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Glycine Modulates Ethanol Inhibition of Heteromeric *N*-Methyl-D-Aspartate Receptors Expressed in *Xenopus* Oocytes

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SUMMARY

Ethanol inhibits *N*-methyl-D-aspartate (NMDA) receptor-mediated responses at pharmacologically relevant concentrations, suggesting that inhibition of NMDA receptors may underlie some of the actions of ethanol in the central nervous system. We examined the ability of glycine to modulate ethanol inhibition of four recombinant heteromeric NMDA receptors (NR1a/NR2A through NR2D) expressed in *Xenopus* oocytes. Ethanol dose-response analysis revealed enhanced inhibitory efficacy of ethanol in the presence of subsaturating glycine concentrations at the NR1/NR2A, NR1/NR2C, and NR1/NR2D receptors. When assayed over a range of glycine concentrations, ethanol exhibited both glycine-reversible and glycine-independent inhibition of NMDA receptors. In contrast, ethanol inhibition of recombinant NMDA receptors was independent of NMDA concentration. Glycine reversal of ethanol inhibition suggested that ethanol might lower the affinity of glycine for the NMDA receptor and thereby decrease response magnitude. Consistent with this hypothesis, ethanol significantly reduced glycine affinity at NR1/NR2A and NR1/NR2C receptors. Evaluation of the glycine-independent component of ethanol inhibition demonstrated that in the presence of saturating concentrations of glycine, the NR1/NR2A and NR1/NR2B receptors were more sensitive to ethanol than the NR1/NR2C and NR1/NR2D receptors. Activation of the NR1/NR2D heteromers by NMDA and low concentrations of glycine elicited responses characterized by an initial peak followed by a lower-amplitude plateau response, which is consistent with glycine-sensitive desensitization as previously described for native NMDA receptors. In addition, nondesensitizing NR1/NR2B responses elicited in the presence of subsaturating concentrations of glycine were frequently converted into desensitizing responses by the addition of ethanol, an effect that was reversed with increasing glycine concentrations. The ability of ethanol to promote glycine-sensitive desensitization further suggests an interaction between glycine and ethanol inhibition of the NMDA receptor. Taken together, the results of the present report demonstrate that ethanol inhibition of NMDA receptors has both glycine-reversible and glycine-independent components, suggesting two distinct molecular mechanisms for ethanol inhibition of NMDA receptors.

NMDA receptors play an important role in synaptic transmission and neural plasticity at many sites in the mammalian central nervous system (1) and contribute to epileptiform activity and neuronal cell death in a number of experimental and pathological conditions (2). Two NMDA receptor subunit families (NR1a through NR1h and NR2A through NR2D) have been identified in rat (3–6) and mouse (7–10). Alternative splicing of a single NR1 gene generates eight subunit isoforms with distinct functional properties (11, 12). Heterogeneity within the NR2 subunit family results from expression of four closely related but distinct genes (4–6, 7–10). Native NMDA receptors are believed to be heteromeric complexes, formed from combinations of NR1 and NR2 subunits, with the NR2 subunit imparting distinct functional and pharmacological properties (4, 6, 8, 9, 13).

NMDA receptors have been implicated in mediating some of the central actions of ethanol (14–16). Ethanol specifically inhibits native NMDA receptors at concentrations that are pharmacologically relevant (14, 15, 17, 18), although the precise mechanism by which ethanol inhibits the NMDA receptor has yet to be determined. The actions of ethanol at the NMDA receptor may be modulated by glycine. Glycine has been shown to reverse ethanol inhibition of NMDA receptors in certain preparations, including inhibition of NMDA-mediated responses in cultured cerebellar neurons (15, 19, 20). However, other investigators have reported that glycine does not reverse ethanol inhibition of native NMDA receptors (21, 22). We examined the ability of glycine to modulate ethanol inhibition of heteromeric NMDA receptors expressed in *Xenopus* oocytes and now report that ethanol inhibition of NMDA receptors displays both glycine-reversible and glycine-independent components. In addition, we

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ABBREVIATIONS:: NMDA, *N*-methyl-D-aspartate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

demonstrate that glycine-sensitive desensitization of heteromeric NMDA receptors can be modulated by ethanol.

Materials and Methods

cDNAs. pN60 containing the 4.2-kb NR1 subunit cDNA (3) inserted into pBluescript (Stratagene, La Jolla, CA) was the generous gift of Dr. Shigetada Nakanishi (Kyoto University Faculty of Medicine, Kyoto, Japan). This cDNA corresponds to the predominant splice variant found in the central nervous system, NR1a (11). cDNAs encoding the NR2A, NR2C (4), and NR2D (5) were the generous gift of Dr. Peter Seeburg (University of Heidelberg, Heidelberg, Germany). The NR2D subunit cDNA was subcloned into pcDNA1/Amp (Invitrogen, San Diego, CA). The NR2B [5′UTR]cDNA was the generous gift of Drs. Dolan Pritchett and David Lynch (University of Pennsylvania, Philadelphia, PA) and contains the NR2B subunit cDNA (4) from which most of the upstream 5′ untranslated sequences have been removed to allow more efficient translation in *Xenopus* oocytes.

***In vitro* transcription.** Plasmids were linearized with *Not*I (NR1), *Eco*RI (NR2A, NR2C, NR2D), or *Sal*I (NR2B) and transcribed *in vitro* with T3 (NR2A, NR2C), SP6 (NR2B), or T7 (NR1, NR2D) RNA polymerase with the use of the Message Machine transcription kit (Ambion Inc., Austin, TX).

Translation in *Xenopus* oocytes. Oocytes were removed from mature female *Xenopus laevis* (Xenopus One, Ann Arbor, MI) and dissociated in 2 mg/ml collagenase (Type IA, Sigma Chemical Co., St. Louis, MO) in Ca^{2+}-free OR-2 (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6), and the remaining follicle layer was removed manually. Isolated stage V and stage VI oocytes were maintained in ND-96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6) supplemented with 2.5 mM sodium pyruvate and 50 mg/ml gentamicin. NMDA receptor subunit RNAs were dissolved in sterile distilled H_2O. NR1 and NR2 RNAs were mixed in a molar ratio of either 1:1 or 1:3 to minimize the formation of NR1 homomers. Fifty nanoliters of the final RNA mixture were microinjected (15–30 ng total) into the oocyte cytoplasm as previously described (13). Oocytes were incubated in ND-96 solution at 17° before electrophysiological assay (2–8 days). Where indicated, oocytes were injected with 50 nl of BAPTA (40 mM; Sigma) to chelate intracellular Ca^{2+} at 3–6 hr before recording (23).

Electrophysiology. Electrophysiological responses were measured with a standard two-microelectrode voltage-clamp (model OC-725A oocyte clamp, Warner Instruments, Hamden, CT). Electrodes were filled with 3 M KCl and had resistances of 0.6–3 MΩ. The recording chamber was continuously perfused with 116 mM NaCl, 2 mM KCl, 2 mM $BaCl_2$, and 5 mM HEPES, pH 7.4 (Ba^{2+}-Ringer solution), and all drugs were dissolved in the same solution. Resting potentials were more than −20 mV in the presence of Ba^{2+}, corresponding to resting potentials of more than −30 mV in Ca^{2+} buffers.[1] Electrophysiological recordings were performed in Ba^{2+}-Ringer solution to minimize the contribution of the Ca^{2+}-activated Cl^- current to the whole-cell current response to NMDA (24). Although the magnitude of the current responses to bath application of NMDA and glycine in Ba^{2+}-containing buffers was smaller than that observed in the presence of Ca^{2+}, the degree of inhibition of NMDA-mediated responses by ethanol was not affected (data not shown; see also Ref. 25). Response magnitude was determined by the steady state plateau response elicited by bath application of 100 μM NMDA (unless otherwise indicated) and the indicated glycine concentration at a holding potential of −60 mV. The presence of the plateau response was taken as an indication of a lack of significant activation of the endogenous Cl^- current by Ba^{2+} in these cells. The effects of 100 mM ethanol (0.5% or 500 mg/dl, unless otherwise indicated) on the steady state current response was determined by coapplication of ethanol with NMDA and glycine. Because the different heteromers show variability in the level of NMDA receptor expression, coupled with the inherent variability among frogs in expression, electrophysiological recordings were conducted on different days (1–5 days after injection) to allow similar response magnitudes (Fig. 1). Response amplitudes for the four heteromers were usually 50–250 nA. Attempts were made to keep response magnitudes within this range to minimize activation of the endogenous Cl^- current. Dose-response analysis for glycine or NMDA was determined in the presence of 100 μM NMDA or 10 μM glycine, respectively, and was fit (GraphPad Prism, ISI Software, San Diego, CA) according to the following equation: $I = I_{max}/[1 + (EC_{50}/A)^n]$, where I is the current response, I_{max} is the maximal current response, n is the Hill coefficient, A is the agonist concentration, and EC_{50} is the agonist concentration producing a half-maximal response. Data were analyzed by analysis of variance followed by the Tukey-Kramer *post hoc* multiple-comparisons test or by Student's t test (Instat, ISI Software, San Diego, CA).

Results

Fig. 1 shows the effect of 100 mM ethanol on current responses to bath application of 100 μM NMDA and 10 μM glycine in individual oocytes expressing heteromeric NMDA receptors. Dose-response analysis of ethanol inhibition of recombinant NMDA receptors expressed in *Xenopus* oocytes

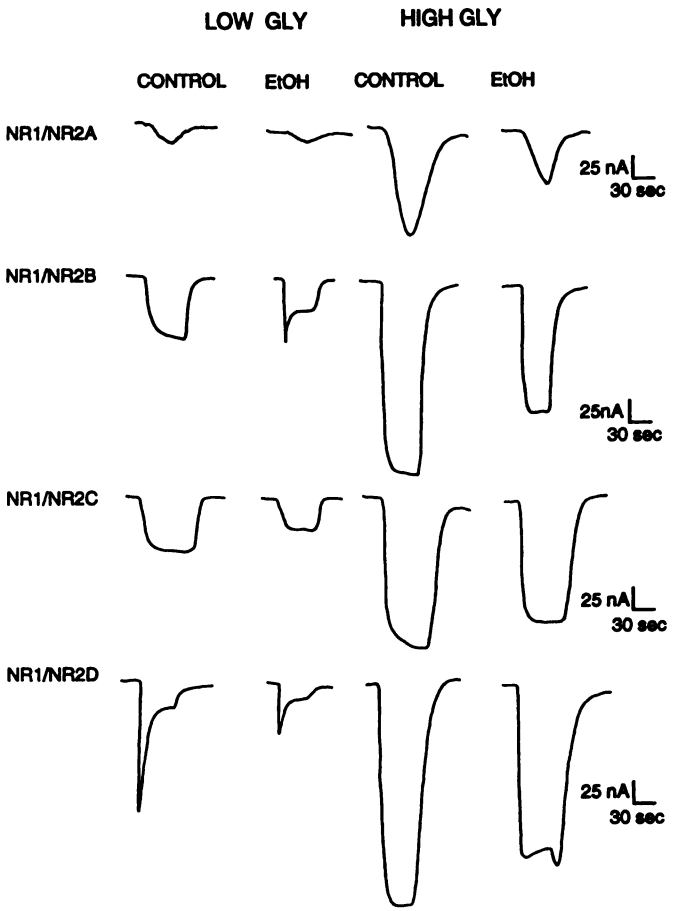

Fig. 1. Recombinant heteromeric NMDA receptors expressed in *Xenopus* oocytes are inhibited by ethanol. Shown are current traces from individual oocytes expressing the indicated heteromeric NMDA receptor. *CONTROL*, Response in the absence of ethanol. *EtOH*, Response in the presence of 100 mM ethanol. Current responses were obtained by bath application of 100 μM NMDA and 10 μM glycine (*HIGH GLY*) or 100 μM NMDA and 1 μM glycine (NR1/NR2A-C) or 0.03 μM glycine (NR1/NR2D) (*LOW GLY*).

[1] R. A. Morrisett, H. C. Larson, and D. T. Monaghan, unpublished observations.

revealed that ethanol inhibition was enhanced in the presence of low (subsaturating) concentrations of glycine (Fig. 2). This effect was particularly pronounced for the NR1/NR2C and NR1/NR2D heteromers, suggesting glycine reversal of ethanol inhibition.

To determine whether ethanol inhibition of NR1/NR2 heteromers was reversible by glycine, we assayed ethanol inhibition of the four heteromeric NMDA receptors over a range of glycine concentrations. As illustrated in Fig. 3, increasing glycine concentrations reduced the percent inhibition by ethanol at NR1/NR2 heteromers, indicating glycine-reversible ethanol inhibition. This effect was most pronounced at the NR1/NR2A, NR1/NR2C, and NR1/NR2D heteromers. In contrast, inhibition of NR1/NR2B receptors by ethanol appeared to be less dependent on glycine concentration (Fig. 3), although the data indicate a marked trend between subsaturating glycine concentrations and enhanced NMDA receptor inhibition by 100 mM ethanol. The inhibition of maximal current responses elicited by 100 μM NMDA and 10 μM glycine by 100 mM ethanol (Figs. 3 and 4) indicates that saturating concentrations of glycine did not completely reverse the inhibition of the recombinant NMDA receptors, which is consistent with an additional glycine-insensitive component to the inhibition of NR1/NR2 heteromers by ethanol. In contrast, inhibition of NR1/NR2 heteromers by ethanol was independent of NMDA concentration; inhibition of the recombinant NMDA receptors by ethanol was not significantly altered by increasing concentrations of NMDA in the presence of 10 μM glycine.

The interaction between ethanol and glycine at heteromeric NMDA receptors suggests that ethanol may act as a competitive inhibitor of glycine at these heteromers, thereby reducing the magnitude of the observed response. Therefore, ethanol would be expected to decrease the apparent affinity

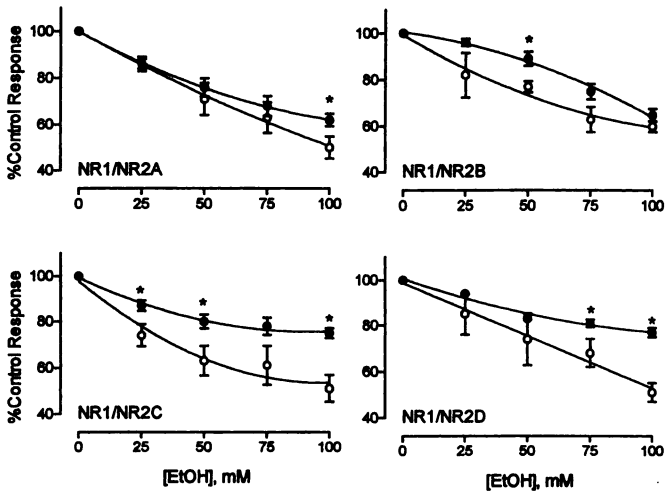

Fig. 2. Dose-response curves for ethanol (*EtOH*) inhibition of heteromeric NMDA receptors expressed in *Xenopus* oocytes. ●, Current responses determined in the presence of 100 μM NMDA and 10 μM glycine. ○, Current responses determined in the presence of 100 μM NMDA and 1.0 μM glycine (NR1/NR2A through NR2C) or 0.03 μM glycine (NR1/NR2D). Values are expressed as the mean percent control current response determined in voltage-clamped oocytes in the absence of ethanol. Statistical analysis was performed at each ethanol concentration to compare responses in high glycine with responses in low glycine. *, significantly different at $p < 0.05$ by student's *t* test. Values represent the mean ± standard error for 9–27 oocytes taken from at least three different frogs.

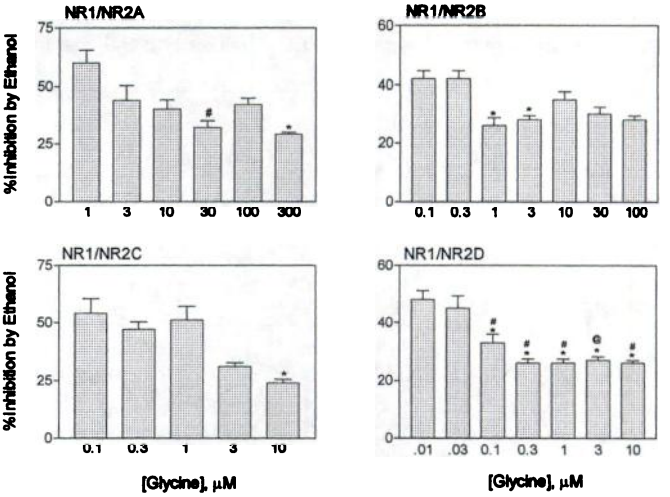

Fig. 3. Effect of glycine concentration on ethanol inhibition of NMDA receptors expressed in *Xenopus* oocytes. Values are expressed as the percent inhibition (mean ± standard error) of the current response elicited by 100 mM NMDA in the presence of the indicated concentration of glycine. Each bar indicates the mean inhibition for 4–12 oocytes. The following comparisons were statistically significant by analysis of variance with Tukey-Kramer test *post hoc* as described in Materials and Methods. NR1/NR2A: *, different from 1.0 μM ($p < 0.05$); #, different from 1.0 μM ($p < 0.01$). NR1/NR2B: *, different from 0.1 μM and 0.3 μM ($p < 0.05$). NR1/NR2C: *, different from 0.1 μM ($p < 0.01$), 0.3 μM ($p < 0.05$), and 10 μM ($p < 0.001$). NR1/NR2D: * different from 0.01 μM ($p < 0.001$); #, different from 0.03 μM ($p < 0.001$); @, different from 0.03 μM ($p < 0.05$).

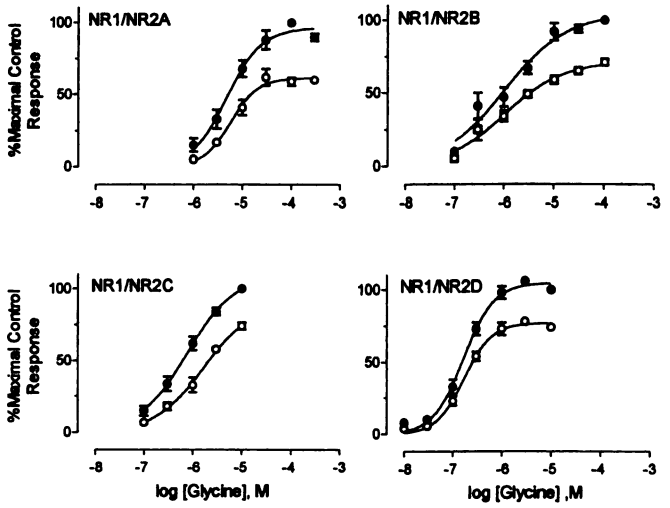

Fig. 4. Comparison of dose-response curves for glycine coactivation of recombinant heteromeric NMDA receptors expressed in *Xenopus* oocytes in the absence (●) and presence (○) of 100 mM ethanol. Glycine dose-response curves were generated in the presence of 100 μM NMDA. Dose response were fit according to the equation $I = I_{max}/[1 + (EC_{50}/A)^n]$ as described in Materials and Methods. Responses were standardized to the maximal response observed in the absence of ethanol. *Points*, mean ± standard error for 3–6 oocytes taken from one frog. Experiments were repeated on two or three separate frogs.

of glycine for the heteromeric NMDA receptor. To test this possibility, EC_{50} values and Hill coefficients for glycine were determined by dose-response analysis in the presence and absence of ethanol (Fig. 4, Table 1). Although ethanol did not significantly alter the Hill coefficient for glycine at the NR1/NR2A, NR1/NR2B, NR1/NR2C, and NR1/NR2D receptors, the EC_{50} value for glycine was significantly increased at the

TABLE 1

Comparison of glycine and NMDA EC_{50} values and Hill coefficients for NR1/NR2 NMDA receptor heteromers expressed in *Xenopus* oocytes

	Glycine				NMDA			
	Control		Ethanol		Control		Ethanol	
	EC_{50}	*n*	EC_{50}	*n*	EC_{50}	*n*	EC_{50}	*n*
NR1/NR2A	4.82 ± 0.07	1.23 ± 0.21	5.73 ± 0.06*	1.56 ± 0.28	50.9 ± 7.3	2.0 ± 0.1	56.1 ± 6.9	2.0 ± 0.2
NR1/NR2B	1.07 ± 0.18	0.70 ± 0.17	1.08 ± 0.13	0.74 ± 0.14	26.6 ± 4.3	1.5 ± 0.2	28.4 ± 5.0	2.4 ± 0.6
NR1/NR2C	0.76 ± 0.02	0.89 ± 0.03	1.66 ± 0.12*	0.87 ± 0.11	21.1 ± 0.7	1.9 ± 0.4	22.7 ± 2.8	2.0 ± 0.3
NR1/NR2D	0.16 ± 0.05	1.39 ± 0.18	0.17 ± 0.03	1.53 ± 0.16	10.6 ± 1.1	1.7 ± 0.1	12.3 ± 3.1	2.3 ± 0.6

NR1/NR2A and NR1/NR2C receptors in the presence of ethanol (Fig. 4, Table 1). In contrast, neither the EC_{50} value nor the Hill coefficient for NMDA at the NR1/NR2A, NR1/NR2B, NR1/NR2C, and NR1/NR2D receptors were altered by 100 mM ethanol (Fig. 5, Table 1).

Examination of the glycine-independent component of ethanol inhibition demonstrated that in the presence of saturating glycine concentrations, the NR1/NR2A and NR1/NR2B receptors were inhibited by ethanol to a significantly greater extent than were the NR1/NR2C and NR1/NR2D receptors (Fig. 6). In contrast, in the presence of subsaturating concentrations of glycine, ethanol inhibition of NR1/NR2A, NR1/NR2C, and NR1/NR2D receptors was significantly enhanced (Fig. 6), such that the four NR1/NR2 heteromers were similarly inhibited by ethanol.

To determine whether any residual endogenous oocyte Cl^- current could contribute to the observed effects of glycine on ethanol inhibition of NMDA receptors, oocytes expressing NR1/NR2C heteromers were injected with BAPTA to chelate intracellular Ca^{2+}. As shown in Fig. 7, responses elicited by 100 μM NMDA and 0.3 μM glycine were inhibited by ethanol to a greater extent than were responses elicited by 100 μM NMDA and 10 μM glycine in both control and BAPTA-injected oocytes. These data demonstrate that glycine-reversible inhibition of NR1/NR2C receptors is unaffected by BAPTA injection.

Activation of the NR1/NR2D receptors by NMDA and subsaturating concentrations of glycine (0.01–0.1 μM) revealed responses that were characterized by an initial peak that was followed by a smaller plateau, which is consistent with apparent desensitization of the receptor (Fig. 8A). This re-

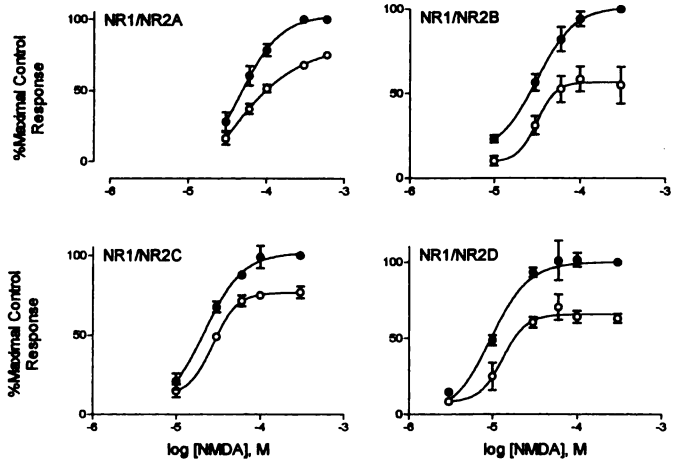

Fig. 5. Comparison of dose-response curves for NMDA activation of recombinant heteromeric NMDA receptors expressed in *Xenopus* oocytes in the absence (●) and presence (○) of 100 mM ethanol. NMDA dose-response curves were generated in the presence of 10 μM glycine. Dose response were fit according to the equation $I = I_{max}/[1 + (EC_{50}/A)^n]$ as described in Materials and Methods. Responses were standardized to the maximal response observed in the absence of ethanol. *Points*, mean ± standard error for 3–6 oocytes taken from one frog.

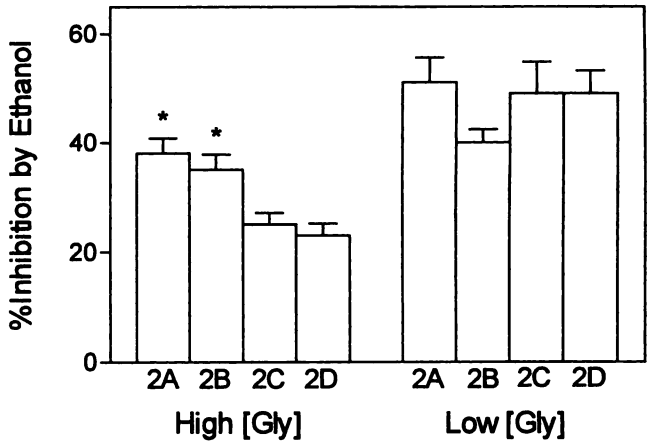

Fig. 6. Comparison of inhibition of heteromeric NMDA receptors containing the indicated NR2 subunit by 100 mM ethanol in the presence of 100 μM NMDA and high concentrations of glycine (10 μM) or low glycine concentrations [1 μM (NR1/NR2A-C) or 0.03 μM (NR1/NR2D)]. *, significantly different from NR2C and NR2D ($p < 0.05$ by analysis of variance with Tukey-Kramer multiple-comparisons test *post hoc*). *Bars*, mean ± standard error for 9–27 oocytes taken from at least three different frogs

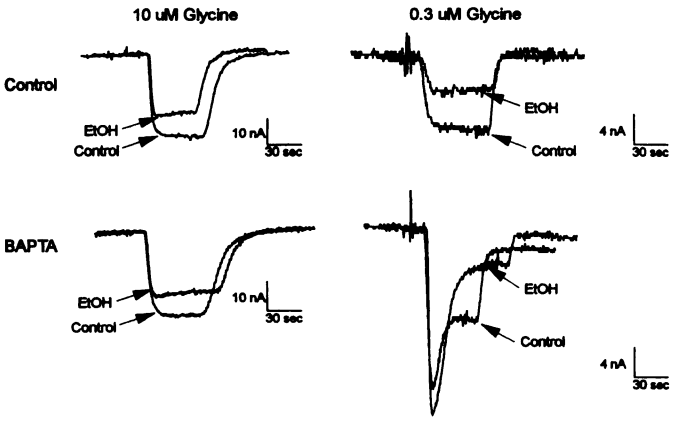

Fig. 7. Glycine-reversible ethanol inhibition of NR1/NR2C receptors in BAPTA-injected oocytes. Oocytes were injected with of BAPTA 2 days after the injection of NR1 and NR2C subunit RNAs. Oocytes were incubated for 3–6 hr before electrophysiological recording. Responses to 100 μM NMDA in the presence of 10 or 0.3 μM glycine were recorded in Ba^{2+}-Ringer solution. Traces obtained in the presence of 100 mM ethanol are superimposed for comparison. *Top*, Control oocyte not injected with BAPTA. *Bottom*, BAPTA-injected oocyte. Both cells were taken from the same batch of RNA-injected oocytes.

sponse has been previously described for native NMDA receptors as glycine-sensitive desensitization (26, 27). Glycine-sensitive desensitization of the NR1/NR2D receptor was observed at glycine concentrations that were correlated with enhanced ethanol sensitivity. Although the peak response was most frequently insensitive to ethanol, the plateau phase was consistently reduced by 25–100 mM ethanol. In the presence of higher glycine concentrations (i.e., concentrations above the EC_{50}), NR1/NR2D-mediated responses exhibited only a plateau phase, which was sensitive to ethanol. Analysis of the NMDA-elicited responses for the four heteromeric receptors revealed that glycine-sensitive desensitization was infrequently observed at any glycine concentration tested for the NR1/NR2A, NR1/NR2B, or NR1/NR2C receptors (Table 2). However, apparent desensitization was observed at the NR1/NR2B heteromer in the presence of ethanol and subsaturating concentrations of glycine (0.1–0.3 μM; Fig. 8B, Table 2). Similarly, nondesensitizing plateau NR1/NR2D responses, elicited by NMDA and subsaturating concentrations of glycine, could often be converted to desensitizing responses in the presence of ethanol (Fig. 8C). Desensitization was not observed at any of the heteromeric receptors in the presence of low concentrations of NMDA (data not shown). Furthermore, injection of oocytes expressing NR1/NR2C heteromers with BAPTA, before electrophysiological recording, did not abolish the appearance of glycine-sensitive desensitization (Fig. 7). Taken together, these data are consistent with glycine-sensitive desensitization at the NR1/NR2D receptor that may also become evident at the NR1/NR2B receptor due to an interaction with ethanol.

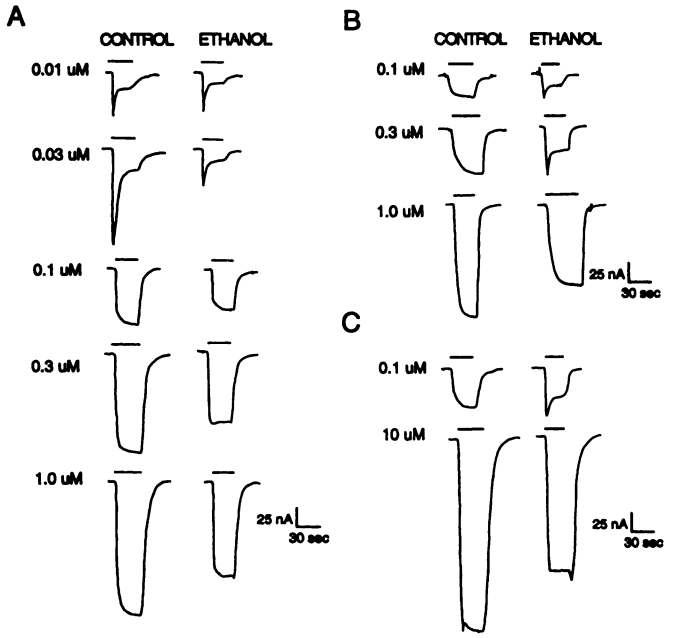

Fig. 8. Glycine-sensitive desensitization of recombinant NMDA receptor heteromers expressed in *Xenopus* oocytes. Shown are current traces from individual oocytes expressing NR1/NR2D (A), NR1/NR2B (B) or NR1/NR2D (C) receptors. The *left trace* of each pair corresponds to the current response in the absence of ethanol while the *right current trace* corresponds to the current response in the presence of 100 mM ethanol. Current responses were obtained by bath application of 100 μM NMDA and the indicated glycine concentration. *Bars over the current traces*, drug application.

Discussion

NMDA receptors have been implicated in mediating some of the central nervous system effects of ethanol. Recent studies have demonstrated that the NMDA receptor is a specific target for ethanol (14–16, 25, 28, 29). However, little is known about the precise mechanism of action of ethanol at the NMDA receptor. The data in the present report demonstrate that ethanol inhibition of recombinant NMDA receptors is modulated by glycine, but not by NMDA, and that this effect is most striking at the NR1/NR2A, NR1/NR2C, and NR1/NR2D receptors. The reversal of ethanol inhibition by glycine was not complete, however, suggesting that there are two components to ethanol inhibition of these receptors: a glycine-reversible component and a glycine-independent component. Inhibition of NR1/NR2B heteromers also varied with the glycine concentration, although the effect was less pronounced than that observed for the NR1/NR2A, NR1/NR2C, and NR1/NR2D receptors.

We previously reported that the pharmacology of heteromeric NR1/NR2C receptors closely parallels the native pharmacology of NMDA receptors in the cerebellum (13, 30, 31). It is interesting to note that the NR2C subunit mRNA localizes almost exclusively to the cerebellar granule cell layer (4, 6, 13, 32). Thus, if the properties of the NR1/NR2C receptor that we report are also observed in native NMDA receptor complexes, ethanol inhibition of cerebellar NMDA receptors should be dependent on glycine. Glycine has been demonstrated to reverse ethanol inhibition of NMDA receptor-mediated Ca^{2+} influx (15) and cGMP formation (19) in cultured cerebellar granule cells. Similarly, in cultured neonatal neurons, glycine reverses ethanol inhibition of NMDA receptor-mediated Ca^{2+} influx (33), possibly reflecting the involvement of the NR2D subunit, which is widely expressed in developing brain. Interestingly, glycine also reverses ethanol inhibition of NMDA-stimulated dopamine release from adult rat striatum (34), an observation potentially accounted for by the presence of NR2D transcripts in the substantia nigra (6).

Other studies have reported a lack of involvement of glycine in modulating ethanol inhibition of the NMDA receptor. Chandler *et al.* (21) reported that glycine does not reverse the ability of ethanol to attentuate NMDA-mediated excitotoxicity in primary cultures from neonatal brain. Furthermore, glycine does not reverse ethanol inhibition of NMDA responses in isolated hippocampal neurons (22). The varied ability of glycine to reverse ethanol inhibition of the NMDA receptor may be related to differences in endogenous glycine level in these studies. The glycine concentration may be especially critical for responses mediated by NR2C- or NR2D-containing receptor complexes, which have a high affinity for glycine. In the present study, the ability of glycine to reverse ethanol inhibition of NR1/NR2 heteromers was greatest at glycine concentrations below the EC_{50}. Alternatively, discrepancies in studies of glycine reversal of ethanol inhibition of NMDA-mediated responses may be due to differences in subunit composition between native brain receptors and recombinant receptors expressed in heterologous systems. In addition, recent studies have indicated that heteromeric complexes composed of more than one type of NR2 subunit can preferentially form in heterologous expression systems (35, 36) and that multiple NR2 subunits coexist in the same receptor complex (46).

TABLE 2

Percentage of oocytes expressing NR1/NR2 heteromers showing desensitization at different glycine concentrations

	10% to 20% Maximal response		30% to 40% Maximal response		70% to 80% Maximal response		100% Maximal response	
	Control	Ethanol	Control	Ethanol	Control	Ethanol	Control	Ethanol
					%			
NR1/NR2A	13 (2/15)	8 (1/12)	0 (0/5)	0 (0/5)	0 (0/6)	33 (2/6)	0 (0/7)	14 (1/7)
NR1/NR2B	0 (0/5)	40 (2/5)	14 (1/7)	57 (4/7)	0 (0/12)	12 (1/8)	0 (0/3)	0 (0/3)
NR1/NR2C	9 (2/23)	10 (2/19)	14 (2/14)	16 (2/12)	9 (1/11)	20 (2/12)	2 (3/45)	8 (3/37)
NR1/NR2D	92 (12/13)	92 (12/13)	55 (5/9)	78 (7/9)	50 (3/6)	50 (3/6)	10 (2/19)	16 (3/19)

The percentage of oocytes exhibiting glycine-sensitive desensitization was determined by analysis of individual oocyte responses to 100 μM NMDA and different concentrations of glycine in the absence (control) or presence (ethanol) of 100 mM ethanol. Equally efficacious glycine concentrations are presented for the different heteromeric NMDA expressed in *Xenopus* oocytes to reflect the differences in glycine affinity (see Table 1). Glycine concentrations represented are 10% to 20% maximal response, 1 μM (NR1/NR2A), 0.1 μM (NR1/NR2B and NR1/NR2C), and 0.03 μM (NR1/NR2D); 30% to 40% maximal response, 3 μM (NR1/NR2A), 0.3 μM (NR1/NR2B and NR1/NR2C), and 0.1 μM (NR1/NR2D); 70% to 80% maximal response, 30 μM (NR1/NR2A), 10 μM (NR1/NR2B), 3 μM (NR1/NR2C), and 0.3 μM (NR1/NR2D); and 100% maximal response, 100 μM (NR1/NR2A and NR1/NR2B) and 10 μM (NR1/NR2C and NR1/NR2D). The absolute number of oocytes showing glycine-sensitive desensitization relative to the total number of oocytes examined are shown in parentheses.

The mechanism by which ethanol inhibits NMDA receptor-mediated responses is unclear. However, the results of the present study suggest that at least some of the action of ethanol may be due to effects on the glycine binding domain of the receptor complex. At least for the NR1/NR2A and NR1/NR2C receptors, ethanol appears to reduce glycine affinity, which could account for the glycine-reversible component of ethanol inhibition. Interestingly, glycine has also been reported to modulate the interaction of ifenprodil with the NR1/NR2B heteromer expressed in *Xenopus* oocytes (23). In the presence of low concentrations of glycine, ifenprodil is a more potent antagonist at the NR1/NR2B NMDA receptor (23).

In the presence of saturating concentrations of glycine, the NR1/NR2C and NR1/NR2D receptors were significantly less sensitive to inhibition by ethanol. This finding is consistent with previous reports of differential sensitivity of recombinant NR1/NR2 heteromers to ethanol (25, 29) and extends these reports to include the NR1/NR2D heteromer. These findings, together with the report that the different NR1 splice variants also differ in their ethanol sensitivity (28), suggest that differences in subunit composition may account for regional variation in ethanol sensitivity of native NMDA receptors. Ethanol selectively inhibits subpopulations of NMDA receptors in the medial septum (38). In addition, NMDA receptors in both the inferior colliculus and hippocampus are more sensitive to ethanol than NMDA receptors in the lateral septum (39). Also, cerebellar NMDA receptors expressed in oocytes are less sensitive to ethanol than are NMDA receptors in the hippocampus (20). The differential expression of NMDA receptor subunit mRNAs (4, 5, 32, 40) and of NMDA receptor subtypes (13) may underlie regional differences in the ability of ethanol to inhibit NMDA receptors.

In the presence of subsaturating concentrations of glycine, NR1/NR2D-mediated responses exhibited glycine-sensitive desensitization. In addition, NR1/NR2B-mediated responses elicited in the presence of ethanol and subsaturating glycine concentrations frequently exhibited glycine-sensitive desensitization. Desensitization was most pronounced at concentrations of glycine that correlated with enhanced ethanol inhibition and was not observed at low concentrations of NMDA in the presence of 10 μM glycine. These data are consistent with previous reports of glycine-sensitive desensitization of NMDA receptors from embryonic hippocampal neurons (26, 27), known to express NR2B and NR2D mRNAs (5, 32). The endogenous oocyte Ca^{2+}-activated Cl^- current is unlikely to be a factor in glycine-sensitive desensitization of the NR1/NR2B or NR1/NR2D receptors due to the replacement of Ca^{2+} by Ba^{2+} in the recording solution (34). Furthermore, glycine-sensitive desensitization was blocked by increasing glycine concentrations. In contrast, increasing glycine concentrations would enhance, rather than inhibit, Ca^{2+}-activated Cl^- currents, which in general are associated with larger-magnitude current responses.[2] In addition, glycine-sensitive desensitization was observed in BAPTA-injected oocytes. We observed apparent desensitization of heteromeric NMDA receptors expressed in oocytes in the presence of the glycine site antagonist 7-chlorokynurenate but not with antagonists that interact with the glutamate binding site,[3] further suggesting an involvement of glycine in the observed desensitization. Finally, the observation that ethanol can promote glycine-sensitive desensitization is consistent with an effect of ethanol, either directly or indirectly, on the glycine binding domain of the NMDA receptor.

Recent evidence has demonstrated the presence of a glycine binding site on the NR1 subunit. Transfected 293 cells expressing the NR1 subunit alone, but not the NR2A subunit alone, can bind the glycine site ligand [^{3}H]DCK with an affinity similar to that observed in cells expressing both NR1 and NR2A subunits (41, 42). In addition, site-directed mutagenesis of the NR1 subunit has been used to identify amino acids that are important for glycine binding to this subunit (43). However, different NR1/NR2 heteromers have distinct glycine affinities (Table 1; Refs. 7 and 8), demonstrating that the NR2 subunit plays a role in determining glycine affinity. We have shown that glycine can modulate ethanol sensitivity of NR1/NR2 heteromers expressed in *Xenopus* oocytes. Thus, ethanol may allosterically modulate the glycine binding domain or may interfere with NR2 subunit interaction with NR1 subunits. We have also shown that the magnitude of the glycine-independent component of ethanol inhibition is dependent on the specific NR2 subunit in the heteromeric com-

[2] A. L. Buller and D. T. Monaghan, unpublished observations.
[3] A. L. Buller and D. T. Monaghan, unpublished observations.

plex. Taken together, the data in the present report demonstrate that the four heteromeric NMDA receptor complexes differentially express glycine-reversible and glycine-independent components of ethanol inhibition and suggest that there are at least two independent mechanisms for ethanol inhibition of NMDA receptors.

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