Ethanol Inhibition of Glycine-Activated Responses in Neurons of Ventral Tegmental Area of Neonatal Rats

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Ye, Jiang Hong, Liang Tao, Li Zhu, Krešimir Krnjević, and Joseph J. Mcardle. Ethanol inhibition of glycine-activated responses in neurons of ventral tegmental area of neonatal rats. J Neurophysiol 86: 2426–2434, 2001. The brain is particularly sensitive to alcohol during the period of its rapid growth. To better understand the mechanism(s) involved, we studied ethanol effects on glycine-activated responses of ventral tegmental area (VTA) neurons isolated from the newborn rat, using whole cell and gramicidin perforated patch-clamp techniques. Previously we reported that 0.1–40 mM ethanol enhances glycine-induced responses of 35% of VTA neurons (Ye et al. 2001). We now direct our attention to the inhibitory effects of ethanol observed in 45% (312 of 694) of neonatal VTA neurons. Under current-clamp conditions, 1 mM ethanol had no effect on the membrane potential of these cells, but it decreased glycine-induced membrane depolarization and the frequency of spontaneous action potentials. Under voltage-clamp conditions, 0.1–10 mM ethanol did not elicit a current but depressed the glycine-induced currents. The ethanol-induced inhibition of glycine current was independent of membrane potential (between −60 and +60 mV). Likewise, ethanol did not alter the reversal potential of the glycine-activated currents. Ethanol-mediated inhibition of glycine current depended on the glycine concentration. While ethanol strongly depressed currents activated by 30 μM glycine, it had no appreciable effect on maximal currents activated by 1 mM glycine. In the presence of ethanol (1 mM), the EC₅₀ for glycine increased from 32 ± 5 to 60 ± 3 μM. Thus ethanol may decrease the agonist affinity of glycine receptors. A kinetic analysis indicated that ethanol shortens the time constant of glycine current deactivation but has no effect on activation. In conclusion, by altering VTA neuronal function, ethanol-induced changes in glycine receptors may contribute to neurobehavioral manifestations of the fetal alcohol syndrome.

INTRODUCTION

Ethanol, a brain depressant and an addictive drug, is strongly teratogenic. As the brain is particularly sensitive to neurotoxic effects during its rapid growth, fetal exposure to alcohol could lead to learning and memory deficits associated with Fetal Alcohol Effects and/or Fetal Alcohol Syndrome (Clarron and Smith 1978). The mechanisms underlying ethanol’s effects on the developing human brain, however, are poorly understood.

According to Ikonomidou et al. (2000), potentiation of GABA₄ receptors by ethanol contributes to widespread apoptotic neurodegeneration in the developing rat forebrain. A role for glycine receptors (GlyR) in this process is not known. Like GABA (Krnjević 1997), glycine activates Cl⁻ selective channels (Werman et al. 1968) and is a major inhibitory neurotransmitter in the mature CNS. The GlyR consists of α and β subunits, which are heterogeneous and undergo developmental changes. For example, the α2 subunit is present in the fetus until 2–3 wk after birth. Afterward the α1 subunit becomes dominant (Becker et al. 1988). The physiological and pharmacological properties of GlyRs, including ethanol sensitivity, depend on their subunit composition (Akagi and Miledi 1988; Eggers et al. 2000; Ye 2000). These properties are likely different for adult and immature types of receptors. In addition, whereas activation of GlyRs of adult mammalian CNS results in neuronal hyperpolarization, activation of GlyRs of the developing CNS results in membrane depolarization and neuronal excitation, owing to a more positive chloride equilibrium potential (Cherubini et al. 1991; Ye 2000).

Several studies have shown that ethanol enhances glycine-activated currents in different preparations: e.g., synaptoneurosomes (Engblom and Akerman 1991), cultured neurons from chicks (Celentano and Wong 1994) and mice (Aguayo and Pancetti 1994; Aguayo et al. 1996). Xenopus oocytes, as well as mammalian cell lines expressing homomeric GlyRs (Mascia et al. 1996a,b; Valenzuela et al. 1998; Q. Ye et al. 1998) and freshly isolated hypoglossal motoneurons (Eggers et al. 2000). However, data from upper brain stem neurons of neonatal animals are lacking.

The ventral tegmental area (VTA) contains the cells of origin of the mesolimbic system. It plays a pivotal role in the mediation of the rewarding effects of drugs of abuse, including ethanol (Gatto et al. 1994; Wise 1996). In our recent experiments, glycine elicited responses from most (82%) VTA neurons (Ye et al. 2000). The ventral tegmental area (VTA) contains the cells of origin of the mesolimbic system. It plays a pivotal role in the mediation of the rewarding effects of drugs of abuse, including ethanol (Gatto et al. 1994; Wise 1996). In our recent experiments, glycine elicited responses from most (82%) VTA neurons (Ye et al. 2000). In the present study, we report that ethanol (0.1–10 mM) also reduces responses to glycine in 45% (312/694) of neonatal VTA neurons. Thus ethanol can depress their excitability.

METHODS

Isolation of neurons and electrophysiological recording

The care and use of animals and the experimental protocol of this study were approved by the Institutional Animal Care and Use Com-
mittee of the University of Medicine and Dentistry of New Jersey (protocol No. 0752). Sprague-Dawley rats (5–14 days old) were decapitated as described earlier (Ye 2000). The brain was quickly dissected, placed into ice-cold saline saturated with 95% O₂–5% CO₂, glued to the chilled stage of a vibratome (Campden Instruments), and sliced to a thickness of 300–400 μm. Slices were transferred to the standard external solution—containing 1 mg pronase/6 ml and saturated with O₂—and incubated at 31°C for 20 min. After an additional 20 min incubation in 1 mg thermolysin/6 ml, the VTA was identified medial to the accessory optic tract and lateral to the fasciculus retroflexus under a dissecting microscope. Micro-punches of the VTA were isolated and transferred to a 35-mm culture dish. Mild trituration through heat-polished pipettes of progressively smaller tip diameters dissociated single neurons. Within 20 min of trituration, isolated neurons attached to the bottom of the culture dish and were ready for electrophysiological experiments. Based on morphology under the light microscope, the cells acutely isolated from VTA were of two types: bipolar and multipolar. The majority was bipolar with one to three dendritic processes emerging from each end of the fusiform soma (20–40 μm in length and 15–25 μm in diameter). The multipolar neurons were larger with a diameter of 35–60 μm and four to five major dendrites. Most of the cells were tyrosine hydroxylase-positive, which is in good agreement with the recent report of Brodie et al. (1999). There were no appreciable differences in the response of these two groups of neurons to ethanol.

The saline in which the brain was dissected contained (in mM) 128 NaCl, 5 KCl, 1.2 NaH₂PO₄, 26 NaHCO₃, 9 MgCl₂, 0.3 CaCl₂, and 2.5 glucose. The pH was adjusted to 7.4 with HCl. The standard external solution contained (mM) 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES. The pH was adjusted to 7.4 with Tris base and the osmolality to 320 mM with sucrose. With 100 mM ethanol, the pH and the osmolality of the solution were unchanged. Patch pipette solutions contained (mM) 150 KCl and 10 HEPES for gramicidin-perforated patch recording and 120 CsCl, 21 TEA-Cl, 4 MgCl₂, 11 ethyleneglycol bis-(-aminoethyl ether)-N,N,N,N’-tetraacetic acid (EGTA), 10 CaCl₂, 10 HEPES, and 2 Mg-ATP for conventional whole cell recording. The pH was adjusted to 7.2 with Tris base, and the osmolality to 280 mM with sucrose. The patch electrodes had a resistance between 3 and 5 MΩ when filled with the preceding solutions. The gramicidin-perforated-patch technique (Abe et al. 1994) was used to record glycine-induced whole cell responses. Gramicidin enters the membrane lipid bilayer to form transmembrane pores which are impermeable to chloride; this preserves the normal internal chloride concentration and is important for the study of chloride channels. The gramicidin stock solution of 10 mg/ml was prepared in methanol (J. T. Baker, Phillipsburg, NJ). It was diluted in the patch solution to a final concentration of 50–100 μg/ml just before the experiment. The pipette tip was filled with gramicidin-free solution by brief immersion prior to back filling. After establishing the giga-seal in the cell-attached configuration by gentle suction, no further negative pressure was applied. The progress of perforation was monitored by measuring the decrease in membrane resistance with further negative pressure was applied. The progress of perforation was monitored by measuring the decrease in membrane resistance with repeated 10-mV hyperpolarizing voltage steps from a holding potential (V₉⁵) of −50 mV. The entry into the perforated-patch mode was signaled by an increase in the amplitude of the capacitive transient. In most experiments, the series resistance before compensation was 15–25 MΩ. Routinely, 80% of the series resistance was compensated; hence, there was a 3 mV error for 1 nA of current.

### Chemical applications

Solutions of agonist, antagonists, alcohol, and gramicidin were prepared on the day of experimentation. Glycine, strychnine, gramicidin, and N,N,N,N’-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) were obtained from Sigma Chemical (St Louis, MO). TPEN was prepared in dimethyl sulfoxide (DMSO) and diluted to its final concentration in standard external solution. The final concentration of DMSO was always <0.1%; it did not induce any ionic current and had no effect on the glycine response at the concentrations used. Ethanol (100% and 95%, prepared from grain) was obtained from Pharmco (Brookfield, CT, or Bayonne, NJ), or from U.S. Industrial Chemicals, Division of National Distillers of Chemical (New York, NY), and was stored in glass bottles. We found no significant difference between the data collected using ethanol from different manufacturers. Solutions were applied to a dissociated neuron with a superfusion system via a multi-barreled pipette as described previously (Ye et al. 2001). The tip of the superfusion pipette was usually placed 50–100 μm away from the cell, a position that allowed rapid as well as uniform drug application while preserving the neuron’s mechanical stability. This system allows complete exchange of solutions in the vicinity of the neuron within 20 ms. The speed of solution exchange was measured by reducing the external Na⁺ concentration from 140 to 10 mM (plus 130 mM N-methyl-d-glucamine, NMDG) during a kainate application. Since kainate currents do not desensitize, the rate of decrease of kainate responses reflects the rate of solution change (Ye et al. 2001). In the later half of the study, we replaced all plastic in the system with Teflon and glass and eliminated all metals.

### Data analyses

Whole cell current decays were fitted by a Chebyshev algorithm (pClamp). Concentration-response data were analyzed with a nonlinear curve-fitting program (Sigma Plot, Jandel Scientific). Data were statistically compared using Student’s t-test at a significance level of P < 0.05, otherwise as indicated. For all experiments, average values are expressed as mean ± SE with the number of neurons indicated in parentheses. To obtain a concentration-response relationship for VTA glycine receptors, all neurons were exposed to three or four concentrations of glycine, in the range of 0.003–1 mM. For each concentration, four to six responses from a given neuron were normalized to the peak current evoked by 30 μM glycine. The normalized values from three to five neurons at each concentration of glycine were averaged. Using a Simplex algorithm (Sigma plot, Jandel Scientific), these averages were then fitted to the Hill equation: \[ I = I_{\text{max}}/[1 + (EC_{50}/C)^n] \], where \( I, I_{\text{max}}, C, EC_{50} \) and \( n \) are glycine-activated current, maximal glycine-activated current, glycine concentration, the concentration for 50% of maximum response, and the Hill coefficient, respectively.

### RESULTS

#### Ethanol can decrease glycine-induced depolarization and neuronal excitability

We first studied ethanol’s effects under current-clamp conditions with the gramicidin-perforated patch technique. In agreement with our earlier report (Ye 2000), glycine elicited depolarization and, occasionally, action potentials in VTA.
neurons from neonatal rats (Fig. 1). This depolarization is explained by a reversal potential for glycine’s action on neonatal neurons that is much more positive (near −25 mV) than the resting potential (−68 ± 2.5 mV, n = 5). In accord with our recent reports (Ye 2000; J. H. Ye et al. 1998), all these effects of glycine were antagonized by 0.1 μM strychnine (data not shown).

To determine whether ethanol has a direct effect, we routinely applied ethanol alone to the cells before it was co-applied with the agonist (as shown in Fig. 1A). When a direct effect of ethanol was detected, we preequilibrated the cell with ethanol before it was co-applied with glycine, using the protocol illustrated in Fig. 6A. As demonstrated in Fig. 1, ethanol alone did not alter the membrane potential. However, when co-applied with 10 μM glycine, ethanol reduced both the amplitude of the depolarization and the number of action potentials. On average, the steady depolarization induced by 10 μM glycine was 13 ± 3 mV in the absence and 3 ± 3 mV in the presence of 1 mM ethanol; these values are significantly different (P < 0.01; n = 5). Moreover, 10 μM glycine evoked a significantly greater number of action potentials in the absence of 1 mM ethanol (2 ± 1) than in its presence (0 ± 1; for n = 6, P < 0.01).

Ethanol inhibits glycine-activated currents

Glycine elicits responses from most VTA neurons (82%) of neonatal rats (Ye et al. 2001). We also reported that acute applications of 0.1–40 mM ethanol enhanced glycine responses in 35% of VTA neurons (Ye et al. 2001). In the present experiments, we found that 0.1–10 mM ethanol inhibited glycine-induced currents of many neurons. Figure 2A shows typical examples of currents activated by 30 μM glycine alone (A, a) and in the presence of 0.1, 1, and 10 mM ethanol (A, b–d, respectively); the currents recovered to the control level after washout of ethanol (A, e). At concentrations between 0.1 and 3 mM, ethanol reduced glycine currents in a concentration-dependent manner; but 10 mM or higher concentrations were equally or less effective (see following text). Similar results were obtained when ethanol was applied in the reversed order of concentration that is from higher to lower. Such inhibition of glycine-activated current by ethanol occurred in 45% (312/694) of the neurons tested. Figure 2B is a plot of the means of normalized ethanol inhibition as a function of ethanol concentration. On average, 3 and 10 mM ethanol decreased the peak current induced by 30 μM glycine to 67 ± 6% (n = 18) and 69 ± 5% (n = 48) of control, respectively. At even higher ethanol concentrations (100 mM), there was no inhibitory effect on glycine-activated currents. The mechanisms underlying the disappearance of the inhibitory effect at higher ethanol concentrations are unclear and currently under study.

Age-dependence of ethanol’s action

The preceding experiments were performed on neurons from neonatal rats (<14 days). GlyRs are known to be heterogeneous and to change during development. To determine whether developmental changes of GlyRs affect their response to ethanol, we tested ethanol’s effects on VTA neurons isolated from more mature (27- to 34-day-old) rats prepared in an identical manner. Ethanol (1 mM) potentiated, had no effect and inhibited GlyRs in 72% (28/39), 23% (9/39), and 5% (2/39) of the neurons, respectively. This is in sharp contrast to the neonatal rats, where ethanol potentiated, had no effect and inhibited the GlyRs in 35, 20, and 45% of the neurons, respectively.

FIG. 1. Ethanol depresses glycine-induced excitation. Ethanol depresses membrane depolarization and action potential firing elicited by glycine in a current-clamped ventral tegmental area (VTA) neuron of a 6-day-old rat. Gramicidin perforated-patch recordings, with KCl based pipette solution show voltage responses to 1 mM ethanol alone (A) and 10 μM glycine in the absence (B and D) and presence of 1 mM ethanol (C). Traces A–D were consecutive records from the same cell and are on the same time base. For all figures, the bars under (or above) the voltage (or current) traces indicate the periods of perfusion of the indicated chemicals. Initial membrane potential was −75 mV.

FIG. 2. Ethanol depresses peak amplitude of glycine-activated current. Data in Figs. 2–7 were recorded under conventional whole cell conditions. A: current traces were obtained at V_m = −50 mV from VTA neuron of a 7-day-old rat. Glycine current was elicited by 30 μM glycine alone (A, a and e) or together with 0.1 (A, b), 1 (A, c), and 10 mM (A, d) ethanol. B: concentration-response relation for ethanol-induced depression of glycine-activated current. Data are normalized peak currents (±SE) in response to 30 μM glycine plus different concentrations of ethanol (in brackets are numbers of neurons tested). Peak currents were normalized with respect to peak current evoked by 30 μM glycine alone.
Radiation stabilizer manufactured by Ciba-Geigy (Summit, NJ). BTMPS (Tinuvin 770), a sterically hindered amine light and plastic product, bis(2,2,6,6-tetramethyl-4-piperidinyl) sebacate unexpectedly. It is possible that the inhibition resulted from a... 

Additional control experiments

The inhibitory effect of ethanol on glycine response was unexpected. It is possible that the inhibition resulted from a plastic syringes with glass ones and installed metal-free Teflon tubing in the perfusion system. In these experimental conditions, ethanol caused a similar depression of glycine currents recorded in 260 neurons.

The initial experiments on 494 neurons were performed with 100% ethanol prepared from grain (U.S. Industrial Chemicals, Division of National Distillers of Chemical). Complete dehydration of ethanol is known to introduce contaminants. So we repeated the experiments on 200 neurons with 95% ethanol prepared from grain (Pharmco). To ensure that the measurement and other conditions were the same, in some of the experiments we examined the inhibitory effect of ethanol from both sources in the same neurons. There were no significant differences: 100 and 95% ethanol (both at 10 mM) inhibited currents activated by 30 μM glycine to 68 ± 5 and 69 ± 5%, respectively (n = 7, P > 0.05). Thus the inhibition of GlyR cannot be attributed to contaminants introduced during complete dehydration.

Ethanol inhibition of GlyRs is not due to zinc contamination

High levels of contaminating zinc may be present in ethanol from commercial sources. Because zinc is a potent modulator of glycine receptors (Laube et al. 1995, 2000), we compared the effects of zinc with those of ethanol. As illustrated in Fig. 3, zinc had biphasic effects: at concentrations of 0.5–10 μM, zinc potentiated, but at concentrations >50 μM, it depressed the glycine-activated current. These results agree with a recent report of biphasic effects of zinc on human GlyRs (Laube et al. 2000). By contrast, glycine-induced responses are depressed by low (0.1–10 mM) and enhanced by high ethanol (1–40 mM) concentrations. In view of the opposite concentration dependence of the effects of zinc and ethanol, contamination by zinc cannot explain our observations.

In addition, ethanol was tested in the presence of TPEN, a chelator of zinc (and other heavy metals). In agreement with a recent study on Zebrafish hindbrain (Suwa et al. 2001), applications of TPEN (100 μM) alone reduced the glycine-activated current, indicating that traces of heavy metals potentiate glycine in control conditions. On average, 100 μM TPEN, 1 mM ethanol, and TPEN + ethanol depressed currents activated by 30 μM glycine by 17 ± 5, 13 ± 5, and 31 ± 6% (n = 3), respectively. After subtracting the effect of TPEN, ethanol...
depressed glycine currents to 14 and 13%, respectively, in the presence or absence of TPEN (P > 0.5, Fig. 3, C and D). These results are further evidence that ethanol’s actions on the GlyRs cannot be attributed to zinc contamination.

**Ethanol-mediated inhibition of glycine-activated current is independent of membrane voltage**

In view of recent evidence that the GlyR-channel is voltage dependent (Legendre 1999) and may include an alcohol-receptor site (Wick et al. 1998), we examined the voltage dependence of the depressant action of ethanol on glycine-activated currents. As illustrated in Fig. 4 (A and B), the current-voltage relations obtained with a voltage ramp were linear and ethanol decreased glycine-activated current to a similar extent at all voltages between +60 and −60 mV. Thus ethanol’s effect on glycine-activated current is independent of membrane potential.

Furthermore, in the presence of ethanol, glycine-activated currents remained selectively permeable to Cl− since their reversal potential remained close to the calculated Nernst potential for Cl− (−1 mV in our experimental conditions). As a control for the presence of a time-dependent component of current that is slower than the voltage ramp, we also studied the effect of ethanol while holding the membrane potential constant for >3 min. As illustrated in Fig. 4C, ethanol inhibited the glycine-activated current equally when the membrane was held at −30 and +30 mV.

**Ethanol inhibition of glycine-activated current depends on glycine concentration**

Ethanol might inhibit glycine-activated current by decreasing the affinity of the receptor for glycine, and/or by decreasing the efficacy of glycine at the receptor. To explore these possibilities, we tested ethanol on currents induced by a wide range of glycine concentrations (3–1,000 μM). Typical glycine-activated current records, obtained in the absence and presence of 1 mM ethanol, are shown in Fig. 5. Ethanol had a greater effect on the current induced by 30 μM glycine (Ab) than on the current induced by 300 μM glycine (Bb). On average, 1 mM ethanol decreased the peak current activated by 10, 30, and 1,000 μM glycine to 66 ± 2 (n = 7), 75 ± 3 (n = 7) and 97 ± 4% (n = 6) of control, respectively. As can be seen from the corresponding concentration-response curves in Fig. 5C, the EC50 of glycine was significantly greater in the presence (60 ± 3 μM, n = 4, P < 0.01) than in the absence (32 ± 5 μM, n = 4) of 1 mM ethanol. Thus ethanol lowered the apparent affinity of glycine for GlyRs.

**Ethanol’s effects on the kinetics of glycine-activated current**

As changes in either agonist affinity or channel opening probability can alter the EC50 of agonists (see Colquhoun 1998), we analyzed the kinetics of glycine-activated current. To allow accurate measurement of time constants within the limits of the fast perfusion system (time constant of ~10 ms), glycine was applied at concentrations <30 μM. To ensure that the measurement of the rate of activation and deactivation was not influenced by the rates of onset and offset of ethanol’s action, we applied ethanol for 2 s before and after the application of glycine.

**FIG. 4.** Ethanol-induced depression of glycine-activated current is independent of membrane voltage. Glycine current-voltage relation was studied in whole cell recording with pairs of voltage ramps (from −60 to +60 mV) applied at rate of 1 mV/10 ms, as illustrated in A. The 1st voltage ramp measured background/leakage current: the data plotted in B were obtained by subtracting the currents elicited by the 1st ramp from the currents elicited by the 2nd ramp during the application of 30 μM glycine, in the absence (C) and presence of 1 mM ethanol (D). 

**B.** Current-voltage plots show that ethanol depressed glycine-activated current equally at all potentials, without changing the apparent reversal potential. Similar data were obtained from three other cells. 

**C.** Current-voltage data, normalized to values at +60 mV. 

In agreement with previous observations (Akaike and Kaneda 1989; Harty and Manis 1998), after a brief latent period, the onset of the inward current following glycine concentration jumps could be fitted by a single exponential function (Fig. 6A). The approximately linear relation between 1/τon and the concentration of glycine (Fig. 6B) is consistent with a one-binding site model, τon = 1/(Ck_on + k_off) and τoff = 1/k_off (where C is the concentration of glycine). The slope of these curves gave an estimated value for the rate of association of glycine (k_on) that was not changed significantly by ethanol: k_on was close to 9.9 × 10^4 mol−1 s−1 in 0, 0.1, and 1 mM ethanol.
We also measured the deactivation time constant ($t_{\text{off}}$) for glycine-activated channels from the time course of responses when glycine was rapidly washed from the external medium. The values of $t_{\text{off}}$ obtained by fitting single exponentials to the current decay after glycine concentration jumps did not change significantly with glycine concentration (Fig. 6C). In contrast, ethanol decreased the $t_{\text{off}}$ of current activated by 5 μM glycine from a control value of 340 ± 25 ms in the absence of ethanol to 290 ± 22 ms in 0.1 mM ethanol and 230 ± 25 ms in 1 mM ethanol. Thus the deactivation time constant was highly dependent on ethanol’s concentration (ANOVA, $P < 0.01; n = 6$) but was independent of glycine’s concentration (ANOVA, $P > 0.5; n = 6$). Furthermore, the $y$ intercept of these plots was used to estimate the dissociation rate ($k_{\text{off}}$), which was substantially increased by ethanol (Fig. 6C): 2.9, 3.7, and 4.4 s$^{-1}$ for 0, 0.1, and 1 mM ethanol, respectively (ANOVA, $P < 0.05; n = 6$). Hence the apparent dissociation constant ($K_D$) for glycine increased from ~29 to 45 μM. These values are close to those obtained directly from the glycine dose-response curves.

**Ethanol does not increase receptor desensitization**

The depression of glycine-activated currents by ethanol could result from increased receptor desensitization. Indeed, previous authors suggested that faster desensitization contributes to alcohol modulation of the nicotinic acetylcholine (nACh) receptor (Nagata et al. 1996). To test this hypothesis, we studied the desensitization of glycine-activated current in the absence and presence of ethanol. As shown in Fig. 7, the decay rate of current activated by 10 μM glycine was reduced rather than increased by 1 mM ethanol. The decay of glycine-activated currents both in the absence and presence of ethanol could be fitted with single exponentials. The ratio of the decay
One may argue that if two effects occur by actions at distinct actions near EC$_{50}$, potentiations are harder to record because they tend to ensure that the effects of glycine were reproducible.

Precautions: We took several precautions: we (1) replaced all plastic tubing with Teflon and glass and ensured there was no metal in the perfusion system; (2) used 95% ethanol prepared from grain, stored in glass bottles, and did control experiments with ethanol from different sources; (3) tested ethanol in the presence of TPEN, a zinc chelator; (4) demonstrated that the inhibitory effect of low (1 mM) ethanol does not occur in VTA neurons from older animals, prepared in an identical manner; and (5) tested a substantial number of neurons at each concentration of ethanol to ensure that the effects of glycine were reproducible.

**DISCUSSION**

Our principal finding is that 0.1–10 mM ethanol decreased glycine-induced responses in VTA neurons of neonatal rats. This is the first report that ethanol can inhibit GlyRs of native neonatal central neurons. Our study confirms and extends previous findings on interactions between ethanol and GlyRs obtained from recombinant expression systems or native preparations by electrophysiological recording or neurochemical methods (Aguayo and Pancetti 1994; Aguayo et al. 1994; Celentano et al. 1998; Engblom and Akerman 1991; Harty and Manis 1998; Mascia et al. 1998; Mihic et al. 1997; Wick et al. 1998; Ye et al. 2001). In addition, our results suggest that ethanol binds to a site on the GlyR that can allosterically reduce the affinity of this receptor for glycine.

**Ethanol inhibition of the glycine-activated current of VTA neurons is genuine**

Because this finding differs radically from previous results (see following text), and there is a strong historical precedent for artifacts in alcohol studies, it was important to rule out spurious effects due to the presence of contaminants and other possible artifacts. We therefore took the following additional precautions: we (1) replaced all plastic tubing with Teflon and glass and ensured there was no metal in the perfusion system; (2) used 95% ethanol prepared from grain, stored in glass bottles, and did control experiments with ethanol from different sources; (3) tested ethanol in the presence of TPEN, a zinc chelator; (4) demonstrated that the inhibitory effect of low (1 mM) ethanol does not occur in VTA neurons from older animals, prepared in an identical manner; and (5) tested a substantial number of neurons at each concentration of ethanol to ensure that the effects of glycine were reproducible.

**Glycine concentration and ethanol’s effects**

Ethanol potentiations are more easily observed at low concentrations of agonist, typically EC$_{20}$. With glycine concentrations near EC$_{50}$, potentiations are harder to record because they are smaller and receptors are more prone to desensitization. One may argue that if two effects occur by actions at distinct sites, agonist concentrations near EC$_{50}$ may bias the measurement in the direction of negative receptor modulation (inhibition). However, in our previous experiments, we clearly found ethanol potentiation of glycine responses in neurons exposed to 30 μM glycine. This suggests that applying glycine at concentrations near the EC$_{50}$ does not preclude detection of potentiation by ethanol. The inhibitory effect of ethanol observed in the current study was therefore probably not due to masking of a potentiating action by receptor desensitization.

**Comparison with previously reported interactions between ethanol and glycine**

Many studies on several preparations have shown that ethanol and other anesthetics enhance neuronal responses to glycine (Aguayo and Pancetti 1994; Aguayo et al. 1996; Celentano et al. 1988; Eggers et al. 2000; Engblom and Akerman 1991; Mascia et al. 1996a,b; Mihic 1999; Mihic et al. 1997; Tapia et al. 1998; Valenzuela et al. 1998; Ye et al. 2001). In our own recent study (Ye et al. 2001), 0.1–40 mM ethanol potentiated, depressed, or had no effect on GlyR-mediated responses of 35, 45, and 20% of neonatal VTA neurons. This is in good agreement with reports that ethanol potentiates, inhibits, or has no effect on GABA$_A$ receptor-mediated synaptic responses of neurons from different brain regions, as well as within a single neuronal population (Harris 1999; Weiner et al. 1997).

The factors underlying the variability of the ethanol-GABA$_A$ receptors interaction are unclear. Several factors, such as the subunit composition of the receptor, its phosphorylation state, and the methods for GABA and ethanol application, have been suggested. Similar factors may also be involved in ethanol-GlyR interactions. Since most (72%) adult VTA neurons showed potentiation by ethanol and only 5% (2 neurons) showed an inhibition, the difference in ethanol effects could be due to the known developmental shift from α2 to α1 subunit-containing receptors. This possibility is currently under investigation using compounds, such as cyanotriphenylborate, which distinguish between α1 and α2 subunit-containing receptors (Rundstrom et al. 1994).

Alternatively, the various responses to ethanol could indicate regulation of the channel protein by phosphorylation (Mascia et al. 1998; Swope et al. 1999). This possibility is supported by an earlier finding that ethanol’s effect on glycine-evoked responses seems to depend in part on the phosphorylation state of GlyRs (Mascia et al. 1998). Our preliminary results indicate that protein phosphorylation is indeed involved in ethanol-GlyR interactions in VTA neurons (unpublished data). In addition, we previously found that the equilibrium potential of chloride shifts from $-29$ mV in neonatal to $-50$ mV in adult VTA neurons (Ye 2000). This will certainly affect the driving force of Cl$^-$. The impact of this on the results needs further study.

**Mechanism of ethanol actions**

Ethanol may alter the permeability of Cl$^-$ channels. However, the fact that the reversal potential of glycine currents remained unchanged indicates that ethanol does not alter the ion selectivity of the channel. A related question is whether ethanol’s effects are voltage dependent. As ethanol is not charged at physiological pH, any voltage dependence would result from an ethanol-induced conformational change of the
glycine receptor channel that affected its voltage sensitivity. This is unlikely because ethanol’s inhibition of glycine-activated current was independent of membrane voltage.

More significant is the fact that in the presence of ethanol, the glycine concentration response curve shifted to the right in a parallel manner without a change in the maximal value. A simple explanation would be that ethanol binds to a site on the GlyR that can allosterically reduce the affinity of the receptor for glycine. This possibility is supported by two findings: that ethanol depressed glycine-activated currents by shifting the dose-response curves and that it enhanced the rate of offset of glycine responses. Thus the effects of ethanol may be attributed to faster dissociation of glycine from its binding site, even though other possibilities are not excluded.

The absence of ethanol effects on $\tau_{on}$ can result from either no change in the rate constants of binding and unbinding (in the standard equation for binding described earlier) or in a more complex kinetic modification with no changes in the balance between these rate constants. Furthermore a decrease in the deactivation time constant could also come about in several different ways. For example, ethanol could increase the agonist dissociation rate constant, as in the ethanol-mediated inhibition of P2X purinoceptor function in bullfrog dorsal root ganglion neurons (Li et al. 1998).

Physiological consequences of ethanol inhibition of GlyR responses

Recent studies have revealed dramatically different effects of both GABA$_A$ and glycine during early development. Neocortical cells have a relatively high intracellular [Cl$^-$. Therefore in contrast to their inhibitory effects in adult neurons, both glycine and GABA induce an outward flux of Cl, resulting in neuronal depolarization and excitation (Cherubini et al. 1991; Ye 2000). Glycine-induced membrane depolarization could result in the activation of voltage-gated Ca$^{2+}$ channels and NMDA receptor channels, thus raising intracellular Ca$^{2+}$. As an important second messenger, cytoplasmic Ca$^{2+}$ plays a critical role in many neuronal functions, including a trophic function at early stages of neuronal development (Cherubini et al. 1991; Reichling et al. 1994). In addition, recent evidence indicates that Ca$^{2+}$ influx, triggered by the activation of embryonic GlyRs, is required for the synaptic localization of GlyR and gephyrin, its anchoring protein (Betz et al. 1999). This Ca$^{2+}$ influx is crucial for the formation of gephyrin and GlyR clusters at developing postsynaptic sites (Kirsch and Betz 1998). Thus GlyR-mediated excitatory responses during embryonic development play an important role in synaptogenesis and functional verification—an essential step in the proper targeting of postsynaptic receptors to developing synaptic connections (Betz et al. 1999).

By dampening growth-promoting increases in cytoplasmic [Ca$^{2+}$], ethanol-mediated inhibition of glycine may be responsible for abnormal CNS development. Currently, the mechanisms underlying Fetal Alcohol Syndrome and/or Fetal Alcohol Effect remain obscure. It is thought that the brain is particularly sensitive to the neurotoxic effects of ethanol during the period of rapid growth and synaptogenesis, which occurs postnatally in rats but prenatally (during the last trimester of gestation) in humans. During this period, transient ethanol exposure can delete millions of neurons from the developing brain (Ikonomidou et al. 2000). Alcohol has other negative effects, such as causing neurons to grow incorrectly. Because even very low concentrations of ethanol can inhibit GlyR-mediated excitatory responses, this effect may be particularly significant for such neurotoxic effects of ethanol.

In conclusion, 0.1–10 mM ethanol depressed glycine-induced excitatory responses in 45% of neurons freshly dissociated from the VTA of neonatal rats. This effect may be due to an ethanol-induced decrease of glycine affinity for its receptor. This finding may shed light on the role of the GlyR in the neurotoxic effects of alcohol observed in Fetal Alcohol Syndrome and/or Fetal Alcohol Effect.

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