Factors That Enhance Ethanol Inhibition of N-Methyl-D-Aspartate Receptors in Cerebellar Granule Cells

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ABSTRACT

The objective of this study was to identify factors that influence ethanol (EtOH) inhibition of the N-methyl-D-aspartate receptor (NMDAR) in primary cultured cerebellar granule cells. Several factors contributing to the inhibitory effects of EtOH on NMDAR function were assessed using both whole-cell and perforated patch-clamp recordings. The NMDAR subunit composition was examined by Western blot analysis using NR2 subunit-specific antibodies and pharmacological manipulation with the NR2B-specific antagonist infenprodil. Western blot analysis indicated that NMDAR subunit composition changed from a combination of NR2A and NR2B containing NMDARs to primarily NR2A with increasing days in vitro (DIV). Although the NR2B subunit was detectable until 21 DIV, there was a significant decrease in ifenprodil sensitivity after 7 DIV. EtOH sensitivity did not change with an increasing DIV. A high concentration of glycine reversed EtOH inhibition of steady-state, but not peak, NMDA-induced current during whole-cell recordings. Significant glycine reversal of effects of a low concentration of EtOH on peak current was observed under perforated patch-clamp conditions. A 30-s EtOH pretreatment significantly enhanced EtOH inhibition of NMDA-induced peak current. Collectively, these results indicate that EtOH sensitivity of the NMDAR in primary cultured cerebellar granule cells is not related to subunit composition nor ifenprodil sensitivity, involves a kinetic interaction with glycine, and can be enhanced by a slowly developing transduction mechanism that occurs within tens of seconds.

It has been shown both in vitro (Dildy and Leslie, 1989; Hoffman et al., 1989; Lima-Landman and Albuquerque, 1989; Lovinger et al., 1989; Woodward and Gonzales, 1990) and in vivo (Simson et al., 1991) that ethanol (EtOH) at physiologically relevant concentrations inhibits the N-methyl-D-aspartate receptor (NMDAR). Ethanol inhibits N-methyl-D-aspartate (NMDA)-elicited biochemical responses (Dildy and Leslie, 1989; Hoffman et al., 1989); NMDAR mediated neurotransmission (Woodward and Gonzales, 1990; Simson et al., 1991) and directly inhibits receptor function as measured using electrophysiological techniques (Lima-Landman and Albuquerque, 1989; Lovinger et al., 1989). Single-channel analysis suggests that EtOH acts on an allosteric site on the NMDAR and inhibits the channel by reducing agonist efficacy (Lima-Landman and Albuquerque, 1989; Wright et al., 1996).

The NMDAR has binding sites for the coagonist glycine, Mg$^{2+}$ and Zn$^{2+}$ ions, as well as protons and polyamines that can modulate receptor function. These allosteric sites have been investigated as possible targets for EtOH (Rabe and Tabakoff, 1990; Dildy-Mayfield and Leslie, 1991; Morrissett et al., 1991; Peoples and Weight, 1992; Woodward, 1994a; Chu et al., 1995; Peoples et al., 1997). However, EtOH seems to act independently of most if not all of these sites. There is some disagreement as to whether EtOH acts through the coagonist glycine site on the NMDAR. Hoffman and coworkers (1989) were the first to observe an interaction between EtOH and glycine on the NMDAR. It was speculated that the inhibiting effects of EtOH on the NMDAR could be mediated by decreasing glycine potency (Rabe and Tabakoff, 1990). Further studies supported this interaction by showing that glycine concentrations above 10 µM decreased the inhibitory effects of EtOH on NMDA-stimulated calcium influx ([Ca$^{2+}$]) in primary cultured cerebellar granule cells (CGCs) (Rabe and Tabakoff, 1990) and in acutely dissociated rat brain cells (Dildy-Mayfield and Leslie, 1991). In contrast, others have reported that glycine could not reverse EtOH inhibition of NMDA-induced increases in [Ca$^{2+}$], in primary cultured cortical cells (Bhave et al., 1996; Cebers et al., 1996) or in primary cultured CGCs (Cebers et al., 1996). Glycine concentrations greater or equal to 3 µM have been shown to completely reverse the inhibitory effects of EtOH on NMDA-mediated dopamine release from striatal slices (Woodward and Gonzales, 1990) but not for NMDA-mediated norepinephrine release from rat cortical (Gonzales and Woodward, 1990) and hippocampal slices (Woodward, 1994b). In exper-

ABBREVIATIONS: CGC, cerebellar granule cell; DIV, days in vitro; EtOH, ethanol; NMDAR, N-methyl-D-aspartate receptor; NMDA, N-methyl-D-aspartate; Pk, peak; SS, steady state; WC, whole cell; PP, perforated patch.
ments that have examined recombinant receptor function, it has been reported that high glycine concentrations significantly reverse EtOH inhibition of NMDA-induced current in *Xenopus* oocytes expressing heteromeric NMDARs containing the NR2A, NR2C, and NR2D but not the NR2B subunits (Buller et al., 1995). In contrast, high concentrations of glycine did not reverse EtOH inhibition of NMDA-induced current, and EtOH did not change the EC<sub>50</sub> of glycine for receptors on primary cultured hippocampal neurons using whole-cell (WC) patch-clamp electrophysiological techniques (Peoples et al., 1997) or in oocytes expressing NR1 with NR2A or NR2C (Mirshahi and Woodward, 1995).

These differences may be explained in part by the different brain regions examined and different methods used. Assessment of the actions and interactions of EtOH with glycine on the NMDAR expressed in primary cultured CGCs using patch-clamp electrophysiological techniques has not been reported. Previous examination using this methodology in other neurons has indicated no interaction between glycine and EtOH (Peoples et al., 1997). Whether this is due to methodology used or the type of neuron studied remains to be determined. When using the WC patch-clamp method, the cell membrane is ruptured and the internal milieu of the cell is disrupted. In the biochemical assays described above, the cell membrane and internal milieu remain intact. It is possible that this disruption in the integrity of the cell could explain the different results.

One purpose of this study was to assess the possible interaction of glycine and the inhibitory effects of EtOH on NMDAR function using WC and perforated patch (PP) electrophysiological techniques in primary cultured CGCs. High glycine concentrations did significantly reverse EtOH-induced inhibition of NMDA-mediated currents in our CGC cultures under most conditions, but reversal was more extensive under PP clamp conditions. We also observed that a 30-s pretreatment with either 10 or 100 mM EtOH significantly enhanced inhibition of NMDA-induced peak current.

### Materials and Methods

#### Preparation and Maintenance of CGC Cultures

All drugs were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Primary neuronal cultures were prepared using cerebellar tissue from 6- to 8-day-old Sprague-Dawley rats. The tissue was removed, pooled, minced, and then treated with 0.125% trypsin for 3 min at 37°C. After digestion, the cells were dissociated by trituration and plated onto poly-D-lysine (5 μg/ml)-coated 35-mm dishes at 1 × 10<sup>6</sup> cells/35-mm dish in plating medium. The plating medium consisted of minimum essential medium (Gibco/BRL, Gaithersburg, MD) supplemented with 2 mM L-glutamine, 130 μM DNase, 10% heat-inactivated horse serum, and 10% fetal bovine serum (all serum was purchased from Gibco/BRL). After 18 to 20 h, this medium was replaced with feeding medium (minimum essential medium containing 5% fetal bovine serum, 2 mM L-glutamine, 25 mM KCl, penicillin/streptomycin (100 U/100 μg/ml), and a fluoride-oxyuridine/uridine, 35 μM/15 μM mixture). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. Neurons were maintained for up to 42 days in vitro (DIV).

To minimize excitotoxic cell death due to the introduction of fresh medium, we did not exchange the medium, but the existing medium was supplemented with 0.5 ml of fresh feeding medium every 7 days. Representative dishes of cells at 10, 19, 28, and 31 DIV were tested for cell viability by the trypan blue exclusion method. Cells at 10, 19, and 28 DIV were >90% phase bright. The mean number of cells in three nonoverlapping fields was 650, 399, and 645; the number of trypan-stained cells in these three fields was 3, 1, and 1, respectively. Cells at 31 DIV were >80% phase bright, and the mean number of cells counted equaled 770 with 1 trypan-stained cell. These data indicated that our CGCs remained healthy throughout the duration of the study.

#### Immunodetection

Construction and characterization of the commercially available NMDA NR2A and NR2B antibodies (Chemicon International Inc., Temecula, CA) have been described previously (Snell et al., 1996). Techniques for preparation of tissue derived from the rat hippocampus, HEK 293 cells or cultured cells, as well as Western blot analysis have also been described (Snell et al., 1996; Popp et al., 1998).

#### Whole-Cell Patch-Clamp Recordings

Culture dishes were placed on the stage of an inverted microscope (Nikon Inc., Garden City, NY) and superfused at 1 to 2 ml/min with external medium (150 mM NaCl, 2.5 mM CaCl<sub>2</sub>, 10 mM HEPES, 10 mM glucose, and 200 mM tetrodotoxin, pH adjusted to 7.4 with NaOH and osmolality adjusted to 333–336 mmol/kg with sucrose). All recordings were performed at room temperature using the Axopatch 200 patch-clamp amplifier (Axon Instruments, Foster City, CA). The internal patch-electrode solution consisted of: 100 mM N-methyl-D-glucamine, 100 mM MesOH, 40 mM CsF, 10 mM HEPES, 1 mM MgCl<sub>2</sub>, 5 mM QX-314 (Research Biochemicals Inc., Natick, MA), and 5 mM EGTA (pH adjusted to 7.4 with CsOH and osmolality adjusted to 314–317 mmol/kg with sucrose).

#### Perforated Patch-Clamp Recordings

In PP experiments, both the patch-electrode and external solutions were the same as those used in the WC experiments. Amporpheticin B from a stock solution of 5 mg/100 μl in dimethyloxide was the permeablizing reagent used in these experiments. The stock solution was prepared and diluted 100-fold into the patch-electrode solution so as to permeabilize the membrane patch at the tip of the electrode. The final concentration of amorphopicin B was 0.005 mg/ml. The stock solution could be used for up to 8 h, and dilutions to fill the electrodes were prepared every 1 to 1.5 h. For both WC and PP experiments, the electrode tip resistance was approximately 5 megohms. After formation of the gigaohm seal, the cells usually opened within 2 min, as indicated by a decrease in series resistance to final values averaging 15 megohms. Series resistance in WC experiments was 10 to 12 megohms. The holding potential in all experiments was ~60 mV.

#### Ifenprodil and EtOH Experiments

In all experiments, drugs were dissolved in the external medium and delivered by gravity from solution-containing reservoirs placed above the preparation, gated by plastic stopcocks, and connected to a linear array of microcapillary tubes (0.32 mm i.d.). The array was moved manually to apply different solutions to cells, and the duration of drug application lasted between 5 and 10 s. This system allowed for rapid solution superfusion of the neuron being studied and rapid solution exchange (~150 ms). Currents were low pass filtered at 1 kHz using a 3-pole Bessel filter. Signals were digitized and current traces measured using pClamp 6.0 software (Axon Instruments Inc., Foster City, CA). Ifenprodil (RBI) was made from stock solutions originally dissolved in dH<sub>2</sub>O that had been stored at ~20°C. In these experiments, 10 μM ifenprodil was applied simultaneously with 100 μM NMDA and 10 μM glycine. To determine the effects of ifenprodil on NMDA-induced currents, steady-state (SS) current values were used. These values were obtained by measuring the difference between two cursors placed at time points immediately before and 4 to 6 s after drug application. The exact time point was consistent within a given cell. The value of this SS current was normalized to the mean SS value obtained from several NMDA-induced currents just before ifenprodil application. Ethanol (10, 25, 50, 100, and 200 mM) (Aaper Alcohol and Chemical Co., Shelbyville, KY) was also coapplied with 100 μM NMDA and 10 μM glycine during the simultaneous application protocol. To determine the effects of EtOH on NMDA-induced current, peak and SS amplitudes were measured unless otherwise stated. Values used were the mean amplitude obtained from several EtOH applications, which had been normalized to mean NMDA peak cur-
rent amplitudes obtained before and after a specific EtOH application. In the experiments that included the effects of EtOH on SS currents, the values were obtained in an identical manner. As in the ifenprodil experiments, SS current values were obtained at the same time within a given cell, between 4 and 6 s after the onset of drug application.

Glycine Experiments. Peak current amplitudes were used in construction of the glycine dose-response curves. In these experiments, 100 μM NMDA was combined with different glycine concentrations (10 nM to 3.33 μM), and the responses (2–3) were compared with maximum responses elicited by 100 μM NMDA and 10 μM glycine. Glycine dose-response curves for both young and old neurons consisted of data derived from cells from more than one culture batch. Glycine dose-response curve fits were generated using the following equation,

\[ I_{\text{exp}} = \frac{I_{\text{max}}}{1 + (\text{EC}_{50} / \text{glycine concentration})^n} \]

where \( n \) is the Hill slope exponent, \( I_{\text{exp}} \) is the expected current at a given glycine concentration, and \( I_{\text{max}} \) is the maximum current. The concentration of glycine used in the experiments examining EtOH effects at low glycine concentrations was based on the glycine EC50 values obtained from the dose-response studies for 6/7 and 28-day neurons. In these experiments, peak and SS current values were used to compare percentage of EtOH inhibition of NMDA-induced current under low and high glycine conditions in both the WC and PP modes.

Ethanol Pretreatment Experiments. In these studies, the individual cell being voltage clamped was exposed to one of two EtOH concentrations (10 and 100 mM) for 30 s before application of that particular EtOH concentration along with 10 μM NMDA and 10 μM glycine. Peak as well as SS current values were compared between the simultaneous and pretreatment applications for each EtOH concentration, and percentage of inhibition by EtOH was calculated from these values. The order of pretreatment and simultaneous application of EtOH was varied from cell to cell.

Changes in the current decay rate, a measure of desensitization, were calculated in two ways: steady-state/peak (SS/Pk) current amplitude ratio and measurement of the rate of onset of NMDAR desensitization in the continuous presence of agonist (\( \tau_{\text{decay}} \)). Measurement of \( \tau_{\text{decay}} \) for the NMDA-induced currents was performed using pClamp 6 software (Axon Instruments), with \( \tau_{\text{decay}} \) estimated from single exponential nonlinear curve fits using the following equation,

\[ \frac{dI}{dt} = \frac{-(1-k)}{Ae^{-t/\tau} + C} \]

where \( A \) is the amplitude relative to the offset value at the start of the fit region, \( \tau \) is the time constant of decay (\( \tau_{\text{decay}} \)), and \( C \) is the steady-state asymptote. As in the determination of SS current values, the end of the “fit region” (achievement of SS) was 4 to 6 s after the onset of application.

Statistics. All data values are expressed as mean ± S.E.

Ethanol Experiments. Differences in EtOH inhibition of NMDA-induced current among the four age groups were analyzed using ANOVA. The experimental design was a completely randomized factorial design with two treatment levels, DIV and EtOH concentration. DIV was further divided into four age-in-culture groups: 6/7, 10/11, 14, and 28 DIV and five EtOH concentration groups: 10, 25, 50, 100, and 200 mM.

Ifenprodil Experiments. Whenever possible the effect of ifenprodil on NMDA-induced current was recorded for each cell, and the data were analyzed comparing the change in ifenprodil-induced inhibition with age in culture by one-way ANOVA and group differences assessed using \( q \) values obtained from the Tukey-Kramer post hoc test. Regression analysis was used to determine the relationship between ifenprodil and EtOH effects. The correlation coefficient used was the Pearson \( r \).

Glycine Experiments. In the low glycine WC and PP experiments, when applicable, The Dunn’s Multiple Comparison test was used to assess whether younger neurons differed from older neurons in percentage of EtOH inhibition of NMDA-induced current. Differences in percentage of EtOH inhibition of NMDA-induced peak or SS current were assessed by two-way ANOVA. Variables were EtOH concentration (10 or 100 mM EtOH) and glycine concentration (high or low). Data acquired using either the WC or PP clamp methods were analyzed in this manner. The Multiple t test was the post hoc test used to identify individual group differences when the ANOVA resulted in a significant \( F \) value.

Ethanol Pretreatment Experiments. In the EtOH pretreatment experiments, differences due to age in culture were assessed using the Dunn’s Multiple Comparison test. Further analysis consisted of assessing differences in percentage of EtOH inhibition of NMDA-induced peak current by two-way ANOVA, with EtOH concentration (10 and 100 mM) as one variable and treatment (simultaneous and pretreatment) on EtOH inhibition of NMDA-induced peak and SS current within each EtOH concentration. Once again, individual post hoc differences were determined by the Multiple t test. We used a repeated measures design and thus used the Statview II, one-sample t test (ABACUS Concepts, Inc., Berkeley, CA) to compare the percent change induced by EtOH from control values. Comparisons were done for each EtOH concentration under each glycine concentration and under simultaneous or pretreatment conditions. The One-sample t test was used to determine if the differences attributable to EtOH on SS/Pk current ratios and on \( \tau_{\text{decay}} \) were significant relative to control values.

Results

Identification of the NR2 Subunit Present in Primary Cultured CGCs. NMDARs containing only the NR2B subunit are more potently inhibited by the antagonist ifenprodil than NMDARs containing other NR2 subunits (Williams, 1993). Representative responses demonstrating ifenprodil inhibition of NMDA-induced current are shown in Fig. 1A. The graph in Fig. 1B depicts the percentage of inhibition of SS NMDA-induced current by ifenprodil. The percentage of inhibition in 6/7-day-old neurons was 60.87 ± 3.39. By 10 DIV ifenprodil-induced inhibition significantly decreased to 24.86% ± 5.22 (F = 27.5, df = 3/107, \( P < .0001 \), one-way ANOVA). The magnitude of inhibition did not change with additional time in culture (21.9% ± 7.74 for 14 DIV and 17.31% ± 3.69 for ≥ 28 DIV). Post hoc analyses using the Tukey-Kramer test revealed that the decrease seen in ifenprodil inhibition was significant (\( P < .001 \)) when comparing 6/7 DIV to all later ages. The \( q \) values were 9.2, 5.6, and 11.6 for 6 days compared with 10 days, 14 days, and 28 days, respectively. There was no significant difference in inhibition among the 10-, 14-, and 28-day groups.

The largest ifenprodil inhibition of NMDA-induced current in these CGCs was considerably lower than values previously published for both 1- to 2-week cortical (Lovinger, 1995) and striatal (Popp et al., 1998) primary cultured neurons and
indicates that some NR2B NMDA subunit is present in our young CGCs (6/7 days) along with another NR2 subunit. These data also suggest that the relative proportion of these subunits changes with age in culture with an initial rapid change occurring between days 6 and 10.

The results from Western blot analysis confirm the presence of the NR2B and another NR2 subunit, the NR2A. This supports the change in NR2 subunit development indicated by the electrophysiological studies. Immunodetection of the different NR2 subunits was performed using cultures from 3, 6/7, 10/11, 14, 21, 28, and 35 DIV. Each age group was represented by at least three different culture batches. Figure 2 is a representative depiction of the immunodetection results obtained from cultures at 3, 10, 14, 28, and 35 DIV. As can be seen in Fig. 2, probing our cultures with subunit-specific antibodies for the NR2A and NR2B NMDAR subunits revealed the presence of the NR2B early in culture (3–14 DIV). Although the disappearance of the NR2B band varied between 14 and 21 DIV across the different culture batches probed, no NR2B was detectable after 21 DIV in any culture batch. As can be seen in the autoradiograph, the NR2A subunit could be detected by 10 DIV and in most cases was detectable by 6 DIV (data not shown). Expression increased linearly with age in culture for the NR2A subunit.

We report that ifenprodil sensitivity decreased between 6/7 and 10 DIV, a time when both the NR2A and NR2B subunits are present as indicated by immunodetection. Furthermore, ifenprodil sensitivity did not decrease with additional age in culture, even after the NR2B was no longer detected. The observation that ifenprodil sensitivity did not decrease with additional age in culture suggests that although the NR2B is present, the ifenprodil pharmacology of the NMDAR contained in these neurons seems to be more influenced by the NR2A between 6 and 21 DIV. A similar phenomenon has been reported in developing rat cortical neurons containing mixed NR2A/NR2B NMDARs (Stocca and Vicini, 1998).

These Western blot and pharmacological results parallel some aspects of the in vivo developmental profile of cerebellar NMDARs (Wang et al., 1995). However, in vivo, the late development of the NR2A is accompanied by increased expression of NR2C mRNA (Monyer et al., 1992; Watanabe et al., 1994) and NR2C-containing functional receptors (Farrant et al., 1994). However, when our CGC cultures were probed with a pan NR2 antibody, we did not detect another NR2 subunit (data not shown). This is consistent with previous observations that NR2C is not expressed in CGCs grown under depolarized conditions (Bessho et al., 1994).

**EtOH Sensitivity across Development of Primary Cultured CGCs.** We have previously reported changes in the EtOH sensitivity of primary cultured cortical neurons with increasing DIV (Lovinger, 1995). Western blot analysis of those cultures showed that the NR2B was detectable 1 week through 4 weeks in culture with the NR2A detectable at 3 and 4 weeks in culture (R.L.L. and D.M.L., unpublished data), a time during which EtOH inhibition dropped. However, unlike the cortical cultures, there was no change in EtOH sensitivity with age in culture for the CGC neurons. The main effects of age-in-culture and EtOH concentration on EtOH inhibition of NMDA-induced current as well as the
interaction between these two variables was analyzed using two-way ANOVA. The graph in Fig. 3A shows the mean percent inhibition of NMDA-induced peak current increasing with increasing EtOH concentration ($F = 113.22, df = 4/252, P \leq .0001$). There was no difference in the percentage of EtOH inhibition across age in culture ($F = 1.56, df = 4/252, P \leq .185$), and no interaction between EtOH concentration and age was observed ($F = 1.24, df = 16/252, P \leq .236$). Figure 3, B and C, shows representative current traces from a 6/7- and a >28-day-old CGC, respectively, and the effect of a 50 mM EtOH concentration on NMDA-induced current is illustrated. In these neurons, the inhibition produced by 50 mM EtOH was 18% and 16%, respectively.

We observed a subpopulation of neurons, approximately 14%, that were relatively insensitive to the inhibitory effect of EtOH. In 11 of 64 neurons, NMDAR function was potentiated by 10 mM EtOH. Potentiation ranged from 0.7 to 10.4% above baseline peak current amplitude. In four other neurons, NMDAR function was not inhibited by 10 mM EtOH. In addition, 7 of 57 neurons exhibited less than 15% inhibition by 100 mM EtOH. These EtOH “resistant” neurons were present at all ages in culture. This phenomenon has been previously reported at low EtOH concentrations in CGCs (Engblom et al., 1997) as well as other neuronal types (Lima-Landman and Albuquerque, 1989; Lovinger et al., 1989; Woodward and Gonzales, 1990).

**Relationship between Ifenprodil Sensitivity and EtOH Sensitivity in Primary Cultured CGCs.** Previous studies have reported a positive relationship between EtOH sensitivity and ifenprodil sensitivity (Lovinger, 1995; Fink and Göthert, 1996; Yang et al., 1996; Engblom et al., 1997). We had identified two populations of neurons that differed significantly in their sensitivity to ifenprodil. The next step was to determine whether these populations of neurons differed in their EtOH sensitivity. Because the results of ANOVA indicated that time in culture was not related to EtOH sensitivity, further statistical analysis was performed with the time in culture variable collapsed. To determine the relationship between ifenprodil sensitivity and EtOH sensitivity, linear regression analysis was performed on all neurons for each EtOH concentration. There was no correlation between ifenprodil sensitivity and EtOH sensitivity at any concentration of EtOH ($r^2 = 0.012, n = 60; 0.0, n = 50; 0.0, n = 59; 0.049, n = 50$; and $0.015, n = 33$ for 10, 25, 50, 100, and 200 mM EtOH, respectively). The two cells shown in Fig. 3, B and C, are good examples of this lack of correlation between EtOH and ifenprodil sensitivity. Although inhibition by EtOH was comparable in these two cells, ifenprodil inhibited current by 60% in the 6-day-old neuron as opposed to 23% in the 28-day-old neuron.

**Effect of Glycine Concentration on EtOH Inhibition of NMDA-Induced Current.** One factor that could contribute to variability in the inhibitory effect of EtOH observed in CGCs is an interaction with glycine. Published data have indicated the presence of such an interaction (Hoffman et al., 1989; Rabe and Tabakoff, 1990; Woodward and Gonzales, 1990; Dildy-Mayfield and Leslie, 1991; Buller et al., 1995). Therefore, the next group of experiments was designed to examine the effect of glycine concentration on EtOH inhibition of NMDA-induced current.

The affinity for glycine is altered by the type of NMDA NR2 subunit expressed, in that NMDARs containing the NR2B exhibit higher glycine affinity than those containing the NR2A (Stern et al., 1992). In our CGC cultures, the ratio of NR2A/NR2B subunit changed with DIV. It was therefore necessary to determine whether glycine affinity differed between young and old CGCs. Concentration-response analysis for glycine in the presence of 100 μM NMDA revealed not only a different potency of glycine when comparing 6/7-day relative to ≥28-day-old cultured CGCs but also revealed an apparent biphasic concentration-response curve in younger neurons and a monophasic relationship in older neurons (Fig. 4). The glycine $EC_{50}$ value in CGCs ≥28 DIV was 912 nM, and there were two $EC_{50}$ values for glycine in 6/7 DIV: 34.8 nM and 1 μM.

Results from our glycine concentration response analyses are similar to those observed by Kew et al. (1998) in cortical neurons. Like these investigators, we believe that the biphasic glycine concentration response we observed in our young CGCs reflects the function of a mixed population of receptors containing the NR2A or NR2B subunits. The single low potency site in old CGCs is most likely due to receptors containing predominantly NR2A. This hypothesis is quite consistent with our ifenprodil inhibition and immunological data, which indicate that NMDARs in our CGCs contain a mix of NR2A and NR2B subunits early in development but switch to predominant NR2A expression later in development.
different techniques used to examine NMDAR function. Neurochemical assays use intact neurons that have not been subjected to intracellular dialysis such as that which occurs during traditional WC recording. Thus, we compared EtOH-glycine interactions in cultured CGCs using WC patch-clamp and PP-clamp techniques. Perforated patch allows "near noninvasive" recordings of WC currents, and thus might approximate a noninvasive biochemical assay.

Age in culture did not change the relationship between glycine concentration and EtOH inhibition of NMDA-induced peak currents regardless of electrophysiological method used (analysis results not shown). Therefore, further statistical analysis was collapsed across the variable age in culture. EtOH inhibition was concentration dependent; therefore, the effects of glycine concentration and patch-clamp method were assessed using a low (10 mM) and a high (100 mM) EtOH concentration. Under WC patch-clamp conditions, the percentage of inhibition of NMDA-induced peak current produced by 10 or 100 mM EtOH was not diminished in the presence of a high glycine concentration (Table 1). However, under high glycine conditions and using the perforated patch-clamp method, the inhibitory effect of EtOH on peak current was significantly reduced from 14 ± 2.8 to 4.11 ± 1.43% (F = 10.26, df = 1/46, P ≤ .002, simple main effects test) (Table 1). Representative traces depicting 10 mM EtOH-induced inhibition of NMDA receptor-mediated currents using the PP method under low glycine conditions are shown in Fig. 5A. A high concentration of glycine reversed this inhibition to almost zero (Fig. 5B). At 100 mM EtOH, glycine concentration had no effect on EtOH inhibition of NMDA-induced peak current assessed using the PP conditions (Table 1).

However, 10 μM glycine did significantly reduce EtOH inhibition of NMDA-induced SS current under most conditions (Table 1). Inhibition of SS current by 10 mM was reduced to almost zero under high glycine conditions using both whole-cell (F = 8.84, df = 1/61, P ≤ .005, simple main effects test) and perforated patch-clamp (F = 5.44, df = 1/46, P ≤ .02, simple main effects test) techniques. Using the perforated patch-clamp technique, a high concentration of glycine significantly decreased the inhibitory effect of 100 mM on NMDA-induced SS current (F = 6.85, df = 1/61, P ≤ .01, simple main effects test) (Table 1). However, in whole-cell experiments exposure to a high concentration of glycine had no effect on 100 mM EtOH inhibition of NMDA-induced SS current (F = 2.34, df = 1/61, P ≤ .13, simple main effects test) (Table 1). Significant results from post hoc comparisons

![Figure 4](image_url) **Fig. 4.** Glycine dose-response curves for young (6/7 day) and old (≥28 day) CGCs. The dose-response curves were generated by comparing peak current resulting from coapplication of 100 μM NMDA and glycine concentrations ranging from 20 nM to 3.33 mM for 6/7 day-old CGCs and from 100 nM to 3.33 μM for ≥28-day-old CGCs normalized to responses in the presence of 10 μM glycine. The EC50 value for old CGCs was 912 nM, and there were two EC50 values for young CGCs: 34.8 nM and 1 μM.

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<th>EtOH effect on NMDA-induced current under high and low glycine conditions assessed using PP or WC patch-clamp electrophysiological techniques</th>
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<td>Values are mean percentage of change ± S.E. in amplitude of NMDA-induced peak and SS currents in the presence of 10 or 100 mM EtOH. WC data listed in this table were obtained from cells in all experiments in which the whole-cell patch-clamp method was used. Significance (*) of individual comparisons using post hoc Multiple t test are indicated.</td>
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* All individual comparisons between high and low glycine concentrations within each treatment method at either 10 or 100 mM EtOH: * P ≤ .05; ** P ≤ .01; *** P ≤ .005.
at individual EtOH and glycine concentrations are given in Table 1.

**Effect of EtOH Pretreatment on Inhibition of NMDA-Induced Current.** We compared the effect of simultaneous coapplication of EtOH and 100 μM NMDA (simultaneous application) with inhibition produced by coapplication of EtOH and these agonists immediately following a 30-s exposure to EtOH alone (pretreatment application). Experiments were first performed in the presence of a high concentration of glycine (10 mM). The effect of high (100 mM) and low (10 mM) EtOH concentration across method of application was also assessed. Thirty-second pretreatment with EtOH alone significantly enhanced the inhibitory effect of EtOH on NMDA-induced peak current ($F = 98.28$, df = 1/48, $P \leq .0001$, two-way ANOVA). We next performed the post hoc analysis using the Multiple $t$ test to compare inhibition with pretreatment versus simultaneous application at each EtOH concentration. Exposure to 10 mM EtOH alone before coapplication of EtOH and agonists increased inhibition of peak current from 11.1 ± 2.0% observed with simultaneous coapplication of EtOH and agonists to 31.1 ± 1.85 ($t = 7.56$, $P \leq .001$) (Fig. 6A). The pretreatment protocol also significantly increased inhibition of peak current by 100 mM EtOH over simultaneous coapplication of EtOH and agonists from 30.47 ± 2.2 to 49.0 ± 1.73% ($t = 7.2$, $P \leq .001$) (Fig. 6A). EtOH inhibition, using the pretreatment protocol, still significantly increased with increasing EtOH concentration ($F = 91.59$, df = 1/48, $P \leq .0001$, two-way ANOVA). This enhanced inhibition of peak NMDA-induced current produced by EtOH pretreatment was seen irrespective of the order in which the protocols were applied, and inhibition was completely reversible. Pretreatment did not significantly enhance inhibition of SS current ($F = 2.2$, df = 1/48, $P = .14$, two-way ANOVA).

Similar experiments were also performed in the presence of a low glycine concentration. Enhanced EtOH inhibition due to pretreatment was also observed under low glycine conditions. Both young and old CGCs were pretreated for 30 s with either 10 or 100 mM EtOH before exposure to EtOH plus agonists. The Dunn’s Multiple Comparison test indicated that there was no significant effect of age in culture on percentage of EtOH inhibition regardless of EtOH concentration or method of EtOH application (data not shown). Therefore, further analysis on the effect of EtOH pretreatment on NMDA-mediated current under low glycine conditions was performed combining data from young and old neurons.

Two-way ANOVA (EtOH concentration × treatment protocol) indicated that pretreatment significantly increased
EtOH inhibition of NMDA-induced peak current under low glycine conditions \( (F = 62.32, df = 1/44, P \leq .0001) \). The magnitude of inhibition was concentration dependent \( (F = 98.06, df = 1/44, P \leq .0001) \). Post hoc comparisons indicated that the percentage of inhibition produced by a 30-s pretreatment with 10 mM EtOH alone before exposure to EtOH and agonists \( (22.4 \pm 2.64) \) was significantly greater than inhibition during simultaneous coapplication of EtOH and agonists \( (1.0 \pm 2.8, t = 6.45, P \leq .001) \) (Fig. 6B). Thirty-second pretreatment with 100 mM EtOH alone before application of EtOH and agonists also enhanced percent inhibition from 27.26 \( \pm 1.8 \) to 44.13 \( \pm 2.7 \) \( (t = 4.92, P \leq .001) \) (Fig. 6B). Inhibition of SS current was not significantly enhanced by EtOH pretreatment for either EtOH concentration \( (F = 0.69, df = 1/48, P = .4, \text{two-way ANOVA}) \). Figure 6C contains representative examples of the enhanced EtOH inhibition of NMDA-mediated peak current produced by 30-s EtOH pretreatment.

**Glycine Interaction with Effects of EtOH Pretreatment on Inhibition of NMDA-Induced Current**. To assess if high glycine concentrations could reverse the inhibitory affects of EtOH on NMDA-mediated peak and SS currents under pretreatment conditions, we analyzed the data using a two-way ANOVA (glycine concentration \( \times \) application protocol) for either SS or peak current within each EtOH concentration. The percentage of inhibition of SS current decreased under high glycine conditions during both simultaneous and pretreatment conditions (data not shown). However, in only one instance was this decrease significant. A high concentration of glycine \( (F = 13.19, df = 1/46, P = .0007) \) did significantly reverse the inhibitory effects of 100 mM EtOH on NMDA-induced SS current under pretreatment conditions from 48.4 \( \pm 6\% \) inhibition to 24.7 \( \pm 6\% \) inhibition \( (t = 3.3, df = 1/46, P \leq .01) \) but not under simultaneous treatment conditions \( (t = 1.28, df = 1/46) \). Inhibition of SS current by 10 mM EtOH was not affected by glycine concentration \( (F = 2.22, P = .1, \text{two-way ANOVA}) \) under simultaneous or pretreatment \( (P = 0.53, P = .5, \text{two-way ANOVA}) \) conditions. However, analyzing the data using the One-sample \( t \) test to determine whether SS current in the presence of EtOH differed from control values indicated that 10 mM EtOH significantly inhibited SS current by 12.0 \( \pm 5\% \) using the simultaneous protocol under low glycine conditions \( (t = 2.45, df = 1/13, P \leq .05) \). Under high glycine concentrations, no significant change in SS current was observed in the presence of 10 mM EtOH \( (5.9 \pm 7\% \) change, \( t = 0.93, df = 1/13) \). This indicates that high glycine reversal of inhibition was observed under some conditions in this phase of the study. Additionally, the high concentration of glycine did not reverse the inhibitory effects using the pretreatment or simultaneous protocols at 10 or 100 mM EtOH on NMDA-induced peak current (analyses not shown). The lack of a significant glycine-induced alteration in EtOH inhibition of SS current under most conditions in this experiment is probably attributable to the smaller sample size used in comparison with the experiments presented in Table 1.

**EtOH Alters Decay of NMDAR-Mediated Current**. We examined the effect of EtOH on two indicators of macroscopic current desensitization: SS/Pk ratio and \( \tau_{\text{decay}} \). Steady-state-Pk ratio is a measurement of the extent of receptor desensitization at equilibrium, whereas \( \tau_{\text{decay}} \) is the rate of onset of this desensitization. This increase in the SS-Pk ratio is indicative of a decrease in receptor desensitization. We observed a differential effect by EtOH on macroscopic channel kinetics in the presence of different glycine concentrations. There was an increase in the SS-Pk ratio in the presence of EtOH under all high glycine conditions (repeated measures \( t \) values \( t = 2.24, 3.27, 2.36; df = 12 \) for 10 mM EtOH simultaneous application, 10 mM EtOH pretreatment application, and 100 mM EtOH simultaneous application, respectively) with the exception of 100 mM EtOH under the pretreatment condition \( (t = 0.7; df = 11) \) (see Table 2 for values). The time to reach steady state \( (\tau_{\text{decay}}) \) was greater in the presence of EtOH under high glycine conditions, but increases were not significant (analyses not shown). In general, similar increases were not observed in the presence of the low glycine concentration. The only exception to this pattern was a significant increase in SS-Pk observed when 10 mM EtOH was applied using the pretreatment protocol under low glycine conditions \( (t = 2.2, df = 11, P \leq .05) \). Also observed under low glycine conditions was a significant decrease in both SS/Pk ratio and \( \tau_{\text{decay}} \) in the presence of 100 mM EtOH using the simultaneous application protocol \( (t = 2.68 \) and 3.63, respectively, \( df = 11, P \leq .01) \). Table 2 lists the overall mean percentage of change relative to control values within each cell for SS-Pk and \( \tau_{\text{decay}} \).

**Rebound Increase in NMDAR-Mediated Current following EtOH Pretreatment**. We often observed that the magnitude of peak current generated by NMDA and glycine increased immediately upon washout of EtOH following the pretreatment protocol. This “rebound” phenomenon can be seen in the current traces depicted in Fig. 6C. Under high glycine conditions, this phenomenon was observed in 10 of 12 cells, and the range of these changes was large. The mean amplitude for peak current generated by NMDA and high glycine conditions following the end of the pretreatment protocol was 17.5 \( \pm 7 \) greater than NMDA-induced currents before the start of the pretreatment protocol. A similar phenomenon was observed under low glycine conditions with a comparable increase in peak current amplitude \( (19.6 \pm 7) \). This rebound effect was seen following pretreatment with 10 or 100 mM EtOH.

**TABLE 2**

EtOH effects on macroscopic desensitization kinetics of NMDA-induced currents

<table>
<thead>
<tr>
<th>Glycine Condition</th>
<th>10S</th>
<th>10P</th>
<th>100S</th>
<th>100P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS/Pk current (% change)</td>
<td>18.8 ( \pm 9^* )</td>
<td>20.9 ( \pm 6^{**} )</td>
<td>21.2 ( \pm 9^* )</td>
<td>17.1 ( \pm 24 )</td>
</tr>
<tr>
<td>( \tau_{\text{decay}} ) (% change)</td>
<td>12.5 ( \pm 12 )</td>
<td>20.9 ( \pm 16 )</td>
<td>31.3 ( \pm 17 )</td>
<td>39.5 ( \pm 20 )</td>
</tr>
</tbody>
</table>

\* Comparisons between EtOH and control value with repeated measures \( t \) test: \* \( P \leq .05 \); \** \( P \leq .01 \).
The increase in peak current following the end of EtOH exposure using the pretreatment protocol was accompanied in many cells by an increase in the rate of current decay, as seen by a decrease in $\tau_{\text{decay}}$ (see Fig. 6C for example). However, this effect was highly variable between cells and even varied considerably over the course of recordings from a single neuron.

**Discussion**

The most striking finding we have described is the increased sensitivity to EtOH inhibition following EtOH pretreatment. The magnitude of the increase in EtOH inhibition was comparable under low and high glycine conditions and was observed over a range of EtOH concentrations. Sizable inhibition could be observed at quite low EtOH concentrations using this protocol. The high EtOH sensitivity observed in CGCs under these conditions may be especially relevant to intoxication at low brain EtOH levels.

We reported that EtOH inhibition of peak NMDA-activated WC currents was unaffected by glycine concentration in CGCs. This was true for both high and low concentrations of EtOH. This lack of reversal of EtOH inhibition of peak current by high glycine concentrations using WC patch-clamp methodology is similar to results previously reported in primary cultured hippocampal neurons (Peoples et al., 1997). However, we wanted to study receptor function using an electrophysiological technique (PP), which would cause minimal disruption of the intracellular environment as is the case during biochemical assays. Using the PP-clamp method, a high concentration of glycine significantly reversed 10 mM but not 100 mM EtOH inhibition of NMDA-induced peak current.

High glycine concentrations reversed 10 and 100 mM EtOH inhibition of NMDAR steady-state current under both WC and PP-clamp conditions. Glycine reversal was more pronounced when using PP-clamp technique. The peak value of NMDA-induced current is a pre-equilibrium condition determined by a number of factors including the binding and unbinding of agonist, rates of opening and closing of NMDAR channels, and the onset of receptor desensitization. This process occurs within milliseconds and is unlikely to be influenced greatly by the balance between desensitization and resensitization processes. With continued exposure to agonist, more channels enter into the desensitized state. Electrophysiological measurement of NMDAR SS current reflects the achievement of equilibrium between open, closed, and desensitized states of the individual channels. The balance between desensitization and resensitization processes plays a key role in determining SS current levels (Benveniste et al., 1999, Orser et al., 1994). In biochemical assays used in past studies, changes in $[Ca^{2+}]$, were measured seconds after agonist application and therefore most likely reflect steady-state NMDAR function, similar to the SS current through the NMDARs we measure electrophysiologically. Thus our observation of glycine reversal of SS current is consistent with the effects of glycine first observed by Hoffman and coworkers (Hoffman et al., 1989; Rabe and Tabakoff, 1990).

Our findings are consistent with a decrease in the balance of desensitization/resensitization (SS/Pk ratio) of the NMDAR in the presence of EtOH and high glycine. It has been observed that glycine rescues the NMDAR from the desensitized state, leading to an increased SS/Pk current ratio in the presence of glycine (Benveniste et al., 1999), similar to that which we observed in the presence of EtOH and high concentrations of glycine. It is possible that EtOH promotes entry into the desensitized state, as suggested by Wright et al. (1996), and that glycine counteracts this effect. However, inhibition of peak current remains even in the presence of a high glycine concentration, suggesting that inhibition is not solely due to enhancement of desensitization by EtOH. These issues will have to be sorted out in future studies using more rigorous methods for kinetic analysis.

Ethanol sensitivity has been suggested to vary with age in culture (Lovinger, 1995) and with the relative level of NR2B expression as indicated by ifenprodil sensitivity (Lovinger, 1995; Fink and Göhert, 1996; Yang et al., 1996; Engblom et al., 1997). We examined the importance of these two factors in determining EtOH sensitivity of the NMDARs contained in our CGCs. The EtOH sensitivity of our CGCs did not change between 6 and 35 DIV even though there was a change in the relative proportions of the NR2A and NR2B subunits during this time. This suggests that there was no significant difference in EtOH sensitivity attributable to changes in the proportion of NR2A or NR2B subunits contained in these neurons.

Ethanol sensitivity was not related to ifenprodil sensitivity in our CGCs over the developmental time course we examined. Although the highest degree of ifenprodil-induced inhibition reported in these cells was only 61%, this value is comparable with previously reported values (64 ± 4 and 50 ± 5% inhibition) for native NMDARs in primary cultured cortical neurons 3 to 4 weeks in culture, a time at which EtOH sensitivity of the NMDAR was low (Lovinger, 1995). Therefore, it is possible that NMDARs contained in CGCs are generally less sensitive to EtOH. However, EtOH inhibition did not differ significantly from previously published values for NMDARs expressed in primary cultured striatal neurons, which were potently inhibited by 10 μM ifenprodil (Popp et al., 1998). A positive correlation between ifenprodil sensitivity and EtOH sensitivity in primary cultured cortical neurons was previously reported (Lovinger, 1995). We reported no correlation between ifenprodil and EtOH sensitivity at any concentration of EtOH in this study. Once again, this supports the idea that high EtOH sensitivity is not conferred solely by the presence of NR2B but must involve other factors (Lovinger 1995, Yang et al., 1996).

At the time we conducted our functional assays (6/7 DIV), there was a mixture of NR2A and NR2B NMDA subunits present. It is possible that the EtOH and ifenprodil sensitivity of receptors would be greater at earlier DIV (e.g., 3 days), when NR2B expression predominates. This could be evaluated by recording from CGCs at earlier developmental time points. However, we were not able to obtain satisfactory electrophysiological recordings from primary cultured CGCs before 6 DIV and thus could not fully explore this possibility.

Our finding that EtOH sensitivity was not related to ifenprodil sensitivity in CGCs seems to contradict the results of Engblom et al. (1997) who reported no EtOH-induced inhibition of NMDAR function in CGCs grown under depolarizing conditions when the proportion of the NR2A subunit seemed
to be very high. These investigators did observe an increase in both EtOH and ifenprodil inhibition in CGCs grown under nonpolarizing conditions. It is difficult to directly compare the magnitude of EtOH inhibition in the two studies for the following reasons. First, we did not examine CGCs grown under nonpolarized conditions; thus we do not know if this treatment would increase EtOH and ifenprodil sensitivity in our cultures. Second, the methodology used by Engblom et al. (i.e., measurement of Ca$^{2+}$ transients and pre-exposure to NMDA before determination of EtOH and ifenprodil inhibition) differed considerably from that used in the present study. Thus, there are several possible explanations for the apparent discrepancy in the results obtained in the two studies, but exploration of these possibilities is beyond the scope of the present report.

The results of the present experiments indicate that EtOH sensitivity of NMDAR function in CGCs differs from that observed in other cells in several ways. Unlike other neurons, neither age in culture nor the presence of different proportions of the NR2B and NR2A subunits seems to contribute to the EtOH sensitivity in these cells. In CGCs, EtOH affects the apparent rate of desensitization. This differs from previously published data reporting no change in the rate of current decay due to EtOH in rat cortical neurons (Lovinger and Peoples, 1993). EtOH inhibition also seems to involve a glycine-sensitive component in CGCs that has not been consistently observed in forebrain neurons. In addition, perforated patch data indicate the possible involvement of an intracellular factor that can enhance this glycine-sensitive component, at least in the presence of a low EtOH concentration.

The intracellular component could be protein kinase C, as suggested by the findings of Snell et al. (1994), or could be another protein kinase or even a structural protein. EtOH sensitivity in CGCs is also enhanced following a brief pre-exposure to EtOH, an effect not observed for NMDARs in oocytes using a different pre-exposure duration (Mirshahi and Woodward, 1995). Because, in vivo, EtOH is continuously exposed to the brain, the time-dependent effects of EtOH pretreatment and the large inhibition produced by 10 mM EtOH (a concentration below legal intoxication) seem to be extremely important. It will be interesting to identify further interactions between these factors that affect the EtOH sensitivity of these neurons. We have observed pre-treatment-enhanced EtOH inhibition under perforated patch-clamp conditions (R.L.P. and D.M.L., unpublished data). Future experiments will examine the mechanism by which pretreatment increases EtOH inhibition of NMDAR function.

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