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# Ethanol sensitivity of NMDA receptor function in developing cerebellar granule neurons

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**Abstract**

The mechanism by which ethanol inhibits the function of the NMDA subtype of glutamate receptor has not been elucidated. One possibility that has been suggested is that NMDA receptor subunit composition influences the sensitivity of the receptor to ethanol. We have taken advantage of developmental changes in subunit composition of the NMDA receptor in cultured neurons to examine possible changes in the effect of ethanol. We found an increase in expression of the NR2A subunit, and a decrease in expression of the NR2B subunit of the NMDA receptor in primary cultures of cerebellar granule neurons over time in culture, with no significant change in NR1 expression. This change in NR2 subunit expression was associated with the expected changes in functional properties of the NMDA receptor (measured as the NMDA-induced increase in intracellular $Ca^{2+}$), i.e., ifenprodil sensitivity and glycine potency were higher when there was a relatively greater proportion of NR2B in the cultured neurons. However, the potency of ethanol to inhibit NMDA receptor function was *lower* when there was a greater proportion of NR2B subunits. Previous studies showed that ethanol inhibition of NMDA receptor function in cerebellar granule neurons resulted from an ethanol-induced decrease in potency of the co-agonist, glycine, and that this effect of ethanol was blocked by inhibitors of protein kinase C. Our current results suggest that the lower potency of ethanol to inhibit the response of NMDA receptors when cerebellar granule neurons are expressing a greater proportion of NR2B subunits is a result of the *higher* affinity of the NMDA receptors for endogenous levels of glycine at this point in time. 

*Keywords:* NMDA receptor; Cerebellar granule neuron; Ifenprodil; Ethanol

## 1. Introduction

Ethanol is a potent inhibitor of the function of the NMDA subtype of glutamate receptor (Hoffman et al., 1990). However, the molecular determinants of sensitivity to ethanol are not fully understood. The native NMDA receptor is a ligand-gated ion channel which is believed to consist of a heteromeric assembly of subunits (Mori and Mishina, 1995). The NR1 subunit, which has eight splice variants (Nakanishi et al., 1992; Sugihara et al., 1992), is localized throughout the brain, and can form functional channels when expressed in *Xenopus* oocytes (Sugihara et al., 1992). A family of NR2 subunits (NR2A-D) has also been identified (Ishii et al., 1993). These subunits are discretely localized in brain, and do not form functional receptors when expressed individually in oocytes (Ishii et al., 1993). However, when NR1 and NR2 subunits are expressed in combination, receptors are formed that have properties similar to native NMDA receptors (Mori and Mishina, 1995).

The presence of a particular NR2 subunit in a receptor has a significant influence on the pharmacological characteristics of the receptor (Mori and Mishina, 1995). This property has led to investigations of the influence of NMDA receptor subunit composition on ethanol inhibition of the function of recombinant NMDA receptors expressed in *Xenopus* oocytes or mammalian cells (Koltchine et al., 1993; Masood et al., 1994; Lovinger, 1995; Buller et al., 1995; Chu et al., 1995). In general, such studies have not demonstrated substantial differences in the response of the NMDA receptors to ethanol, although receptors composed of NR1/NR2C or NR1/NR2D subunits are less sensitive to ethanol inhibition than those comprising NR1/NR2A or NR1/NR2B subunits (Kuner et al., 1993; Masood et al., 1994; Chu et al., 1995). On the other hand, comparisons of the inhibitory effect of ethanol with the inhibitory effect of ifenprodil, a non-competitive antagonist that selectively

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inhibits the function of NMDA receptors that contain the NR2B subunit (Williams, 1993; Blevins et al., 1997), have led to the hypothesis that, in *native* receptors, the presence of the NR2B subunit may be a necessary (although not sufficient) component of NMDA receptors that are sensitive to ethanol inhibition (Lovinger, 1995; Yang et al., 1996). To further assess the factors that affect the ethanol sensitivity of native NMDA receptors, we have, in the present study, evaluated the effect of ethanol and other modulatory agents on NMDA receptor function in cerebellar granule neurons, during a period of neuronal development in which changes in NMDA receptor subunit expression occur.

## 2. Materials and methods

Dizocilpine, glycine, ifenprodil, kainate, and NMDA were obtained from Research Biochemicals (Natick, MA). Fura-2-acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR). Ionomycin was obtained from Calbiochem (San Diego, CA). Basal medium Eagle (BME), fetal bovine serum and TRIzol were from Gibco-BRL (Gaithersburg, MD). The RPA II kit was obtained from Ambion (Austin, TX). Polyclonal antibodies for the NMDA receptor NR2A and NR2B subunits were kindly provided by Dr. M.D. Browning (Department of Pharmacology, University of Colorado Health Sciences Center, Denver, CO), and monoclonal antibody for the NMDA receptor NR1 subunit (that recognizes all splice variants) was purchased from Pharmingen (San Diego, CA). Renaissance™ chemiluminescence reagents and Reflection™ film were purchased from Dupont-New England Nuclear (Boston, MA). All other commercial products were from Sigma (St. Louis, MO). Amino acid analysis to assess glycine levels was performed by Commonwealth Biotechnologies (Richmond, VA).

### 2.1. Cell culture

Primary cultures of cerebellar granule neurons were prepared from 7-day-old Sprague–Dawley rat pups as described previously (Iorio et al., 1992). For intracellular calcium ion ($[Ca^{2+}]_i$) measurements, cells ($2 \times 10^6$ cells/well) were plated on glass coverslips coated with polyethyleneimine (100 μg/ml). Cells ($2 \times 10^7$ cells/100 mm dish) plated on tissue culture dishes coated with poly-L-lysine (10 μg/ml) were used for the extraction of total protein or total RNA. Experiments were carried out with cells maintained in culture for either 4 or 7 days, as indicated.

### 2.2. Western blot analysis

Cultures were washed twice with ice-cold phosphate-buffered saline, harvested in a buffer containing 2 mM EDTA, 2.3% sodium dodecyl sulfate, 10% glycerol and 62.5 mM Tris (pH 6.9) and then frozen at −80°C (Hoffman et al., 1996). For immunoblotting, the samples were thawed and protein concentrations were determined using the bicinchoninic acid method (Pierce, Rockford, IL). Each sample was then solubilized by addition of dithiothreitol and urea (final concentrations, 150 mM and 2.4 M, respectively) followed by boiling for 3 min. Solubilized samples (20 μg per lane) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 7.5% resolving gels, and proteins were transferred to nitrocellulose membranes (0.22 μm, Schleicher and Schuell, Keene, NH) according to the procedures described by Snell et al. (1996). After blocking, blots were probed with subunit-specific antibodies to the NR1, NR2A or NR2B proteins (Hoffman et al., 1996; Snell et al., 1996). Blots were then incubated with horseradish peroxidase-conjugated goat immunoglobulin G (goat anti-mouse for NR1 or goat anti-rabbit for NR2A and NR2B). Immunoreactive bands were visualized on film using the enhanced chemiluminescence method. Quantitation of protein bands was performed by image analysis (Image 1.39, NIH shareware) as previously described (Snell et al., 1996).

### 2.3. RNase protection assays

Total RNA from cerebellar granule neurons was obtained using the TRIzol reagent. Antisense probes for NR1, NR2A and NR2B were obtained by reverse transcriptase–polymerase chain reaction from rat brain mRNA as previously described (Hoffman et al., 1996), and corresponded to nucleotide sequence 73–348 for NR1, 3979–4396 for NR2A and 3007–3368 for NR2B. The cDNA products were cloned into the Bluescript II SK(−) vector, which was then linearized and used as a template in an [α-$^{32}$P]UTP-labeled cRNA probe synthesis reaction (Maxiscript T7/T3 kit, Ambion). A β-actin probe, obtained from Ambion, was labeled to low specific activity and used to correct for loading artifacts in the assay. Solution hybridization/RNase protection assays were carried out according to the RPA II kit protocol. Protected fragments were separated on a 5% denaturing polyacrylamide gel. Autoradiograms were analyzed using the Bio-Rad GS-250 Molecular Imager and PhosphorAnalyst image analysis software. The results are expressed as the ratio of the volume (area × Phosphor counts) of the NR1, NR2A or NR2B band to the volume of the corresponding β-actin band.

### 2.4. Measurements of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was determined by measuring fura-2 fluorescence, as previously described (Snell et al., 1994a). In brief, cerebellar granule neurons were loaded with 5 μM fura-2-acetoxymethyl ester for 60 min at 37°C. The glass

coverslips were then rinsed with $Mg^{2+}$-free cell buffer (145 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 1 mM $Na_2HPO_4$ and 1 mM $CaCl_2$, pH 7.4) and transferred to a cuvette containing 2 ml of cell buffer maintained at 37°C with constant stirring. Fluorescence was measured using an SLM-Aminco spectrofluorometer (excitation at 345 and 380 nm; emission at 505 nm). The data were stored on a NEC 286 computer using the software from SLM-Aminco (Urbana, IL). Determinations of $R_{max}$ and $R_{min}$ were carried out as previously described (Snell et al., 1994a). Intracellular free calcium concentrations were determined according to Grynkiewicz et al. (1985).

#### 2.4.1. General procedure

Glass coverslips with adherent cells were placed in a cuvette fitted with a superfusion system attached to a peristaltic pump. The coverslip was perfused with cell buffer for 2 min at a flow rate of 4 ml/min and the basal levels of $[Ca^{2+}]_i$ were then recorded for 30 s. At this time, in some experiments, a 50 μl sample of cell buffer was removed and stored at −20°C for later amino acid analysis. NMDA (100 μM) was then added and NMDA-induced changes in the levels of $[Ca^{2+}]_i$ were recorded for 30 s ($S_1$). The NMDA was washed out by superfusing the coverslip with buffer for 3 min. Basal levels of $[Ca^{2+}]_i$ were redetermined for 30 s, in the absence or presence of either ethanol or ifenprodil, and the change in $[Ca^{2+}]_i$ induced by a second addition of NMDA (100 μM) was monitored for 30 s ($S_2$). In some of the experiments, addition of NMDA was followed by the addition of 10 μM glycine, for both the $S_1$ and $S_2$ stimulations (see Fig. 3). In other experiments, cells were perfused with buffer containing 1 μM 5,7-dichlorokynurenic acid and 1 μM glycine during the $S_1$ and $S_2$ stimulations.

For each stimulation, the effect of NMDA on $[Ca^{2+}]_i$ was calculated by subtracting the basal value (before the addition of NMDA) from the peak value obtained after the addition of NMDA. This value is noted as $\Delta[Ca^{2+}]_i$ (nM). In most instances, results are expressed as the ratio of the response to the second stimulation over the response to the initial stimulation ($S_2/S_1$, the '$\Delta[Ca^{2+}]_i$ ratio') (Snell et al., 1994a). Expression of data as the $\Delta[Ca^{2+}]_i$ ratio reduced the variability among the cerebellar granule cell preparations.

The effect of ifenprodil on kainate-induced changes in $[Ca^{2+}]_i$ was studied using an identical protocol, except that 1 μM dizocilpine was present in the cell buffer, to block NMDA receptor activation, and kainate (100 μM) was used for the first and second stimulations.

To generate glycine concentration-response curves for $pA_2$ analysis, the $S_1$ stimulation was carried out using 100 μM NMDA and 10 μM glycine. The washout after $S_1$ was followed by the addition of varying concentrations of glycine in the absence or presence of varying concentrations of 5,7 dichlorokynurenic acid (see Figs. 5 and 6), prior to the second NMDA (100 μM) stimulation ($S_2$).

### 2.5. Statistical analysis and calculations

Statistical analysis of the data was performed by analysis of variance (ANOVA) followed by post-hoc comparisons, or with Student's *t*-test, as appropriate, using the Sigmastat 1.0 statistical program. In experiments where data were expressed as a $\Delta[Ca^{2+}]_i$ ratio, an arcsine $(x/2)$ transformation was performed before statistical analysis. All data are presented as mean ± S.E.M. unless otherwise noted. $P < 0.05$ was taken to be statistically significant.

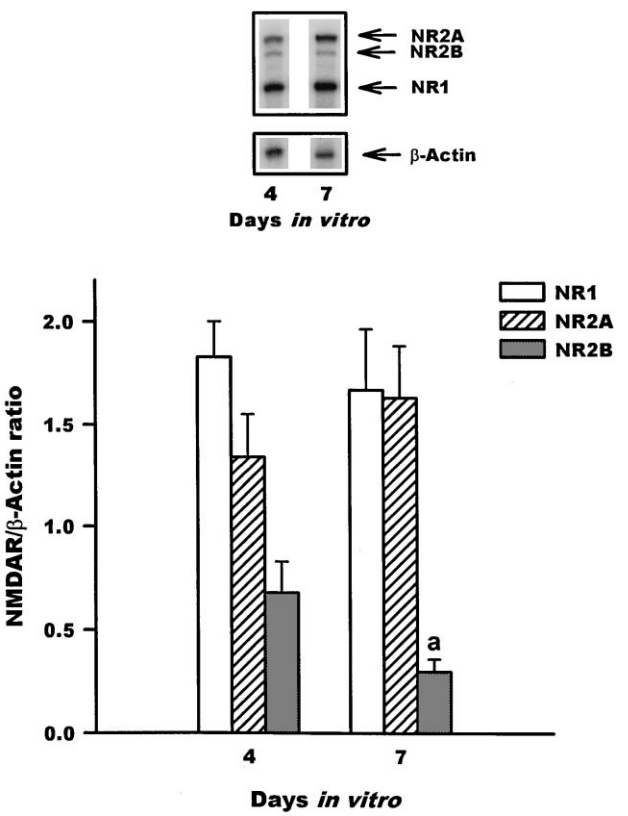


Fig. 1. Expression of mRNAs for NMDA receptor subunits in cerebellar granule neurons. Expression of NMDA receptor (NMDAR) subunit mRNA in cerebellar granule neurons maintained in vitro was assessed using RNase protection assays. Total RNA was extracted from cells, and probes were synthesized, according to the procedures described in Section 2. The RNase protection assays were performed using the RPA II kit (Ambion). The average values from quantitative densitometry of the labeled bands (inset shows a representative RNase protection assay) from five to 15 samples are shown in the graph. The data are presented as the mean ± S.E.M. ratio of the adjusted volume (area × Phosphor counts) of the mRNA bands for NR1, NR2A or NR2B to the band for β-actin. β-actin mRNA levels were not significantly different on days 4 and 7 in culture (densitometric units, mean ± S.E.M.: 4 days, 8835 ± 1297, $n = 5$; 7 days, 9218 ± 1115, $n = 15$). [a] $P < 0.05$ compared to NR2B/β−actin ratio on day 4 in culture (Student's *t*-test).

$IC_{50}$ values were calculated from the best-fit regression lines to the following equation:

$$\text{Response} = \frac{\text{Response}_{max} - \text{Response}_{min}}{\left[1 + \left(\frac{[\text{Drug}]}{IC_{50}}\right)^{n}\right]} + \text{Response}_{min}$$

where $\text{Response}_{max}$ is the maximum $\Delta[Ca^{2+}]_i$ ratio (in the presence of 100 μM NMDA and 10 μM or greater glycine concentration), $\text{Response}_{min}$ is the minimum $\Delta[Ca^{2+}]_i$ ratio (in the presence of added NMDA alone), [Drug] is the concentration of ethanol or ifenprodil, and n is the slope of the regression line.

### 2.5.1. Glycine concentration–response curves

A baseline endogenous concentration of glycine was present in all experiments, as illustrated in Fig. 3 by the presence of a response to the addition of NMDA alone. Iterative curve fitting was therefore used to determine best fit lines for glycine concentration–response data, using a modification of a two-equivalent binding site model for monophasic fits as described by Kew et al. (1998),

$$\text{Response} = \frac{\text{Response}_{max}}{\left(1 + \frac{K_D}{[A + g]}\right)^{2}}$$

where $\text{Response}_{max}$ is the maximum $\Delta[Ca^{2+}]_i$ ratio (in the presence of 100 μM NMDA and 10 μM or greater added glycine concentration), $K_D$ is the dissociation constant for glycine, $[A]$ is the concentration of added glycine, and $g$ represents the endogenous glycine concentration. This model is derived from electrophysiological studies of the activation kinetics of NMDA channels indicating that the binding of NMDA and glycine are independent and that two molecules of glycine, binding with equal affinity (i.e., nH = 1), are required for full activation. The calculated endogenous glycine concentration was added to each glycine concentration used in the concentration–response curve to give the corrected glycine concentration. The data were normalized by dividing by the $\text{Response}_{max}$, and then replotted using the corrected glycine concentrations and fitted with the two-equivalent binding site equation excluding the term for the endogenous glycine ($g$).

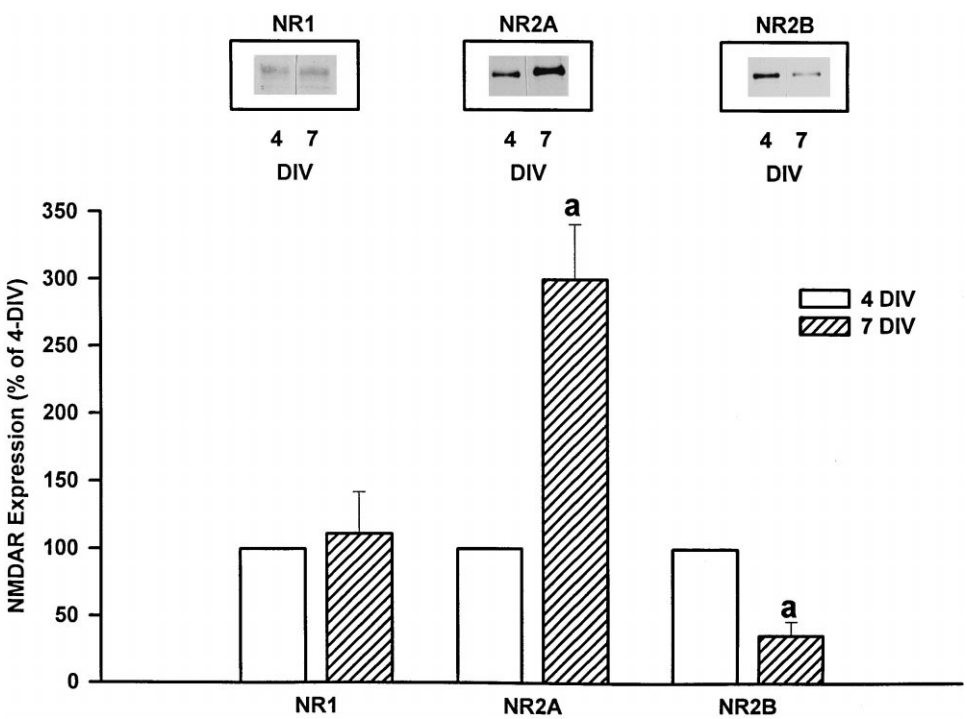


Fig. 2. Expression of NMDA receptor subunit proteins in cerebellar granule neurons. Expression of NMDA receptor (NMDAR) subunit proteins in cerebellar granule neurons was assessed by using subunit-specific antibodies (Snell et al., 1996). Total protein was extracted from the cells on day 4 and day 7 in vitro according to the procedures described in Section 2. Immunoblots for NR1, NR2A and NR2B from one set of cultures extracted on day 4 and day 7 in vitro are shown. Equal amounts of solubilized protein (20 μg) were added to each lane. NR1 monoclonal antibody recognized a major band of about 110 kDa and a doublet of about 100 kDa. All bands were included in quantitation. The NR2A and NR2B polyclonal antibodies each recognized a single band of about 165 kDa. The mean ± S.E.M. values from the quantitative densitometry of immunoreactive bands from 4–5 sets of cultures are shown in the graph. The densitometry values obtained for day 7 samples are expressed as the percent of those obtained for day 4 samples. [a] $P < 0.05$, compared to 100% (one sample *t*-test). DIV, days in vitro.

*2.5.2. Calculation of 5,7-dichlorokynurenic acid affinity*

The procedure described above was repeated for glycine concentration-response data obtained in the presence of 0.3, 1 and 3 μM concentrations of 5,7-dichlorokynurenic acid. $pA_2$ calculations (dose-ratio method) were performed using the corrected glycine concentration-response curves, according to the procedure described by Tallarida and Murray (1984). Dose ratios were calculated by dividing the concentration of glycine required to produce a 72% increase in the NMDA-induced $\Delta[Ca^{2+}]_i$ ratio for each glycine concentration-response curve in the presence of 5,7-dichlorokynurenic acid ($A'$), by the concentration of glycine required to produce the same response in the absence of 5,7-dichlorokynurenic acid ($A$). The size of the NMDA-induced response (72%) fell on the ascending slope of all glycine concentration–response curves and was larger than the smallest measured response used to calculate the best fit line on a given curve. Schild plots were constructed by plotting log $(A'/A-1)$ against -log[5,7-dichlorokynurenic acid]. The $pA_2$ value was obtained from the abscissa intercept of the best-fit linear regression line with unit slope, using the least squares criterion.

## 3. Results

*3.1. NMDA receptor expression in cerebellar granule neurons over time in culture*

Fig. 1 shows the mRNA levels for NR1, NR2A and NR2B in cerebellar granule neurons maintained in culture for 4 or 7 days. Quantitation of the appropriate protected fragments in the RNase protection assay revealed that NR1

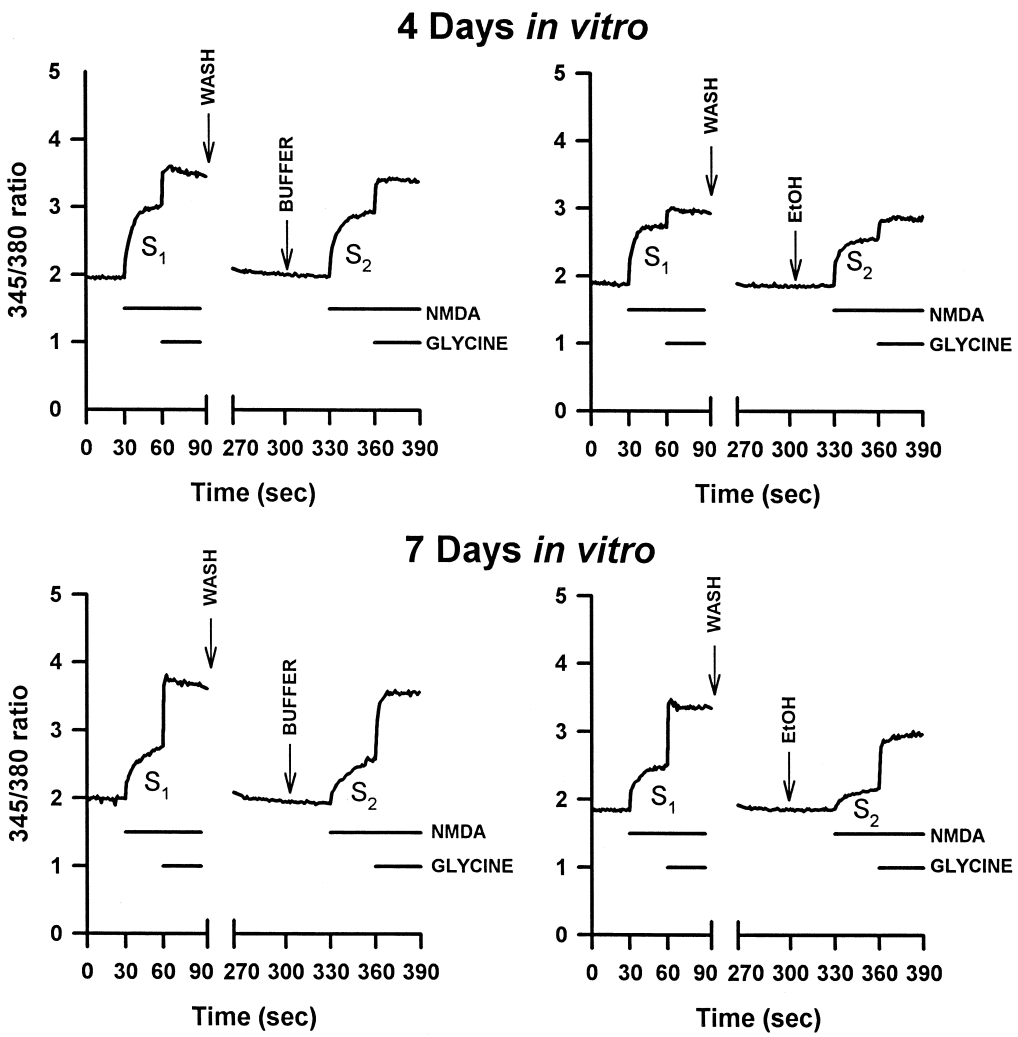


Fig. 3. Effect of ethanol on NMDA-induced increase in $[Ca^{2+}]_i$ in cerebellar granule neurons. Cerebellar granule neurons were cultured on glass coverslips coated with polyethyleneimine, and the NMDA-induced changes in $[Ca^{2+}]_i$ were determined with fura-2 fluorescence, as described in Section 2, on either day 4 or day 7 in vitro. Neurons were exposed to 100 μM NMDA and the change in $[Ca^{2+}]_i$ was monitored for 30 s. The change in $[Ca^{2+}]_i$ was monitored for another 30 s after addition of 10 μM glycine. NMDA was washed out by superfusing the coverslip with cell buffer, and neurons were then re-exposed to NMDA and NMDA plus glycine in the absence or presence of ethanol (EtOH; 100 mM).

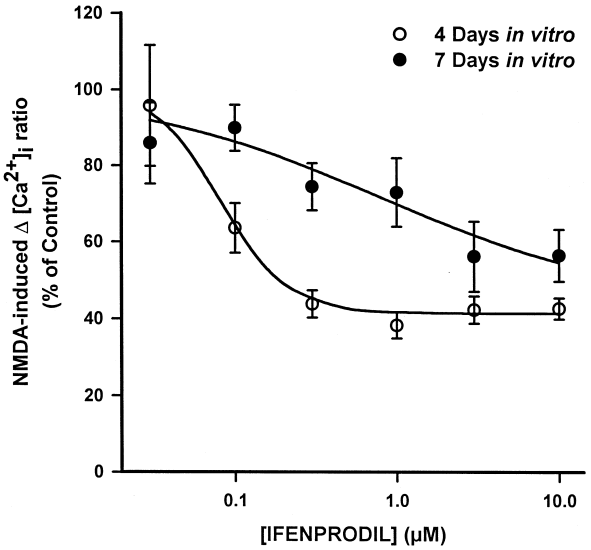


Fig. 4. Concentration dependence of ifenprodil inhibition of the NMDA-induced increase in $[Ca^{2+}]_i$ in cerebellar granule neurons. Cerebellar granule neurons were prepared, and changes in $[Ca^{2+}]_i$ were determined as described in the text and legend to Fig. 3. Cells were exposed to 100 μM NMDA, washed and re-exposed to 100 μM NMDA in the absence or presence of the indicated concentrations of ifenprodil. Data were calculated as the $\Delta[Ca^{2+}]_i$ ratio as described in the text and are presented as percent of control ratio ($\Delta[Ca^{2+}]_i$ ratio obtained in the absence of ifenprodil). Values represent the mean ± S.E.M. of 3–13 observations. ANOVA revealed a significant main effect of developmental stage of cerebellar granule neurons ($F = 18.82$, df = 6,67, $P < 0.001$), a significant main effect of ifenprodil ($F = 23.26$, df = 6,67, $P < 0.001$) and a significant interaction between ifenprodil and the developmental stage of the cerebellar granule cells ($F = 3.24$, df = 6,76, $P < 0.01$). Post-hoc comparisons (Newman–Keuls test) showed significant differences between cells on day 4 and day 7 in culture at 0.1, 0.3, and 1.0 μM ifenprodil ($P < 0.05$). In cerebellar granule neurons on day 4 in culture, post-hoc analysis revealed significant differences between control and 0.3, 1, 3 and 10 μM ifenprodil values; on day 7 in culture, between control and 1, 3 and 10 μM ifenprodil values.

mRNA did not change significantly between days 4 and 7. NR2A mRNA increased by approximately 20% between day 4 and day 7, but this change did not reach statistical significance. On the other hand, NR2B mRNA was significantly *decreased* (by 56%) on day 7, as compared to day 4.

Protein levels of the NMDA receptor subunits, assessed by immunoblotting, are shown in Fig. 2. Quantitative analysis of the immunoblots indicated that the level of the NR1 protein did not change between days 4 and 7 in culture. However, there was a significant increase (about 200%) in the level of NR2A protein on day 7, compared to day 4, and a significant decrease (about 70%) in the level of NR2B protein on day 7, compared to day 4 (Fig. 2).

### *3.2. NMDA receptor function in cerebellar granule neurons over time in culture*

In order to determine whether the observed changes in NMDA receptor subunit proteins over time in culture were associated with functional changes in the receptor, we evaluated the modulation of the NMDA response (increase in $[Ca^{2+}]_i$), in neurons maintained for 4 or 7 days in culture, by agents whose action at the NMDA receptor is known to be influenced by receptor subunit expression. Fig. 3 shows representative fluorometric traces from these neurons. The basal level of $[Ca^{2+}]_i$ in the cerebellar granule neurons was 50 ± 4 nM on day 4 in culture, and 45 ± 4 nM on day 7 in culture (mean ± S.E.M. of 6–7 observations). In none of the experiments described below was this level of basal calcium altered by the inclusion of ethanol or ifenprodil. The increase in $[Ca^{2+}]_i$ produced by 100 μM NMDA without added glycine was significantly higher in neurons maintained in culture for 4 days, compared to 7 days (138 ± 9 nM on day 4 and 103 ± 10 nM on day 7, mean ± S.E.M. of 6–7 observations; $P < 0.05$, Student's *t*-test). However, the total increase in $[Ca^{2+}]_i$ produced by NMDA (100 μM) and 10 μM added glycine was the same on days 4 and 7 (286 ± 32 nM and 286 ± 27 nM respectively, mean ± S.E.M. of 6–7 observations).

#### *3.2.1. Response to ifenprodil*

Ifenprodil is a non-competitive antagonist that has been found to selectively inhibit the function of recombinant NMDA receptors that contain the NR2B subunit (Williams, 1993). The concentrations of ifenprodil used in the current studies were chosen to act preferentially at the 'high-affinity' ifenprodil site, i.e., the site associated with the NR2B subunit. As shown in Fig. 4, ifenprodil inhibited the NMDA response in a concentration-dependent manner in cerebellar granule neurons maintained in culture for 4 or 7 days. However, ifenprodil was a more potent inhibitor in neurons maintained for 4 days in culture, where the $IC_{50}$ was calculated to be 80 nM. The shallowness of the

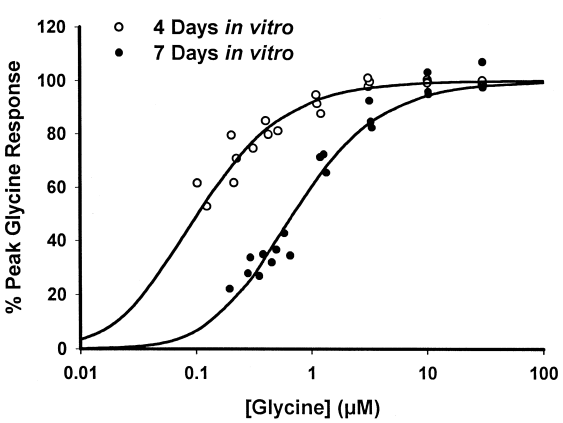


Fig. 5. Estimation of glycine affinity and endogenous glycine concentration. Cerebellar granule neurons were prepared and $[Ca^{2+}]_i$ was measured as described in the text and legend to Fig. 3. Cells were exposed to 100 μM NMDA and the indicated glycine concentrations. Each data point represents a value from a single observation. Glycine concentrations have been corrected for the calculated endogenous glycine level, and best-fit lines were computed using a two-equivalent binding site model, as described in the text.

Table 1
Calculated NMDA receptor glycine dissociation constants and endogenous glycine concentrations in cerebellar granule neuron preparations

| | Day 4 in vitro | Day 7 in vitro |
|---|---|---|
| $K_{Gly}$ (nM) | 43 ± 8 | 288 ± 38[a] |
| Endogenous [Gly] (nM) | 147 ± 34 | 276 ± 46 |

Glycine dissociation constants and endogenous glycine concentrations were calculated using the modified two-equivalent binding site model equation, as described in Section 2.
Values represent mean ± S.E.M. ($n = 3$).
[a] $P < 0.05$, compared to day 4 in vitro (Student's *t*-test).

inhibition curve obtained with cells on day 7 in culture precluded an accurate determination of an $IC_{50}$ value, but an estimated value of 809 nM was obtained. These data are compatible with the conclusion that there are few 'high-affinity' ifenprodil sites on day 7 in culture. Ifenprodil had no effect on the increase in $[Ca^{2+}]_i$ produced by 100 μM kainate on either day 4 or day 7 in culture (data not shown), confirming that ifenprodil, under conditions used for our assays, acted primarily at the NMDA receptor.

### 3.2.2. Response to glycine

The affinity of the NMDA receptor for the co-agonist, glycine, has been reported to be affected by the subunit composition of the receptor, with receptors containing the NR2B subunit showing higher affinity than those containing the NR2A subunit (Laurie and Seeburg, 1994). We evaluated the potency of glycine to increase the response to NMDA, and the potency of the competitive glycine site antagonist, 5,7-dichlorokynurenic acid, to inhibit the NMDA response, in cerebellar granule cells maintained for 4 or 7 days in culture. A glycine concentration–response curve (Fig. 5) was generated as described in Section 2, and the apparent dissociation constant for glycine calculated from these data was significantly lower (i.e., higher apparent affinity) in cells maintained for 4 days in culture than in cells maintained for 7 days in culture (Table 1). The

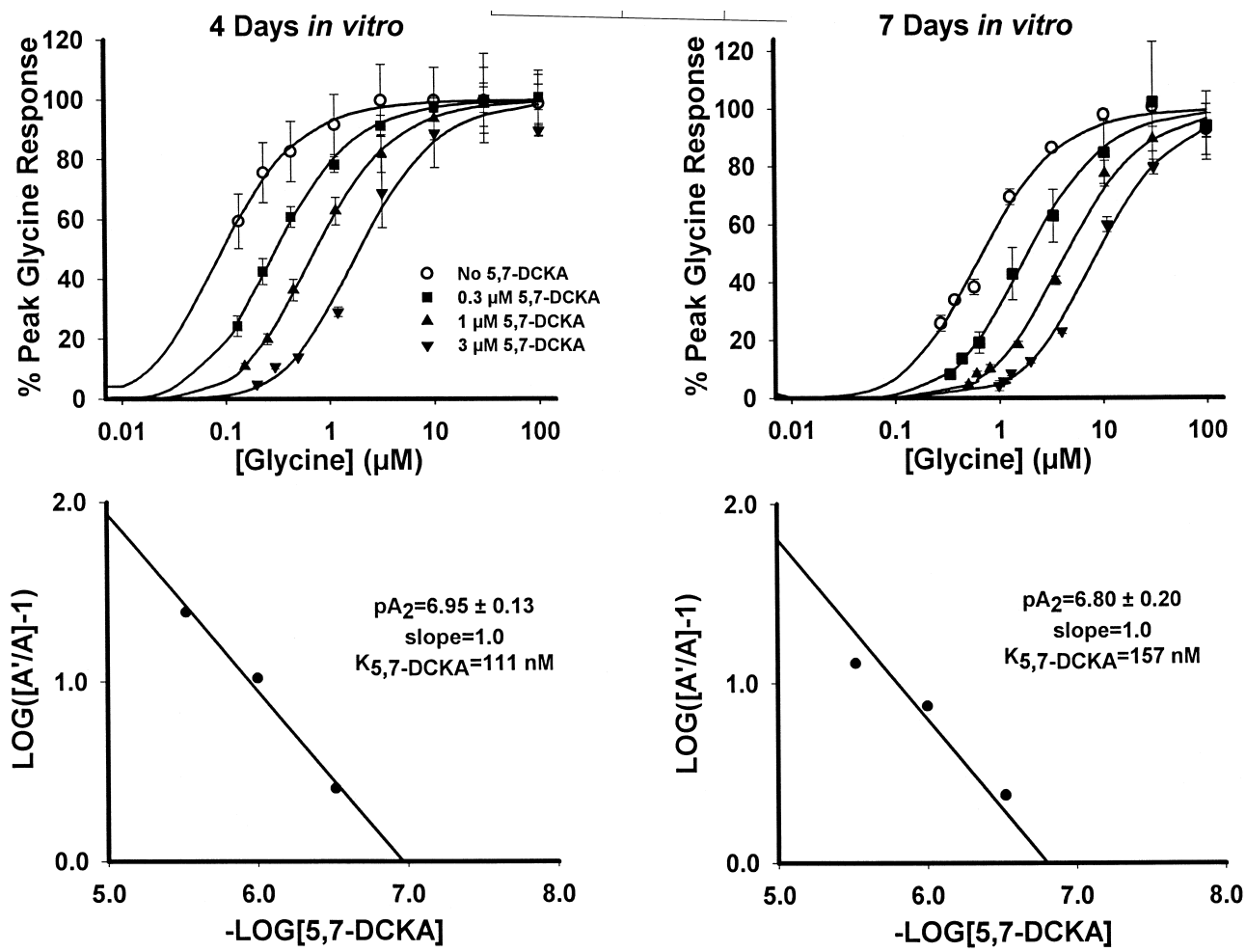


Fig. 6. Estimation of 5,7-dichlorokynurenic acid affinity. Cerebellar granule cells were prepared and $[Ca^{2+}]_i$ was measured as described in the text and legend to Fig. 3. Cells were exposed to 100 μM NMDA and a series of glycine concentrations in the presence of varying concentrations of 5,7-dichlorokynurenic acid (5,7-DCKA) (Panels A and B). These data were used for $pA_2$ analysis as described in the text. The Schild plots are shown in Panels C and D. Data represent the mean ± S.E.M. from three independent observations.

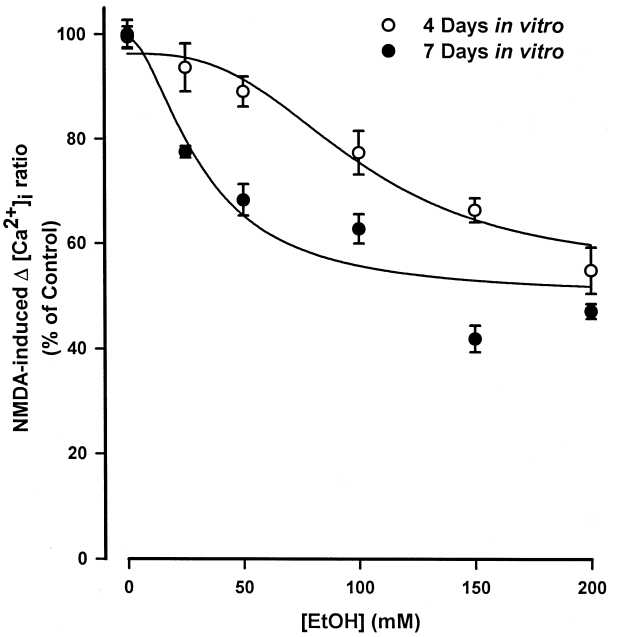


Fig. 7. Concentration dependence of ethanol (EtOH) inhibition of NMDA-induced increase in $[Ca^{2+}]_i$ in cerebellar granule neurons. Cerebellar granule neurons were prepared, and changes in $[Ca^{2+}]_i$ were measured, as described in the text and legend to Fig. 3. Data were calculated as the $\Delta[Ca^{2+}]_i$ ratio as described in the text and are presented as percent of control ratio ($\Delta[Ca^{2+}]_i$ ratio obtained in the absence of ethanol). Values represent the mean ± S.E.M. of 3–34 observations. ANOVA revealed a significant main effect of ethanol ($F = 34.92$, df = 5,143, $P < 0.001$) and developmental stage of cerebellar granule cells ($F = 19.06$, df = 1,143, $P < 0.001$), and a significant interaction between these two factors ($F = 5.38$, df = 5,143, $P = 0.001$). Post-hoc Newman–Keuls analyses showed significant differences between cells on day 4 and day 7 in vitro at 50, 100 and 150 mM ethanol ($P < 0.05$). In cerebellar granule cells on day 4 in culture, post-hoc analysis revealed significant differences between control and 100, 150 and 200 mM ethanol values ($P < 0.05$); on day 7 in culture, post-hoc analysis revealed significant differences between control and 50, 100, 150 and 200 mM ethanol values ($P < 0.05$).

equation used to fit the concentration–response curves also allowed for calculation of endogenous glycine concentrations. As shown in Table 1, these concentrations were not significantly different in cultures maintained for 4 or 7 days in vitro. We also attempted to determine the concentration of endogenous glycine in the cell buffer, at the time that the NMDA response was assessed, by amino acid analysis. However, the glycine levels in the cell buffer at both 4 and 7 days in culture were found to be at or below the threshold for detection (about 200 nM glycine), in agreement with the calculated values. The high salt concentration in the assay buffer precluded amino acid analysis of larger sample volumes following a buffer concentration procedure.

A series of glycine concentration–response curves in the presence of increasing concentrations of 5,7-dichlorokynurenic acid was also generated (Fig. 6). Using the corrected glycine concentration-response curves, as described in Methods, $pA_2$ analysis produced affinity constants for 5,7-dichlorokynurenic acid ($K_{DCKA}$) that were not significantly different on day 4 and day 7 in vitro (day 4: $pA_2 = 6.95 \pm 0.13$; $K_{DCKA} = 111$ nM; day 7: $pA_2 = 6.80 \pm 0.20$; $K_{DCKA} = 157$ nM).

### 3.2.3. Response to ethanol

The response to NMDA was inhibited by ethanol in a concentration-dependent manner in cerebellar granule neurons maintained in culture for 4 or 7 days (Figs. 3 and 7). This inhibition was previously shown to be reversible (e.g., Hoffman et al., 1989). In contrast to the findings with ifenprodil, ethanol was a more potent inhibitor of the NMDA response in cells maintained for 7 days in vitro than in cells maintained for 4 days in vitro ($IC_{50}$, based on the asymptotic maximum inhibition of 45% on day 4 or 50% on day 7: 4 days, 99 mM; 7 days, 18 mM). As

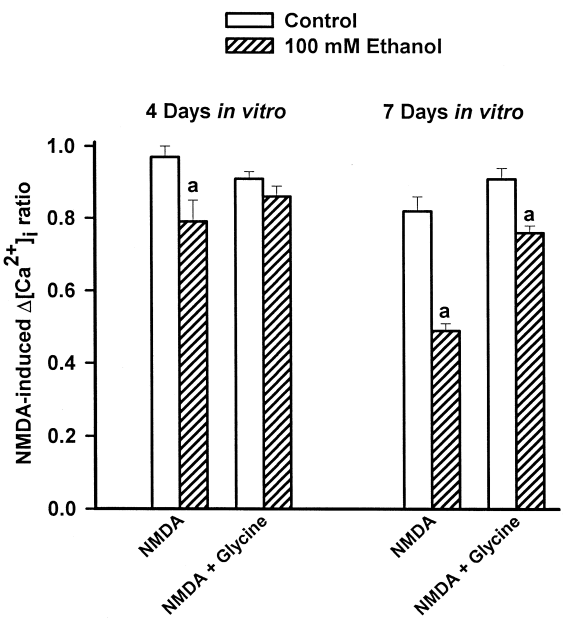


Fig. 8. Reversal of ethanol (EtOH) inhibition of the NMDA response by glycine. Cerebellar granule neurons were prepared, and changes in $[Ca^{2+}]_i$ were measured, as described in the text and legend to Fig. 3. Cells were exposed to 100 μM NMDA in the absence or presence of 10 μM glycine, washed and re-exposed to NMDA and glycine in the absence or presence of 100 mM ethanol. Data were calculated as the $\Delta[Ca^{2+}]_i$ ratio as described in the text. Values represent mean ± S.E.M. of 5–8 observations. ANOVA with repeated measures performed on data obtained with neurons on day 4 in vitro indicated a significant main effect of ethanol ($F = 7.48$, df = 1,24, $P < 0.02$), no significant main effect of glycine ($F = 0.31$, df = 1,24, $P > 0.30$), and a significant ethanol × glycine interaction ($F = 7.29$, df = 1,24, $P < 0.02$). Post-hoc Neuman–Keuls tests revealed significant inhibition by ethanol in the absence, but not in the presence of glycine ($^aP < 0.05$). On day 7 in vitro, ANOVA with repeated measures indicated a significant main effect of ethanol ($F = 70.10$, df = 1,18, $P < 0.001$), a significant main effect of glycine ($F = 39.7$, df = 1,18, $P < 0.001$), and a significant ethanol × glycine interaction ($F = 10.50$, df = 1,18, $P < 0.005$). Post-hoc analysis indicated significant inhibition by ethanol both in the absence and presence of glycine ($^aP < 0.05$). The interaction effect is accounted for by significantly less ethanol inhibition observed in the presence of glycine (see text).

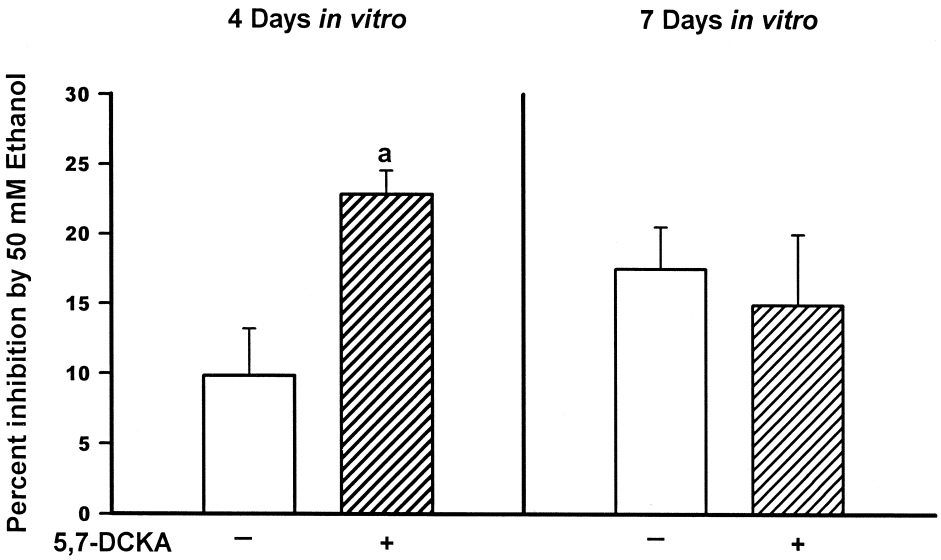


Fig. 9. Ethanol inhibition of the NMDA response in the presence of 5,7-dichlorokynurenic acid. Cerebellar granule neurons were prepared, and $[Ca^{2+}]_i$ was measured, as described in the text and legend to Fig. 3. Cells were exposed to 100 μM NMDA or to 100 μM NMDA in the presence of 1 μM 5,7-dichlorokynurenic (5,7-DCKA) acid and 1 μM added glycine, washed, and re-exposed to NMDA under the same conditions but in the presence or absence of 50 mM ethanol. Data were calculated as the $\Delta[Ca^{2+}]_i$ ratio as described in the text, and are presented as percent inhibition of the control response (no ethanol). Values represent mean ± S.E.M. ($n = 6$ observations). [a] $P < 0.05$, compared to no 5,7-dichlorokynurenic acid (Student's *t*-test).

previously reported (Rabe and Tabakoff, 1990; Snell et al., 1994b), ethanol inhibition of the NMDA response was attenuated in the presence of a high concentration of added glycine (Fig. 8). In cells maintained for 4 days in culture, 100 mM ethanol produced 18 ± 6% inhibition of the NMDA response in the absence of added glycine, and 6 ± 3% inhibition of the response in the presence of 10 μM added glycine ($n = 5$, $P < 0.02$; paired *t*-test). In cells maintained for 7 days in culture, the 49 ± 2% inhibition produced by 100 mM ethanol in the absence of added glycine was reduced to 17 ± 2% in the presence of the added glycine ($n = 8$, $P < 0.001$, paired *t*-test).

We also found that when the competitive glycine site antagonist, 5,7-dichlorokynurenic acid, was added to the perfusion buffer (see Methods and legend to Fig. 9), ethanol (50 mM) inhibition of the NMDA response was increased by 133 ± 18% ($n = 6$) in cells maintained for 4 days in culture, compared to no change or a small decrease in inhibition in cells maintained for 7 days in culture (Fig. 9).

## 4. Discussion

In our cultures of cerebellar granule neurons, both NR2A and NR2B were expressed at both time points studied, and we cannot therefore determine whether NR2B expression is an absolute requirement for substantial inhibition of NMDA receptor function by ethanol. However, in contrast to the implication of some previous reports (see below), i.e., that the function of NMDA receptors containing NR2B subunits is most sensitive to ethanol inhibition, the results presented here indicate that, in cultured cerebellar granule neurons, the sensitivity of NMDA receptors to inhibition by ethanol is greater at a time when there is a higher proportion of NR2A subunits expressed, relative to NR2B subunits.

We examined the developmental profile of NMDA receptor subunit expression in cerebellar granule neurons over a relatively short period in culture, but one during which critical changes in NMDA receptor subunit expression occur. During this period, although we observed no change in mRNA or protein levels of NR1, there was a significant increase in NR2A protein, accompanied by an increase (20%) in mRNA for NR2A. In contrast, there was a significant *decrease* in NR2B mRNA and protein levels over this time period. Resink et al. (1995) reported an increase in NR1 mRNA and protein, and no change in NR2A mRNA and protein, in cerebellar granule neurons on day 9 in culture, compared to day 5. However, our results are more comparable to those of Vallano et al. (1996), who assessed changes in NMDA receptor subunit mRNA levels in cerebellar granule neurons by quantitative reverse transcriptase–polymerase chain reaction methodology. These authors concluded that mRNA for NR2A increased, and mRNA for NR2B decreased, in cells maintained for 4–5 days compared to 7–14 days in culture. The fact that the increase in NR2A mRNA levels between days 4 and 7 in vitro was not statistically significant in our studies may reflect the substantial variability among cultures with respect to the timing of these developmental changes (e.g., Vallano et al., 1996), and/or the possibility that larger changes in mRNA levels preceded the changes in protein levels. It is also possible that changes in NR2A

protein levels could occur without corresponding changes in mRNA levels (e.g., Snell et al., 1996). Nevertheless, a number of studies have shown a developmental increase in NR2A and decrease in NR2B (both protein and mRNA levels) in the rat cerebellum in vivo over a period from postnatal day 7 to 21 (Watanabe et al., 1992; Wang et al., 1995; Wenzel et al., 1997). Thus, our findings are consistent with previous studies indicating that development of the NMDA receptor in primary cultures of rat cerebellar granule neurons mimics in vivo development (Monyer et al., 1994). At 7 days in culture, Vallano et al. (1996) found no detectable levels of mRNA for NR2C in cerebellar granule neurons, and we also found very low levels of mRNA for NR2C, and no detectable NR2C protein (data not shown).

While the stoichiometry of native NMDA receptors has not been definitively determined, the relative increase in NR2A and decrease in NR2B subunit proteins between days 4 and 7 in vitro might be expected to affect the subunit composition of NMDA receptors in the cerebellar granule cells. The changes would presumably lead to a relatively greater proportion of receptors containing NR1/NR2A subunits, compared to NR1/NR2B subunits, on day 7 in vitro, and/or to a change in the subunit composition of heterotrimeric receptors (Wafford et al., 1993; Luo et al., 1997). If this is the case, receptors should have different functional properties on day 7 in culture, compared to day 4. This supposition is supported by our observations of modulation of NMDA receptor function by ifenprodil. Ifenprodil, as mentioned earlier, is an atypical noncompetitive NMDA receptor antagonist (Reynolds and Miller, 1989; Carter et al., 1989; Legendre and Westbrook, 1991). Several studies have shown that heteromeric recombinant NR1/NR2B receptors have higher affinity for ifenprodil than NR1/NR2A receptors (Williams, 1993; Lynch et al., 1995), i.e., that ifenprodil can selectively inhibit the function of NMDA receptors that contain the NR2B subunit. The finding that ifenprodil is a more potent inhibitor of NMDA receptor function in cerebellar granule neurons on day 4 in culture, when NR2B subunit expression is relatively higher, compared to day 7, is consistent with the conclusion that the differences in levels of NMDA receptor subunit proteins on these two days in culture are associated with the expected differences in the properties of the NMDA receptor. Priestley et al. (1996), using voltage-clamp recording techniques, also demonstrated a decrease in ifenprodil sensitivity of NMDA receptor responses in cerebellar neurons over time in culture. In addition, Blevins et al. (1997) found that ifenprodil sensitivity of the NMDA response was positively correlated with the NR2B/NR2A transfection ratio when these subunits were expressed in HEK 293 cells.

The potency of glycine as a co-agonist at the NMDA receptor has also been reported to be influenced by the subunit composition of the receptor. Although the binding site for glycine appears to be localized to the NR1 subunit (Kuryatov et al., 1994; Williams et al., 1996; Uchino et al., 1997), and mutations of particular amino acid residues in this subunit can affect glycine potency (Williams et al., 1996), there is also evidence for an influence of NR2 subunits on glycine potency. Expression studies have demonstrated that NMDA receptors composed of NR1/NR2B subunits have as much as 10-fold higher affinity for glycine than receptors composed of NR1/NR2A subunits, while the affinity for the glycine site antagonist, 5,7-dichlorokynurenic acid, is not affected by this difference in subunit composition (Planells et al., 1993; Laurie and Seeburg, 1994; Priestley et al., 1995). Similarly, Kew et al. (1998) recently reported that the affinity of the NMDA receptor for glycine in cortical neurons of young rats, which express a relatively greater amount of NR2B, was higher than glycine affinity in neurons of older rats, which show a developmental increase in NR2A expression. These findings are consistent with our demonstration of a higher potency for glycine to enhance NMDA receptor function in cerebellar granule cells maintained for 4 days in culture, compared to cells maintained for 7 days in culture, while there was no significant difference in affinity for 5,7-dichlorokynurenic acid at these two time points. Thus, both the studies with ifenprodil and those with glycine support the contention that the differential levels of NR2A and NR2B proteins in the cerebellar granule cells at the different developmental stages are associated with characteristic, subunit-related, functional properties of the NMDA receptor. It should also be noted that our calculated estimates of glycine affinity at the NMDA receptor in cerebellar granule neurons are comparable to those that have been reported by others, using different measurements of NMDA receptor function (Priestley and Kemp, 1993).

In contrast to the results with ifenprodil, ethanol was found to be a more potent inhibitor of the function of the NMDA receptor in cerebellar granule neurons maintained for 7 days in culture, when NR2B subunit levels are relatively lower, compared to 4 days in culture. The ethanol $IC_{50}$ measured in cells maintained for 7 days in culture was similar to that reported previously (Hoffman et al., 1989; Snell et al., 1994b). We also found that ethanol inhibition of the NMDA response in cerebellar granule neurons was reduced in the presence of a high concentration of glycine, at both 4 and 7 days in culture. In our earlier studies, with cerebellar granule neurons maintained in culture for 7 days, ethanol was found to reduce the potency of glycine to enhance the NMDA response (Snell et al., 1994b). Therefore, at low glycine concentrations, ethanol produced a substantial inhibition of NMDA receptor function, but this inhibition was diminished at high glycine concentrations, as also found in the current work. We also previously showed that treatment of cerebellar granule neurons (maintained in culture for 7 days) with phorbol ester resulted in a decreased NMDA response, due to a decrease in glycine potency. The inhibitory effects of

both phorbol ester and ethanol could be blocked by protein kinase C inhibitors (Snell et al., 1994a,b). These data suggested the involvement of protein kinase C in the inhibitory effect of ethanol on NMDA receptor function in intact cerebellar granule neurons.

The facts that: (1) a response to NMDA could be generated in the absence of added glycine; and (2) added glycine could attenuate the inhibitory effect of ethanol, suggested that endogenous levels of glycine, present under our assay conditions, may be a factor influencing both the response of cultured cerebellar granule neurons to NMDA, and the effect of ethanol on the NMDA response. Although the endogenous glycine concentration was too low to be assessed by amino acid analysis, our calculations of endogenous glycine levels indicated no significant difference in these levels between cells maintained for 4 or 7 days in culture, in agreement with an earlier report by Hayashi et al. (1993). On the other hand, the higher affinity of the NR2B-containing NMDA receptor for glycine in cells maintained for 4 days in culture could allow the endogenous glycine to modulate the inhibitory effect of ethanol in these cells, while, in cells grown for 7 days in culture, the lower glycine affinity of the NR1/NR2A subunit-containing NMDA receptors would circumvent such an effect of endogenous glycine. This hypothesis is supported by the experiments in which the competitive glycine site antagonist, 5,7-dichlorokynurenic acid, was added to the cultures. Addition of this competitive antagonist led to greater sensitivity of the NMDA receptor to ethanol inhibition in cells grown for 4 days in culture (an increase in inhibition of 133%) (Fig. 9). There was little or no effect of 5,7-dichlorokynurenic acid on the inhibitory response to ethanol in cells grown for 7 days in culture, where the affinity for glycine was relatively low.

The data suggest that, in cerebellar granule neurons, the affinity of the NMDA receptor for glycine, in combination with the endogenous glycine concentration, is an important determinant of the sensitivity of receptor function to inhibition by ethanol (i.e., the subunit composition determines the glycine affinity, and in turn, determines the potency of ethanol to inhibit receptor function). Consistent with the present results, we previously found that NMDA receptor function (NMDA-induced increase in $[Ca^{2+}]_i$) in cerebral cortical cells maintained for one week in culture was relatively resistant to ethanol inhibition (Bhave et al., 1996). These cells expressed only NR1 and NR2B subunits (Snell, L.D., Bhave, S.V., Tabakoff, B. and Hoffman, P.L., unpublished results), and the high affinity for glycine of the cortical neuron NMDA receptors may have contributed to the observed insensitivity to ethanol. On the other hand, our results appear to differ from those of Engblom et al. (1997), who reported that ethanol did not inhibit NMDA receptor function in cerebellar granule neurons grown in medium containing a high concentration of KCl. These authors suggested that the lack of inhibition under these conditions was due to the presence of receptors containing the NR2A subunit, i.e., that ethanol selectively inhibited receptors that contain the NR2B subunit. However, there was no measure of subunit expression in their study and, as shown here, subunit expression is changing significantly over the period of 4–7 days in culture. Engblom et al. (1997) pooled data taken from cells at 5–7 days in vitro, which could confound their results. In addition, their report of no inhibition of NMDA receptor function in cerebellar granule neurons grown in 25 mM KCl is at odds with previous work (Hoffman et al., 1989; Rabe and Tabakoff, 1990; Snell et al., 1994b; Valenzuela et al., 1998), and may reflect the fact that ethanol was added to the cells *after* NMDA in the studies of Engblom et al. (1997). We also find very little inhibition of NMDA receptor function in cerebellar granule neurons if ethanol is added after the activation of the receptor by NMDA (Snell, L.D., Bhave, S.V., Tabakoff, B. and Hoffman, P.L., unpublished).

Lovinger (1995), using patch clamp techniques, reported that ethanol produced substantial inhibition of NMDA-induced currents in cerebral cortical cells after one week in culture, and that the magnitude of ethanol and ifenprodil inhibition of the NMDA response decreased in parallel over four weeks in culture, as the amount of NR2A expression increased (Zhong et al., 1994; Follesa and Ticku, 1996). These results appear to directly contrast with our finding that ethanol inhibition of NMDA receptor function in cerebellar granule neurons *increased* along with an increase in NR2A protein. The difference between our findings and those of Lovinger (1995) may be related to the difference in assay methodology. In our studies, intact cells are used, in which factors such as protein kinase C remain functional and can play a role in the actions of ethanol, e.g., by affecting, in combination with changes in subunit composition, the potency for glycine at the NMDA receptor. In contrast, Lovinger (1995) measured NMDA-induced currents using the whole cell patch clamp technique, which can reduce or eliminate the influence of intracellular modulators of NMDA receptor activity. The results presented here and prior findings that protein kinase C activity and tyrosine kinase activity may be important in the expression of ethanol's actions (Snell et al., 1994a,b; Miyakawa et al., 1997) suggest that such modulators can interact with NMDA receptors of different subunit composition to determine the sensitivity of the receptor to inhibition by pharmacologically relevant concentrations of ethanol. Interestingly, recent studies by Lovinger and colleagues have confirmed that glycine can attenuate ethanol inhibition of NMDA receptor function in cerebellar granule neurons, and that the effect of glycine is most pronounced when recordings are carried out using the perforated patch clamp recording method, in which intracellular contents are more intact (Lisa Popp, personal communication). Our studies did not distinguish among the splice variants of NR1, and expression studies have indicated only very little effect of NR1 splice variants on sensitivity of NMDA receptors to ethanol (Koltchine et al.,

1993). However, receptors containing these variants do differ in their response to protein kinase C activators (Durand et al., 1992), and, as antibodies become available, it would be of interest to assess changes in expression of NR1 splice variants over time in culture that could also affect the sensitivity of the NMDA receptor to ethanol.

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## References

Bhave, S.V., Snell, L.D., Tabakoff, B., Hoffman, P.L., 1996. Mechanism of ethanol inhibition of NMDA receptor function in primary cultures of cerebral cortical cells. Alcohol. Clin. Exp. Res. 20, 934–941.

Blevins, T., Mirshahi, T., Chandler, L.J., Woodward, J.J., 1997. Effects of acute and chronic ethanol exposure on heteromeric *N*-methyl-D-aspartate receptors expressed in HEK 293 cells. J. Neurochem. 69, 2345–2354.

Buller, A.L., Larson, H.C., Morrisett, R.A., Monaghan, D.T., 1995. Glycine modulates ethanol inhibition of heteromeric *N*-methyl-D-aspartate receptors expressed in *Xenopus* oocytes. Mol. Pharmacol. 48, 717–723.

Carter, C., Rivy, J., Scatton, B., 1989. Ifenprodil and SL 82,0715 are antagonists at the polyamine site of the *N*-methyl-D-aspartate (NMDA) receptor. Eur. J. Pharmacol. 164, 611–612.

Chu, B., Anantharam, V., Triestman, S.N., 1995. Ethanol inhibition of recombinant heteromeric NMDA channels in the presence and absence of modulators. J. Neurochem. 65, 140–148.

Durand, G.M., Gregor, P., Zheng, X., Bennett, M.V.L., Uhl, G.R., Zukin, R.S., 1992. Cloning of the apparent splice variant of the *N*-methyl-D-aspartate receptor NMDAR1 with altered sensitivity to polyamines and activators of protein kinase C. Proc. Natl. Acad. Sci. U.S.A. 89, 9359–9363.

Engblom, A.C., Courtney, M.J., Kukkonen, J.P., Åkerman, K.E.O., 1997. Ethanol specifically inhibits NMDA receptors with affinity for ifenprodil in the low micromolar range in cultured cerebellar granule cells. J. Neurochem. 69, 2162–2168.

Follesa, P., Ticku, M.K., 1996. Chronic ethanol-mediated up-regulation of the *N*-methyl-D-aspartate receptor polypeptide subunits in mouse cortical neurons in culture. J. Biol. Chem. 271, 13297–13299.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of $Ca^{2+}$ indicators with greatly improved fluorescent properties. J. Biol. Chem. 260, 3440–3450.

Hayashi, H., Watanabe, Y., Shibuya, T., 1993. Relation between long-lasting amounts of excitatory amino acid and its neuronal uptake system in cultured cerebellar granule cells under hypoglycemia. Prog. Neuropsychopharmacol. Biol. Psychiatry 17, 463–474.

Hoffman, P.L., Rabe, C.S., Moses, F., Tabakoff, B., 1989. *N*-methyl-D-aspartate receptors and ethanol: inhibition of calcium flux and cyclic GMP production. J. Neurochem. 52, 1937–1940.

Hoffman, P.L., Rabe, C.S., Grant, K.A., Valverius, P., Hudspith, M., Tabakoff, B., 1990. Ethanol and the NMDA receptor. Alcohol 7, 229–231.

Hoffman, P.L., Bhave, S.V., Kumar, K.N., Iorio, K.R., Snell, L.D., Tabakoff, B., Michaelis, E.K., 1996. The 71 kDa glutamate-binding protein is increased in cerebellar granule cells after chronic ethanol treatment. Mol. Brain Res. 39, 167–176.

Iorio, K.R., Reinlib, L., Tabakoff, B., Hoffman, P.L., 1992. Chronic exposure of cerebellar granule cells to ethanol results in increased *N*-methyl-D-aspartate receptor function. Mol. Pharmacol. 41, 1142–1148.

Ishii, T., Moriyoshi, K., Sugihara, H., Sakurada, K., Kadotani, H., Yokoi, M., Akazawa, C., Shigemoto, R., Mizuno, N., Masu, M., Nakanishi, S., 1993. Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. J. Biol. Chem. 268, 2836–2843.

Kew, J.N.C., Richards, J.G., Mutel, V., Kemp, J.A., 1998. Developmental changes in NMDA receptor glycine affinity and ifenprodil sensitivity reveal three distinct populations of NMDA receptors in individual rat cortical neurons. J. Neurosci. 18, 1935–1943.

Koltchine, V., Anantharam, V., Wilson, A., Bayley, H., Treistman, S.N., 1993. Homomeric assemblies of NMDAR1 splice variants are sensitive to ethanol. Neurosci. Lett. 152, 13–16.

Kuner, T., Schoepfer, R., Korpi, E.R., 1993. Ethanol inhibits glutamate-induced currents in heteromeric NMDA receptor subtypes. Neuroreport 5, 297–300.

Kuryatov, A., Laube, B., Betz, H., Kuhse, J., 1994. Mutational analysis of the glycine-binding site of the NMDA receptor: structural similarity with bacterial amino acid-binding proteins. Neuron 12, 1291–1300.

Laurie, D.J., Seeburg, P.H., 1994. Ligand affinities at recombinant *N*-methyl-D-aspartate receptors depend on subunit composition. Eur. J. Pharmacol. 268, 335–345.

Legendre, P., Westbrook, G.L., 1991. Ifenprodil blocks *N*-methyl-D-aspartate receptors by a two-component mechanism. Mol. Pharmacol. 40, 289–298.

Lovinger, D.M., 1995. Developmental decrease in ethanol inhibition of *N*-methyl-D-aspartate receptors in rat neocortical neurons: relation to the actions of ifenprodil. J. Pharmacol. Exp. Ther. 274, 164–172.

Luo, J., Wang, Y., Yasuda, R.P., Dunah, A.W., Wolfe, B.B., 1997. The majority of *N*-methyl-D-aspartate receptor complexes in adult rat cerebral cortex contain at least three different subunits (NR1/NR2A/NR2B). Mol. Pharmacol. 51, 79–86.

Lynch, D.R., Lawrence, J.J., Lenz, S., Anegawa, N.J., Dichter, M., Pritchett, D.B., 1995. Pharmacological characterization of heterodimeric NMDA receptors composed of NR1a and 2B subunits: differences with receptors formed from NR1a and 2A. J. Neurochem. 64, 1462–1468.

Masood, K., Wu, C., Brauneis, U., Weight, F.F., 1994. Differential ethanol sensitivity of recombinant *N*-methyl-D-aspartate receptor subunits. Mol. Pharmacol. 45, 324–329.

Miyakawa, T., Yagi, T., Kitazawa, H., Yasuda, M., Kawai, N., Tsuboi, K., Niki, H., 1997. Fyn-kinase as a determinant of ethanol sensitivity: relation to NMDA-receptor function. Science 278, 698–701.

Mori, H., Mishina, M., 1995. Structure and function of the NMDA receptor channel. Neuropharmacology 34, 1219–1237.

Monyer, H., Burnashev, N., Laurie, D.J., Sakmann, B., Seeburg, P.H., 1994. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12, 529–540.

Nakanishi, N., Axel, R., Shneider, N.A., 1992. Alternative splicing generates functionally distinct *N*-methyl-D-aspartate receptors. Proc. Natl. Acad. Sci. U.S.A. 89, 8552–8556.

Planells, C.R., Sun, W., Ferrer-Montiel, A.V., Montal, M., 1993. Molecular cloning, functional expression, and pharmacological characterization of an *N*-methyl-D-aspartate receptor subunit from human brain. Proc. Natl. Acad. Sci. U.S.A. 90, 5057–5061.

Priestley, T., Kemp, J.A., 1993. Agonist response kinetics of *N*-methyl-D-aspartate receptors in neurons cultured from rat cerebral cortex and cerebellum: evidence for receptor heterogeneity. Mol. Pharmacol. 44, 1252–1257.

Priestley, T., Laughton, P., Myers, J., LeBourdellés, B., Kerby, J., Whiting, P.J., 1995. Pharmacological properties of recombinant human *N*-methyl-D-aspartate receptors comprising NR1a/NR2A and NR1a/NR2B subunit assemblies expressed in permanently transfected mouse fibroblast cells. Mol. Pharmacol. 48, 841–848.

Priestley, T., Ochu, E., Macaulay, A.J., 1996. Time-dependent changes in NMDA receptor expression in neurones cultured from rat brain. Mol. Brain Res. 40, 271–274.

Rabe, C.S., Tabakoff, B., 1990. Glycine site-directed agonists reverse the actions of ethanol at the *N*-methyl-D-aspartate receptor. Mol. Pharmacol. 38, 753–757.

Resink, A., Villa, M., Benke, D., Möhler, H., Balázs, R., 1995. Regulation of the expression of NMDA receptor subunits in rat cerebellar granule cells: effect of chronic $K^+$-induced depolarization and NMDA exposure. J. Neurochem. 64, 558–565.

Reynolds, I.J., Miller, R.J., 1989. Ifenprodil is a novel type of *N*-methyl-D-aspartate receptor antagonist: interaction with polyamines. Mol. Pharmacol. 36, 758–765.

Snell, L.D., Iorio, K.R., Tabakoff, B., Hoffman, P.L., 1994a. Protein kinase C activation attenuates *N*-methyl-D-aspartate induced increases in intracellular calcium in cerebellar granule cells. J. Neurochem. 62, 1783–1789.

Snell, L.D., Tabakoff, B., Hoffman, P.L., 1994b. Involvement of protein kinase C in ethanol-induced inhibition of NMDA receptor function in cerebellar granule cells. Alcohol. Clin. Exp. Res. 18, 81–85.

Snell, L.D., Nunley, K.R., Lickteig, R.L., Browning, M.D., Tabakoff, B., Hoffman, P.L., 1996. Regional and subunit specific changes in NMDA receptor mRNA and immunoreactivity in mouse brain following chronic ethanol ingestion. Mol. Brain Res. 40, 71–78.

Sugihara, H., Moriyoshi, K., Ishii, T., Masu, M., Nakanishi, S., 1992. Structures and properties of seven isoforms of the NMDA receptor generated by alternative splicing. Biochem. Biophys. Res. Commun. 185, 826–832.

Tallarida, R.J., Murray, R., 1984. Manual of Pharmacologic Calculations with Computer Programs. Springer, Berlin.

Uchino, S., Nakajima-Iijima, S., Okuda, K., Mishina, M., Kawamoto, S., 1997. Analysis of the glycine binding domain of the NMDA receptor channel ζ1 subunit. Neuroreport 8, 445–449.

Valenzuela, C.F., Bhave, S., Hoffman, P.L., Harris, R.A., 1998. Acute effects of ethanol on pharmacologically-isolated kainate receptors in cerebellar granule neurons: comparison with NMDA and AMPA receptors. J. Neurochem. 71, 1777–1780.

Vallano, M.L., Lambolez, B., Audinat, E., Rossier, J., 1996. Neuronal activity differentially regulates NMDA receptor subunit expression in cerebellar granule cells. J. Neurosci. 16, 631–639.

Wafford, K.A., Bain, C.J., Le Bourdelles, B., Whiting, P.J., Kemp, J.A., 1993. Preferential co-assembly of recombinant NMDA receptors composed of three different subunits. Neuroreport 4, 1347–1349.

Wang, Y., Bosy, T.Z., Yasuda, R.P., Grayson, D.R., Vicinti, S., Pizzorusso, T., Wolfe, B.B., 1995. Characterization of NMDA receptor subunit-specific antibodies: distribution of NR2A and NR2B receptor subunits in rat brain and ontogenic profile in the cerebellum. J. Neurochem. 65, 176–183.

Watanabe, M., Inoue, Y., Sakimura, K., Mishina, M., 1992. Developmental changes in distribution of NMDA receptor channel subunit mRNAs. Neuroreport 3, 1138–1140.

Wenzel, A., Fritschy, J.M., Mohler, H., Benke, D., 1997. NMDA receptor heterogeneity during postnatal development of the rat brain: differential expression of the NR2A, NR2B, and NR2C subunit proteins. J. Neurochem. 68, 469–478.

Williams, K., 1993. Ifenprodil discriminates subtypes of the N-methyl-D-aspartate receptor: selectivity and mechanisms at recombinant heteromeric receptors. Mol. Pharmacol. 44, 851–859.

Williams, K., Chao, J., Kashiwagi, K., Masuko, T., Igarashi, K., 1996. Activation of *N*-methyl-D-aspartate receptors by glycine: role of an aspartate residue in the M3–M4 loop of the NR1 subunit. Mol. Pharmacol. 50, 701–708.

Yang, X., Criswell, H.E., Simson, P., Moy, S., Breese, G.R., 1996. Evidence for a selective effect of ethanol on *N*-methyl-D-aspartate responses: ethanol affects a subtype of the ifenprodil-sensitive *N*-methyl-D-aspartate receptors. J. Pharmacol. Exp. Ther. 278, 114–124.

Zhong, J., Russell, S.L., Pritchett, D.B., Molinoff, P.B., Williams, K., 1994. Expression of mRNAs encoding subunits of the *N*-methyl-D-aspartate receptor in cultured cortical neurons. Mol. Pharmacol. 457, 846–853.