et al. 1997; Hestrin 1992). In parallel, several studies have shown that changes have been shown to be related to neuronal activity in vitro (Vallano et al. 1996) and in vivo (Carmignoto and Vicini 1992). Previous studies of recombinantly expressed NMDA receptors in HEK-293 cells show distinct offset decay kinetics of currents produced by NMDA applications to cells expressing binary combinations of NR1, NR2A, NR2B, and NR2D subunits (Kohr and Seeburg 1996; Kohr et al. 1994; Monyer et al. 1994) and have produced the initial evidence that receptors comprising NR1 and NR2A subunits are characterized by a faster deactivation time course. However, these studies were performed with long and unphysiological application times, and a systematic evaluation of the deactivation time course of recombinant NMDA receptor may allow for appropriate cross comparisons with native receptors. Additionally, novel and more selective antagonists that uniquely identify specific subunits are becoming available (Ilyin et al. 1996; Williams 1993). It should be feasible to employ these reagents in combination with other unique
identifiers to distinguish functional properties and to understand the effects of coexpression of multiple subunits in more complex receptor heteromers. In the present studies, we further characterize the deactivation time course of recombinant NMDA receptors of distinct subunit compositions in response to brief agonist applications. We also compare and contrast recovery from desensitization produced by prolonged L-glutamate applications between NR1/NR2A and NR1/NR2B transfected cells, and we investigate the deactivation time course obtained with a low-affinity agonist with these subunit combinations. Last, taking advantage of the demonstrated selective antagonism of NMDA receptors consisting of NR1/NR2B subunits by haloperidol (Ilyin et al. 1996), we report properties of heteromers obtained by coexpression of distinct cDNAs encoding combinations of the NR1, NR2A, and NR2B subunits.

**METHODS**

**NMDA receptor expression**

Throughout our work we use for simplicity the terminology of Sugihara et al. (1992) in defining the spliced forms of the NR1 subunits (NR1a, NR1b, NR1c, NR1e, and NR1g). According to the terminology of Hollmann et al. (1993), these subunits are designated as NR1-1α, NR1-1β, NR1-2α, NR1-4α, and NR1-4β. The 4.2-kb full-length NR1α (pNMDAR1-1α; genbank No. U08261) cDNA was directionally subcloned into the vector pUC/CMV (Invitrogen, Carlsbad, CA). The NR1β (pNMDAR1-1β; genbank No. U08263), NR1c (pNMDAR1-2α; genbank No. U08262), NR1e (pNMDAR1-4α; genbank No. U08267) and NR1g (pNMDAR1-4β; genbank No. U08268) were directionally subcloned into pcDNA I/Amp (Invitrogen). The 5.0-kb NR2A and NR2C cDNAs were symmetrically subcloned into the EcoRI site of pcDNA I/Amp. Both the parent NR2A and NR2C plasmids were a generous gift of Dr. Shigetada Nakanishi, Kyoto University Faculty of Medicine, Kyoto. The NR2B expression vector was a generous gift of Dr. Richard Huganir (Johns Hopkins University). The full-length NR2D clone, as modified to increase expression of the guanine and cytosine residues in the region coding for the N-terminal 80 amino acids of NR2D such that the amino acid sequence was not altered (Monyer et al. 1994), was a generous gift of Dr. P. Seeburg (University of Heidelberg, Germany). The NR1b, NR1c, NR1e, and NR1g plasmids were a generous gift of Dr. Jim Boulter (University of California, Los Angeles).

**KIDNEY EMBRYONIC CELL CULTURE AND CDNA TRANSFECTION.** Human embryonic kidney 293 cells (American Type Culture Collection, Rockville MD, ATCC No. CRL1573) were grown in minimal essential medium (MEM; Gibco BRL, Gaithersburg, MD), supplemented with 10% fetal bovine serum, 100 units/ml penicillin (Gibco BRL), and 100 units/ml streptomycin (Gibco BRL), in a 6% CO2 incubator. Exponentially growing cells were dispersed with trypsin, seeded at 2 × 10^4 cells/35-mm dish in 1.5 ml of culture medium and plated on 12-mm glass coverslips (Fisher Scientific, Pittsburgh, PA). HEK-293 cells were transfected with rat NMDA receptor subunit cDNAs using the calcium phosphate precipitation. Mixed plasmids (3 μg total) NR1a-c, e-g, and NR2A, 2B, 2C, or 2D were added to the dish containing 1.5 ml MEM culture medium for 12–16 h at 37°C under 3% CO2. The medium was then removed, and the cells were rinsed twice with culture medium and finally incubated in the same medium for 24–48 h at 37°C under 6% CO2. Studies on the recombinantly expressed receptors were performed within 2–3 days after transfection, and data were obtained for a given subunit combination transfected at least three different times. Cotransfection with pGreen-Lantern-1 (GIBCO, BRL), which encodes a fluorescent protein, allowed ready recognition of transfected cells expressing this marker. Approximately 90% of cells expressing the GreenLantern protein also expressed NMDA receptors.

**ANTIBODY PRODUCTION AND IMMUNOCYTOCHEMISTRY.** NR2A antibody production is previously described (Wang et al. 1995). A monoclonal antibody to NR2B was raised in mice by standard techniques. The antigen used was the same described in Wang et al. (1995). Briefly, primers were designed to amplify fragments encoding portions of the C-termini of the NR2A subunit and the NR2B subunit. The polymerase chain reaction fragments were gel purified and subcloned into the BamHI site of pGEX3B expression vector. The fusion proteins were expressed, purified, and used for the immunization of rabbits (NR2A) or mice (NR2B) by standard procedures. Antibodies were purified by affinity chromatography.

HEK-293 cells transiently cotransfected with the cDNAs encoding the NR1a and NR2A and/or NR2B subunits were cultured on poly-L-lysine-coated coverslips. Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; 100 mM sodium phosphate and 150 mM NaCl pH 7.4) for 10 min at room temperature. Cells were washed three times with PBS, and nonspecific sites were blocked using 10% normal goat serum in PBS containing 0.3% Triton X-100 for 20 min at room temperature. Cells were then incubated with the affinity-purified NR2A and NR2B antibodies for 1 h at room temperature, washed three times in PBS, and then incubated with goat anti-rabbit or anti-mouse [fluorescein isothiocyanate (FITC) or Texas Red-conjugated, Jackson Laboratories, West Grove, PA] secondary antibodies for 40 min at room temperature. The cells were washed three times in PBS, and the coverslips were briefly dipped into distilled water to avoid formation of crystals, mounted on slides using Vectashield (Vector Labs, Burlingame, CA) as mounting medium, and visualized using fluorescence microscopy. Photomicrographs were taken with an inverted Diaphot (Nikon, Melville, NY) fluorescent microscope with a ×10 magnification objective. Successful transfections were evaluated for the NR1a, NR2A, and NR2B subunits with the use of NMDA receptor subunit antibodies. Transfection efficiency was ~30%.

**Electrophysiology**

Transfected HEK-293 cells were studied at room temperature (20–22°C). The recording chamber was continuously perfused with bath solution (in mM) 145 NaCl, 5 KCl, 2 CaCl2, and 5 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)–NaOH (pH 7.4); osmolality was adjusted to 315–325 mosM with sucrose. The patch-clamp technique was used in the whole cell recording and outside-out patch recording configurations with a patch-clamp amplifier Axopatch 1D (Axon Instrument, Foster City, CA) after capacitance and series resistance compensation. Electrodes were pulled in two stages on a vertical pipette puller from borosilicate glass capillaries (Wiretrol II, Drummond, Broomall, PA). Typical pipette resistance was 5–7 MΩ. Intracellular (patch pipette) solutions contained (in mM) 145 CsCl, 5 MgCl2, 5 ethylene glycol-bis-(β-aminoethyl ether)-N,N,N’-N’-tetraacetic acid, 5 ATP, and 10 HEPES, pH 7.2 with CsOH.

**DRUG APPLICATIONS.** L-Glutamate and L-cysteate (Sigma, St. Louis, MO) stock solutions were diluted to their final concentrations in bath solution. For fast application of agonists, a piezoelectric translator (P-245.30 Stacked Translators, Physik Instrumente, Waldbronn, Germany) was used to rapidly move a double barrel theta tubing positioned in front of the excised patch to switch quickly from one barrel containing extracellular to the other with added 1 mM L-glutamate. After each patch recording, on and off rates as well as pulse durations were measured by ‘‘blowing out’’ the patch and recording currents generated by
the liquid junction potential due to a 50:1 dilution of the agonist containing solution (Lester and Jahr 1992). Application duration was also measured in small lifted cells in response to rapid application of extracellular solution with the addition of 10 mM KCl (see Fig. 2, inset). Unless otherwise noted, 10–90% rise and decay times of these currents were typically <0.5 ms. For fast application of L-glutamate, in addition to haloperidol, the solutions in the double barrel pipette were exchanged by means of solenoid valves connected to the tubing. Haloperidol (Sigma, St. Louis, MO) was added to both control and L-glutamate containing solutions. Dimethyl sulfoxide was used as vehicle at a maximal final concentration of 0.01%, which by itself did not modify the peak L-glutamate-induced currents.

**DATA ANALYSIS.** Currents were filtered at 1 kHz with an 8-pole low-pass Bessel filter (Frequency Devices, Haverhill, MA), digitized at 3 kHz using an IBM-compatible microcomputer equipped with a Digidata 1200 data acquisition board (Axon Instruments) and pClamp 6.3 software (Axon Instruments). Off-line data analysis, curve fitting, and figure preparation were performed with Origin (MicroCal Software, Northampton, MA) and pClamp software. Fitting of decay times of the averaged L-glutamate–activated currents was performed using a simplex algorithm based on a least-squares exponential fitting routine. Double exponential equations of the form $I(t) = I_f + I_s \times \exp\left(-\frac{t}{\tau_f}\right) + I_t \times \exp\left(-\frac{t}{\tau_t}\right)$, where $I_f$, $I_s$, and $I_t$ are the amplitudes of the fast and slow decay components, and $\tau_f$ and $\tau_t$ are their respective decay time constants used to fit the data. A comparison of the summed square deviation was used to estimate the quality of single versus double exponential fits. To compare decay time between different subunit combinations we used a weighted mean decay time constant $\tau_w = \left[I_f/(I_f + I_s)\right] \times \tau_f + \left[I_s/(I_f + I_s)\right] \times \tau_t$. Data values are expressed as means ± SE unless otherwise indicated; $P$ values represent the results of independent t-tests.

**RESULTS**

L-Glutamate applications to HEK-293 cells transfected with NMDA receptor subunit cDNAs

We studied L-glutamate–activated currents ($I_{\text{Glu}}$) elicited by rapid agonist applications with a piezoelectric translator as small (<7 pF), lifted HEK-293 cells expressing recombinant NMDA receptor heteromers. Transfected cells were voltage clamped at a holding potential of −50 mV and a solution containing 10 μM glycine, in which nominally Mg$^{2+}$-free was used. We also investigated, for comparison, $I_{\text{Glu}}$ recorded in outside-out excised membrane patches from transfected cells. The reason for this comparison was that often only a few channels were activated in excised patches, making assessment of deactivation quite hard. Brief pulses (1 ms) of L-glutamate (1 mM) in the extracellular solution elicited $I_{\text{Glu}}$ with relatively fast rise times and exponential decay both in small cells and in patches (Figs. 1–6). Averages of 5–10 consecutive responses produced by L-glutamate applications on small HEK-293 cells transfected with NR1a/NR2A, NR1a/NR2B, NR1a/NR2C, and NR1a/NR2D cDNAs are shown in Fig. 1A. Because currents decayed with double exponential kinetic, we chose to use the weighted mean decay time constants ($\tau_w$) deriving from a double exponential fitting (see METHODS) measured in all cells and patches investigated (Fig. 1B). With NR1a/NR2A channels, we observed significantly faster deactivation than with all other cDNA combinations studied. We therefore wanted to verify that substitution of the NR1a subunit with four distinct spliced forms (NR1b, NR1c, NR1e, and NR1g) did not alter the deactivation time constants. The results of this study showed (Fig. 1C) that the faster deactivation times observed with the NR1a/NR2A expressing cells were maintained with different alternatively spliced variants of the NMDA receptor subunit NR1.

A concern of our experimental strategy was that the speed of application in small lifted cells might have been slowed down by the larger cell surface as compared with patches. Figure 2A, inset, shows the average current produced by 1-ms applications of extracellular solution with 10 mM KCl added while recording from a small lifted cell. Although these responses were not as fast as those observed in excised patches, they were sufficiently rapid to produce a rise time <0.5 ms. The validity of data obtained in small lifted cells was further supported by comparing direct deactivation with excised patches (Fig. 1B). $\tau_w$ values were similar in outside-out patches as compared with those observed with small cells, being on average 54 ± 9 ms (mean ± SE; $n = 14$) versus 54 ± 4 ms ($n = 34$) for the NR1a/NR2A combination and 280 ± 28 ms ($n = 16$) versus 287 ± 22 ms ($n = 20$) for the NR1a/NR2B combination.

**Variability in the $I_{\text{Glu}}$ deactivation time constants**

For NR1a/NR2A or NR1a/NR2B channels, we observed a large scatter in $\tau_w$ values (Fig. 1, B and C), with a coefficient of variation of 0.41 for both subunit combinations. In Fig. 2A the detailed characterization of $I_{\text{Glu}}$ deactivation with exponential curves recorded in patches and cells is compared between NR1a/NR2A and NR1a/NR2B channels. Whereas with the NR1a/NR2A subunit combination the relative fractional contribution to peak amplitude of the fast component (%F) was very large, with the NR1a/NR2B channels it was faster and variable from cell to cell (Table 1). Furthermore, the average values of the fast and slow time constant ($\tau_f$ and $\tau_s$) describing the deactivation of $I_{\text{Glu}}$ were significantly slower for the NR1a/NR2A combination than for the NR1a/NR2B combination (Table 1). For both $\tau_w$ and fast and slow decay time constants, we observed significant scatter among the cells investigated. A possible reason for this scatter is seen in Fig. 3, which shows the currents recorded in four distinct patches from transfected cells that illustrate a quite different single channel current opening mode in each patch. In particular, in a few patches ($n = 3$ for NR1a/NR2A and $n = 5$ for NR1a/NR2B) such as *patch 1* in Fig. 3A and *patch 2* in Fig. 3B, NMDA channel openings were characterized by an extremely long-lasting burst of openings often lasting hundreds of milliseconds resulting in longer deactivation times. In most other patches, channel kinetics were more similar to those reported in studies of single channel currents recorded during stationary, long-lasting applications with clearly distinct shorter bursts of openings separated by closures. The reason for this difference is unclear, but it is not likely related to the reported acceleration of the deactivation in outside-out patches with time (Lester et al. 1993). In fact, we also observed time-dependent decreases of deactivation in both patches and small cells, but this was never >20%. Furthermore, our deactivation determinations were always performed during the first few minutes of recordings. Whenever possible, channel current am-
FIG. 1. Deactivation time course of recombinant N-methyl-D-aspartate (NMDA) receptors. 

A: NMDA channel currents activated by 1-ms applications of L-glutamate (1 mM) to small lifted HEK-293 cells transfected with distinct NMDA receptor subunit cDNAs. The average of 5–10 consecutive L-glutamate applications is shown together with the open tip current used to measure the duration of the drug application. The amplitude calibration bar does not apply to these traces.

B: summary scatter plot of the weighted decay time of deactivation ($t_w$) of average L-glutamate–activated currents in cells transfected with NR1a subunit cDNA combined with different NR2 subunit cDNAs. Filled symbols, currents measured in small lifted cells; open symbols, currents in outside-out patches. Bars indicate average values. Average values for NR1a/2A cotransfections were statistically significant with respect to the NR1a/NR2B, NR1a/NR2C, and the NR1a/NR2D cotransfection ($P < 0.05$, independent t-test).

C: summary scatter plot of the weighted decay time of deactivation of average NMDA channel currents recorded in cells transfected with different spliced forms of NR1 subunit cDNAs combined with NR2A subunit cDNAs. Filled symbols, currents measured in small lifted cells; open symbols, currents in outside-out patches. Bars indicate average values. Average values for NR1x/2A cotransfection were not significantly different.

Amplitude was measured in patches with the distinct opening modes. These values were $2.1 \pm 0.4 \text{ pA} (n = 4)$ versus $2.08 \pm 0.2 \text{ pA} (n = 12)$ for the NR1a/NR2A channel and $2.09 \pm 0.3 \text{ pA} (n = 5)$ versus $2.07 \pm 0.2 \text{ pA} (n = 13)$ for the NR1a/NR2B channel. Last, channel currents in successive patches pulled from a cell were characterized by similar opening mode (not shown).

Recovery from desensitization of currents with NR1a/NR2B and NR1a/NR2A receptors

Lester and Jahr (1992) originally proposed that recovery from desensitized states of NMDA receptors underlay the double exponential deactivation time course of NMDA currents. To assess the role of recovery from desensitization in determining the distinct deactivation between NR1a/NR2A and NR1a/NR2B heteromers, we studied the time course of recovery of the current induced by 7-ms saturating pulses of L-glutamate (Fig. 4). Superimposed averaged currents from outside-out patches in response to multiple-pulse applications are shown in Fig. 4, A and B. In Fig. 4C, a summary of the results obtained from all cells tested is shown together with the exponential fit of the time course for recovery. With NR1/NR2B subunits, recovery from desensitization was slower and similar to that observed with native receptors (Lester and Jahr 1992). Replacement of 2 mM CaCl$_2$ with 0.2 mM CaCl$_2$ in nine NR1a/NR2A cells and nine NR1a/NR2B cells did not alter the time course for recovery (data not shown). We also investigated for comparison recovery from desensitization using a low-affinity
agonist for the NMDA receptor, l-cysteate, which in native receptors exhibits a fast and complete recovery from desensitization (Lester and Jahr 1992). With both NR1a/NR2A and NR1a/NR2B heteromers, currents induced by 7-ms saturating paired pulses of l-cysteate (20 mM) were of equal amplitude when applied as close as 25 ms (data not shown). We then compared between nine NR1a/NR2A cells and nine NR1a/NR2B cells the extent of glycine-independent desensitization observed from applications of l-glutamate for 5 s after replacement of 2 mM CaCl₂ with 0.2 mM CaCl₂ to prevent Ca-induced inactivation. As illustrated in Fig. 5 (top panel), the current induced by l-glutamate showed a time-dependent decline in the continuous presence of the agonist. As previously reported in native receptors (Lester et al. 1993), the extent of desensitization increased with the time of recording, especially in excised membrane patches. We therefore compared desensitization observed during the first few minutes of recording. The ratio between the peak current at the beginning of the l-glutamate application and the current after 5 s was very variable from cell to cell (range 1–5) being 2.8 ± 1.4 (mean ± SD) for NR1a/NR2A channels and 1.9 ± 0.9 for NR1a/NR2B channels (mean ± SD). To investigate whether the distinct recovery from desensitization observed comparing NR1a/NR2A and NR1a/NR2B heteromers could completely underlie the distinct deactivation time course observed, we investigated the deactivation produced by single brief pulses of l-cysteate. As shown in Fig. 5 (bottom panels) deactivation of currents elicited by pulses of l-cysteate was faster than that of currents elicited by l-glutamate pulses of the same duration (Fig. 5, middle panels). On average the weighted decay time of deactivation was 10.1 ± 3.1 ms and 24.6 ± 6.5 ms for NR1a/NR2A and NR1a/NR2B heteromers, respectively (n = 5).

Taken together, these results suggest that, although slow recovery from desensitization may partially be responsible for the slow deactivating currents observed with the NR1a/NR2B heteromers, differences in deactivation time constants using a poorly desensitizing agonist, l-cysteate, indicates that other factors must also be involved in deactivation.

**TABLE 1. Deactivation time course of recombinant NMDA receptors**

<table>
<thead>
<tr>
<th>NR1a/NR2A:NR2B</th>
<th>τᵢ, ms</th>
<th>τᵣ, ms</th>
<th>%F</th>
<th>Inhibition, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1:0</td>
<td>33 ± 12*</td>
<td>247 ± 139*</td>
<td>90 ± 8 (59)*</td>
<td>4 ± 16 (24)*</td>
</tr>
<tr>
<td>1:1:1</td>
<td>43 ± 24*</td>
<td>387 ± 206*</td>
<td>79 ± 18 (26)*</td>
<td>25 ± 25 (18)*</td>
</tr>
<tr>
<td>1:1:3</td>
<td>64 ± 37</td>
<td>413 ± 226</td>
<td>67 ± 24 (50)*</td>
<td>34 ± 33 (31)*</td>
</tr>
<tr>
<td>1:1:0</td>
<td>80 ± 35</td>
<td>489 ± 175</td>
<td>62 ± 19 (16)</td>
<td>61 ± 34 (12)</td>
</tr>
<tr>
<td>1:1:1</td>
<td>71 ± 37</td>
<td>538 ± 347</td>
<td>42 ± 29 (33)</td>
<td>78 ± 10 (18)</td>
</tr>
</tbody>
</table>

Values are means ± SD with number of cells in parentheses. A summary of deactivation time course of currents activated by l-glutamate (1 mM, 1 ms) in outside-patches and small lifted HEK-293 cells transfected with combinations of NR1a, NR2A, and NR2B N-methyl-D-aspartate (NMDA) receptor subunit cDNAs. The average of 5–10 consecutive l-glutamate applications was fitted with double exponential curves with decay time constants τᵢ and τᵣ. %F indicates the relative proportion of the fast component to the peak current amplitude and Inhibition is the percent inhibition of peak amplitude induced by 50 μM haloperidol. The number of cells and patches where both haloperidol reduction and kinetics were measured is less than the total number of cells because often the cell was blown off by the flow during or after the haloperidol application of full recovery was not obtained. * Average values that were statistically significant with respect to those of the 1:0:1 combination (P < 0.05 analysis of variance followed by Newman-Keuls test).
FIG. 3. Channel currents in patches from transfected cells. NMDA channel currents activated by 1-ms application of L-glutamate. Consecutive responses (left), and the average current (right) of 10 consecutive L-glutamate applications shown with an indication of the weighted decay time in 4 distinct outside-out membrane patches excised from HEK-293 cells expressing NR1a/NR2A (A) and NR1a/NR2B (B) channels. The open tip current used to measure the duration of the drug application pulse is shown above the 1st channel current trace in each panel. The amplitude calibration bar does not apply to these traces.

l-Glutamate application to HEK-293 cells transfected with NR1a/NR2A/NR2B cDNAs

To allow comparison of deactivation time course between native and recombinant receptors, one has to consider reports that were indicating that a proportion of native receptors comprise heteromers of NR1/NR2A/NR2B subunits (Chazonot et al. 1994; Luo et al. 1997; Sheng et al. 1994). We therefore investigated functional and pharmacological properties of NMDA responses in HEK-293 cells cotransfected with NR1/NR2A/NR2B subunit cDNAs. To dissect out the relative contribution of distinct NMDA receptor heteromers, we took advantage of the observed fast deactivation time course of NR1/NR2A channels and the reported selective blockade of NR1/NR2B channels with haloperidol (Ilyin et al. 1996). We initially verified that haloperidol (50 μM) significantly inhibited the peak I_Glu recorded from cells and patches from cells expressing NR1/NR2B channels (Table 1). In addition to the lack of blockade by haloperidol on receptors produced with NR1/NR2A channels (Table 1), we failed to observe inhibition by haloperidol of I_Glu produced by NR1/NR2C and NR1/NR2D channels (2 ± 1%, n = 12, and 1.6 ± 1%, n = 11, respectively). Haloperidol inhibition of fast I_Glu was similar in outside-out membrane patches and in small lifted cells. As reported previously (Ilyin et al. 1996), haloperidol at higher concentrations not only decreases the channel open probabilities, but also produces a decrease in the channel dwell times in patches from NR1a/NR2B transfected cells. We also observed similar changes in the channel dwell times for the NR1a/NR2B channels and observed a comparable effect with the NR1a/NR2A channels (not shown).

We then investigated functional and pharmacological properties of NMDA responses in HEK-293 cells after trans-
DISCUSSION

We used brief pulses of L-glutamate to mimic synaptic transmission on receptors of defined composition with the aim to correlate functional properties of recombinant NMDA receptors with their composition. The availability of specific

| Table 1. | $I_{\text{glu}}$ in cells transfected with uneven ratios of subunits had time constants with average values intermediate between those seen with binary subunit transfections and those seen with 1:1:1 subunit ratios (Table 1).

To assess the success of multiple cDNA transfection, we also studied NR1/NR2A/NR2B transfected HEK-293 cells with the use of specific antibodies for NR2A subunit raised in rabbits (primary antibody) and Texas Red anti-rabbit secondary antibody, as well as specific antibodies for NR2B subunit raised in mice (primary antibody) and FITC anti-mouse secondary antibody. Photomicrographs of cells transfected in these conditions showed that many cells expressing the NR2A subunit also expressed NR2B subunits (Fig. 7A), indicating that the majority of cells are cotransfected by all three subunits. For example cell 1 showed fast deactivation time course and haloperidol sensitivities characteristic of individual NR1/NR2A or NR1/NR2B heteromers. For example cell 1 showed fast deactivation time course and haloperidol sensitivities similar to NR1/NR2A channels, whereas cells 2 and 3 were characterized by slower $\tau_w$ values and haloperidol sensitivities similar to NR1/NR2B channels. Interestingly, some cells, like the example of cell 4, showed slow deactivation time course similar to NR1/NR2B channels but no haloperidol sensitivity like that of NR1/NR2A channels. As illustrated in Fig. 7, the $\tau_w$ of $I_{\text{glu}}$ were always faster in the presence of haloperidol as compared with controls. The coefficient of variation associated with the decay time measured in these cells was 0.75, significantly greater than that obtained for NR1/NR2A and NR1/NR2B channels. With NR1a/NR2A/NR2B triple combinations, the deactivation time constants were clearly best described by two exponential curves in most cells. The relative fractional contribution to peak amplitude of the fast component and the average fast and slow time constants ($\tau_f$ and $\tau_s$) describing the deactivation of $I_{\text{glu}}$ are reported in Table 1. $I_{\text{glu}}$ in cells transfected with uneven ratios of subunits had time constants with average values intermediate between those seen with binary subunit transfections and those seen with 1:1:1 subunit ratios (Table 1).

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mediated currents (Lester and Jahr 1992). In addition however, the high affinity for the agonist, L-glutamate, also plays a large role in producing long-lasting NMDA receptor-mediated responses at synapses in the CNS as well as in excised membrane patches (Lester and Jahr 1992). We therefore assessed the speed of entry into, as well as the recovery from desensitized states of recombinant NMDA receptors endowed with distinct deactivation time course, namely the NR1a/NR2A and the NR1a/NR2B heteromers. We also compared the current deactivations produced by pulses of a lower affinity agonist, L-cysteate, between these heteromers. The slow exponential component of deactivation was remarkably larger with NR1a/NR2B transfection. This result supports the hypothesis that slow recovery from desensitization is partially responsible for the slow deactivating currents observed with the NR1a/NR2B heteromers. At the same time, however, factors other than recovery from desensitization may be involved since significant differences in both deactivation time constants were observed between NR1a/NR2A and NR1a/NR2B channels with both L-glutamate and L-cysteate as agonists. Among these factors, discrete opening and burst kinetics are unlikely candidates since major differences were not observed between these recombinant receptor pairs (Stern et al. 1992). A more likely factor may be the distinct affinities of recombinant NMDA receptors for L-glutamate that were modestly, but significantly, lower for the NR1a/NR2A heteromers (Laurie and Seeburg 1994a; Priestley et al. 1995).

Deactivation time courses are essential in determining the duration of NMDA-EPSCs (Lester and Jahr 1992), and most NMDA-EPSCs investigated in brain slices or in primary neuronal cultures show biexponential kinetics with variable proportions of the fast and slow components (Carmignoto and Vicini 1992; Flint et al. 1997; Kirson and Yaari 1996; Lester and Jahr 1992; Plant et al. 1997; Spruston et al. 1995). Similarly, rapid applications of L-glutamate on patches excised from neurons in culture or from brain slices exhibited double exponential deactivation kinetics (Carmignoto and Vicini 1992; Lester and Jahr 1992; Spruston et al. 1995). Our results with recombinant receptors demonstrate that double exponential deactivation time course in the range of those reported for native receptors could be observed even with the binary NR1a/NR2B transfection. Interestingly, with channels formed from NR1 and NR2A subunits, we observed extremely fast deactivating responses, that do not appear to directly correlate with those reported for native receptor assemblies. Recent analyses of synaptic currents recorded from cerebellar neurons of transgenic mice lacking the NR2A gene resulted in NMDA-EPSCs with slower deactivation times than those obtained from wild type mice (Takahashi et al. 1996). At the same time, Ebralidze et al. (1996) have shown that NMDA-EPSCs in cerebellar neurons were faster in transgenic mice lacking the NR2C subunit than in wild type mice. A possible explanation for the difference in deactivation time constants between recombinant NR1/NR2A channels and native receptors is that, in vivo, “pure” NR1/NR2A channels are likely to be less prominent than the other heteromeric combinations that produce longer deactivation time constants. Recently, two reports have attempted to correlate the presence of mRNA for NMDA re-

Antibodies for NMDA receptor subunits NR2A and NR2B (Wang et al. 1995) made possible the histochemical and electrophysiological verification of the success of the transient transfection of NMDA receptor subunits in the HEK-293 cells. This approach allowed us to verify that following cDNA transfection, cells were expressing NMDA receptor subunit proteins and at the same time provided further confirmation (Wang et al. 1995) that the antibodies we used were specific for distinct subunits. In previous electrophysiological studies of recombinant NMDA receptors expressed in HEK-293 (Kohr and Seeburg 1996; Kohr et al. 1994; Monyer et al. 1994), the subunit dependence of offset kinetics after long L-glutamate steps was similar to our results with respect to deactivation times after brief L-glutamate pulses. That is, both were fastest with NR1/NR2A channels and slowest with NR1/NR2D channels. However, some reported values were slower than those we observed. Several reasons may account for this discrepancy. Among them, recordings obtained from transfected whole cells may be slower because of the reduced agonist application rate. Additionally, the deactivation offsets measured after long pulses may very well be slower than that observed after a brief L-glutamate pulse, by analogy with what has been shown for y-aminobutyric acid-A receptors (Jones and Westbrook 1995).

NMDA receptor desensitization has been hypothesized to be partially involved in the deactivation of NMDA receptor-mediated currents (Lester and Jahr 1992). In addition however, the high affinity for the agonist, L-glutamate, also plays a large role in producing long-lasting NMDA receptor-mediated responses at synapses in the CNS as well as in excised membrane patches (Lester and Jahr 1992). We therefore assessed the speed of entry into, as well as the recovery from desensitized states of recombinant NMDA receptors endowed with distinct deactivation time course, namely the NR1a/NR2A and the NR1a/NR2B heteromers. We also compared the current deactivations produced by pulses of a lower affinity agonist, L-cysteate, between these heteromers. The slow exponential component of deactivation was remarkably larger with NR1a/NR2B transfection. This result supports the hypothesis that slow recovery from desensitization is partially responsible for the slow deactivating currents observed with the NR1a/NR2B heteromers. At the same time, however, factors other than recovery from desensitization may be involved since significant differences in both deactivation time constants were observed between NR1a/NR2A and NR1a/NR2B channels with both L-glutamate and L-cysteate as agonists. Among these factors, discrete opening and burst kinetics are unlikely candidates since major differences were not observed between these recombinant receptor pairs (Stern et al. 1992). A more likely factor may be the distinct affinities of recombinant NMDA receptors for L-glutamate that were modestly, but significantly, lower for the NR1a/NR2A heteromers (Laurie and Seeburg 1994a; Priestley et al. 1995).

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FIG. 5. Desensitization of recombinant NMDA receptors. NMDA channel currents activated by rapid application of L-glutamate and L-cysteate. Top: average current of 5 consecutive L-glutamate applications for 5 s are compared between small cells expressing NR1a/NR2A channels (A), and NR1a/NR2B channels (B). Average current produced by L-glutamate (middle) or L-cysteate (bottom) applications for 1 ms are compared between outside-out patches expressing NR1a/NR2A (A), and NR1a/NR2B (B) channels. Averages of at least 5 consecutive responses; L-glutamate applications are shown with the weighted decay time. The open tip current used to measure the duration of the drug application pulse is shown above each current trace. The amplitude calibration bar does not apply to these traces.
FIG. 6. Haloperidol fails to inhibit slow deactivating currents in cells expressing NR1a/NR2A/NR2B heteromers. Average of 5–10 consecutive responses produced by L-glutamate (1 mM, 1 ms) applications on 4 small HEK-293 cells transfected with NR1a/NR2A/2B cDNAs are shown (A–D, left) with an indication of $\tau_w$. Fast application of L-glutamate, in addition to haloperidol, was obtained by exchanging the solutions in the double barrel pipette (A–D, middle). Haloperidol (50 $\mu$M) was added in both control and L-glutamate–containing solutions. B and C, right: response obtained in the presence of haloperidol (same traces in the middle panel) at an expanded scaling with an indication of the decay time. In each panel, equal calibration bars apply to both left and middle traces.

Receptor subunit with the decay kinetics of NMDA-EPSCs in neurons in brain slices (Flint et al. 1997; Plant et al. 1997). The results of these studies demonstrate that the abundant presence of the NR2A subunit shortens NMDA-EPSC duration in developing hippocampal neurons (Flint et al. 1997), but it is not per se sufficient to produce fast deactivation time course in medial septal neurons (Plant et al. 1997). Interestingly, in septal neurons, whereas the variable sensitivity to blockade by ifenprodil, indicates the presence of heteromers of distinct NMDA receptor subunit, the decay kinetics of NMDA-EPSCs seems dominated by the NR2B subunit. In addition, Luo et al. (1997) have recently reported that, in the adult rat cerebral cortex, the amount of assembled NR1/NR2A binary complex is quite small relative to NR1/NR2B and NR1/NR2A/NR2B complex.

A total of eight isoforms of the NR1 cDNA is generated by alternative splicing of the primary RNA transcript (Anantharam et al. 1992; Sugihara et al. 1992; Zukin and Bennett 1995). The various spliced mRNAs differ with respect to the presence or absence of one of three exons, exons 5, 21, and 22 in the rodent (Hollmann et al. 1993), which results in the formation of NR1 subunits containing one or more of the exon-encoded cassettes. Although considerable effort has been expended to determine the differential localization of the NR1 mRNA splice variants in the rat brain (Laurie and Seeburg 1994; Laurie et al. 1995), the precise functions of each cassette with respect to the unique properties they may confer (either alone or in combination) to receptors containing each of the eight specific NR1 subunits is currently not completely understood, although a variety of NMDA receptor properties have been shown to vary with the different splice variants (Zukin and Bennett 1995 for review). We therefore wanted to verify whether different forms of alternatively spliced NR1 subunits combined with the NR2A subunit were capable of altering the fast deactivation time course observed with NR1a/NR2A. We selected the NR1b
FIG. 7. Summary of the results obtained with recombinant NR1/NR2A/NR2B receptors. A: NMDA receptor subunits, NR1a/NR2A/NR2B plasmid cDNAs were transiently cotransfected in mammalian HEK-293 cells at different ratios. The transfection was evaluated with the use of specific antibodies for NR2A subunit raised in rabbits (primary antibody) and Texas Red anti-rabbit secondary antibody, as well as specific antibodies for NR2B subunit raised in mice (primary antibody) and fluorescein isothiocyanate anti-mouse secondary antibody. Microphotographs width 500 μm. B: plots of the weighted decay times of average I_{Glu} recorded from HEK-293 cells in cells transfected with combinations of NR1a together with NR2A and NR2B cDNAs in different proportions in function of the percent control response with 50 μM haloperidol. Filled circles identify cells where we observed a >40% reduction of the peak current.

The finding that the current deactivation time course from recombinant NMDA receptors is subunit dependent may be pivotal to investigations of native NMDA receptors in the CNS. At present, however, the critical question of the actual composition of native NMDA receptors remains unanswered, although immunoprecipitation studies show that heteromers can be formed with various subunit mixtures (Chaytor et al. 1994; Luo et al. 1997; Sheng et al. 1994). Therefore the findings of specific kinetic properties associated with specific subunit composition must be considered with respect to properties deriving from mixed NR2 subunit heteromers.

To address this problem, we focused on NMDA receptors formed from the cotransfection of the NR1 with the NR2A and the NR2B subunit cDNAs, because receptors comprising...
coassembly of these subunits have been demonstrated to exist in the brain (Chazot et al. 1994; Luo et al. 1997; Sheng et al. 1994). We confirmed the reported selective inhibition of NR1/NR2B channels by haloperidol (Ilyin et al. 1996), and we took advantage of this property to study the kinetic properties and haloperidol sensitivity deriving from transfections with ternary combinations of subunit cDNA. The average deactivation time course of responses to brief L-glutamate applications to cells triply transfected with NR1/NR2A/NR2B cDNAs are intermediate between those of NR1/NR2A and NR1/NR2B channels and are in agreement with the recent report of offset kinetics of long agonist application by Kohr and Seeburg (1996) on recombinant receptors of similar composition. The electrophysiological results with ternary cDNA transfections show the presence of cells with deactivation time course and haloperidol sensitivities of NMDA receptors characteristic of binary combinations of heteromers comprising, respectively, the NR1/NR2A and NR1/NR2B subunit combinations. This happens in spite of the finding that the majority of cells are cotransfected by all plasmids. We believe this is the case for two reasons. First, when NR1/NR2A/NR2B transfections are investigated separately with the two fluorochromes we used, a cell labeled “red” was most often a cell with a small proportion of green fluorescence and vice versa. Second, the number of cells with functional NMDA receptors that also express the green fluorescent protein is >90% in electrophysiological experiments, indicating largely successful incorporation of all plasmids used. The electrophysiological output of a given cell most likely results from the majority of receptor type (NR1a/NR2A, NR1a/NR2B or NR1a/NR2A/NR2B) expressed in a given cell. Thus in addition to cells that express receptors that are functionally identical to either NR1/NR2A or NR1/NR2B type receptors, we also observed cells that gave responses to brief application of L-glutamate characterized by slow deactivation time course and decreased haloperidol sensitivity. This may relate to the formation of heteromers resulting from the coassembly in the same receptor of NR1/NR2A/NR2B subunits (ternary combination). However, the relative proportion of cells with these characteristics is rather low in our dataset. It is possible that only when the majority of receptors in a cell or an excised membrane patch is a ternary combination, does the formation of NR1/NR2A/NR2B heteromers become detectable electrophysiologically. Furthermore, because our experimental conditions did not allow for a tight control of the expression of NMDA receptor subunits, our results may not apply to neurons. An additional complication to the interpretation of our results is the unknown stoichiometry of subunit copies present in recombinant heteromers formed with NR1/NR2A/NR2B subunit. Further studies with means that may better control subunit assembly in the receptor complex will be necessary to resolve these complex issues.

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