

Ethanol Potentiation of Glycine-Induced Responses in Dissociated Neurons of Rat Ventral Tegmental Area

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ABSTRACT

The potentiation of glycine-induced responses by ethanol (EtOH) was studied in neurons freshly dissociated from the ventral tegmental area (VTA) of 5- to 14-day-old postnatal rats using whole-cell and gramicidin-perforated patch-clamp techniques. Under current-clamp conditions, EtOH increased glycine-induced membrane depolarization and action potential firing. Under voltage-clamp conditions, EtOH (0.1–40 mM) alone did not elicit a current. When coapplied with glycine, EtOH enhanced the glycine-induced current in 35% (180 of 474) of the neurons. The EtOH-induced enhancement of glycine current was independent of membrane potential (between –60 and +60 mV); the reversal potential was not changed. Concentration-response analysis showed that in the presence of EtOH

(10 mM), the EC_{50} for glycine decreased from 25 ± 4 to $14 \pm 3 \mu\text{M}$; the Hill coefficient increased from 1.5 ± 0.2 to 1.9 ± 0.3 . Kinetic analysis of glycine currents indicated that EtOH decreased the time constant of activation and increased the time constant of deactivation of glycine-gated chloride channels. EtOH may accelerate glycine association with its receptor at the agonist binding site and increase the apparent agonist affinity. Our observations suggest that, at pharmacologically relevant concentrations, EtOH alters the function of glycine receptors and thus the excitability of neonatal VTA neurons. This action of EtOH may contribute to the neurobehavioral disturbances associated with fetal alcohol syndrome.

The fetal central nervous system (CNS) is one of the most sensitive targets of ethanol (EtOH). Exposure of the human fetus to EtOH results in a combination of abnormalities termed fetal alcohol syndrome or fetal alcohol effects (Clarren and Smith, 1978). The most common manifestations of fetal alcohol syndrome/fetal alcohol effects are neurobehavioral disturbances, such as hyperactivity, learning disabilities, depression, and psychosis (Clarren and Smith, 1978). The mechanisms underlying EtOH effects on the developing human brain, however, are poorly understood.

According to a very recent report (Ikonomidou et al., 2000), EtOH not only blocks *N*-methyl-D-aspartate-type glutamate receptors but also strongly activates GABA_A receptors, thus triggering widespread apoptotic neurodegeneration in the developing rat forebrain. Like GABA (Krnjević, 1997), glycine is a major inhibitory neurotransmitter in the mature CNS, which activates Cl[–]-selective channels. In the adult mammalian CNS, activation of these channels results in neuronal hyperpolarization. Recent studies have revealed dramatically different effects

of these transmitters in early development. Neonatal cells feature relatively high intracellular [Cl[–]], owing to the active inward transport of Cl[–]. Therefore, in contrast to their effects in adult cells, both glycine and GABA induce an outward flux of Cl[–], resulting in neuronal depolarization and excitation (Cherubini et al., 1991; Ye, 2000).

The glycine receptor/channel (GlyR) consists of α - and β -subunits that combine to form a pentameric receptor complex mediating transmembrane flux of Cl[–] (Betz, 1991). Molecular cloning studies have revealed a developmental heterogeneity of GlyR subunits. For example, the α_2 -subunit appears only during an early developmental stage (from fetus to 2–3 weeks after birth), and is subsequently replaced by the α_1 -subunit (Betz, 1991). According to other studies (Akagi and Milei, 1988; Ye, 2000), the physiological and pharmacological properties of the GlyRs depend on their subunit composition and probably differ in adult and immature types.

In contrast to the numerous studies on GABA receptors, studies of the effects of EtOH on GlyRs are fewer and more limited in scope. Engblom and Akerman (1991) reported that EtOH potentiates glycine-activated Cl[–] uptake into synaptoneuroosomes of whole-rat brain. In addition, central depres-

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ABBREVIATIONS: CNS, central nervous system; EtOH, ethanol; GABA, γ -aminobutyric acid; GlyR, glycine receptor/channel; STR, strychnine; I_{Gly} , glycine-activated current; VTA, ventral tegmental area; V_{H} , holding potential; E_{Gly} , reversal potential of glycine current; τ_{d} , time constant of decay; τ_{on} , activation time constant; τ_{off} , deactivation time constant.

sant effects of EtOH were shown to be enhanced by glycine and the glycine precursor serine (Williams et al., 1995); the specific antagonist strychnine (STR) blocked this action, indicating that glycine enhances EtOH effects via STR-sensitive GlyRs (Schiller et al., 1995). More recently, EtOH's positive modulatory effect on recombinant GlyRs was shown to be determined by a single amino acid in the subunit of the STR-sensitive GlyR (Mascia et al., 1996; Mihic et al., 1997). Electrophysiological studies are supportive, revealing a positive modulation of glycine-activated current (I_{Gly}) by EtOH in cultured neurons from chicks (Celentano and Wong, 1994), mice (Aguayo and Pancetti, 1994; Aguayo et al., 1996), and *Xenopus* oocytes and mammalian cell lines expressing homomeric GlyRs (Mascia et al., 1996; Valenzuela et al., 1998; Ye et al., 1998). 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 EtOH (Gatto et al., 1994; Wise, 1996). Recent experiments in this laboratory have shown that glycine-mediated responses can be recorded in the majority of VTA neurons (Ye, 1999, 2000). Despite the importance of the VTA in the reinforcement of drug abuse, EtOH effects on the GlyRs of VTA have not been studied. In the present article, we show that pharmacologically relevant concentrations of EtOH (0.1–40 mM) greatly potentiate the glycine-activated responses of neonatal VTA neurons and thus enhance their excitability.

Materials and 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 Committee of University of Medicine and Dentistry of New Jersey (protocol number 0752). We performed our experiments on VTA neurons prepared as described in Ye (2000). Briefly, 5- to 14-day-old Sprague-Dawley rats were decapitated. The brain was quickly excised, placed into ice-cold saline saturated with 95% O₂ and 5% CO₂, glued to the chilled stage of a vibratome (Campden Instruments, Leics, UK), and sliced to a thickness of 300 to 400 μm . Slices were transferred to the standard external solution saturated with O₂, containing 1 mg of pronase/6 ml and incubated (31°C) for 20 min. After an additional 20-min incubation in 1 mg of 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 of these tissue punches 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.

The saline in which the brain was dissected contained 128 mM NaCl, 5 mM KCl, 1.2 mM NaH₂PO₄, 26 mM NaHCO₃, 9 mM MgCl₂, 0.3 mM CaCl₂, and 2.5 mM glucose. The standard external solution contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, and 10 mM HEPES. The pH of both solutions was adjusted to 7.4 with Tris base and the osmolarity to 320 mM/kg with sucrose. Patch pipette solutions contained 150 mM KCl, 10 mM HEPES for gramicidin-perforated patch recording, and 120 mM CsCl, 21 mM tetraethylammonium chloride, 4 mM MgCl₂, 11 mM EGTA, 1 mM CaCl₂, 10 mM HEPES, and 2 mM Mg-ATP for conventional whole-cell recording. The pipette solutions were adjusted to pH 7.2 with Tris base and the osmolarity to 280 mM/kg with sucrose. The patch electrode had a resistance between 3 and 5 M Ω when filled with the above-mentioned solutions. The gramicidin-perforated patch technique (Abe et al., 1994) was used to record

the glycine-induced whole-cell responses. The gramicidin stock solution of 10 mg/ml was prepared in methanol (J.T. Baker, Inc., Phillipsburg, NJ). It was diluted in the pipette solution to a final concentration of 50 to 100 $\mu\text{g}/\text{ml}$ just before the experiment. The pipette tip was filled with gramicidin-free solution by brief immersion before 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 repeated 10-mV hyperpolarizing voltage steps from a holding potential (V_H) of -50 mV. The entry into the perforated patch mode was signaled by an increase in the amplitude of the capacitive transient. The access resistance reached a steady level of 20 M Ω within 30 min after making the giga-seal. When conditions stabilized, whole-cell recording began. Throughout all experimental procedures the bath was continuously perfused with the standard external solution. All glycine-induced responses were elicited in this solution at an ambient temperature of 20–23°C.

Currents were recorded under voltage clamp with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) interfaced to a Digidata 1200 (Axon Instruments) and directly digitized with pCLAMP 6 software for further off-line analysis. The junction potential between the patch pipette and the bath solutions was nulled immediately before forming the giga-seal. The liquid junction potential between the bath and the electrode was 3.3 mV as calculated from the generalized Henderson equation using the Axoscope junction potential calculator (Barry, 1996). This value was corrected off-line to accurately estimate the reversal potential of I_{Gly} . In most experiments, the series resistance before compensation was 15 to 25 M Ω . Routinely, 80% of the series resistance was compensated; hence, there was a 3-mV error for 1 nA of current.

Chemical Application. Solutions of glycine, strychnine, gramicidin (Sigma Chemical Co., St. Louis, MO) and EtOH (Pharmco, Bayonne, NJ) were prepared on the day of experimentation. Solutions were applied to a dissociated neuron with a superfusion system via a multibarreled pipette (as described previously; Ye, 2000). The tip of the superfusion pipette was usually placed 50 to 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 change was determined by reducing the external Na⁺ concentration from 140 to 10 mM (plus 130 mM *N*-methyl-D-glucamine) during a kainate application. Because the kainate currents do not desensitize, the rate of decrease of kainate responses reflected the rate of solution change (Ye et al., 1999).

Whole-cell current decays were fit by a Chebychev algorithm (pCLAMP). Statistical analyses of concentration-response data were performed using a nonlinear curve-fitting program (Sigma Plot; Jandel Scientific, San Rafael, CA). Data were statistically compared using Student's *t* test at a significance level of $P \leq 0.05$, otherwise as indicated. For all experiments, average values are expressed as mean \pm S.E.M. with the number of neurons indicated in parentheses. To generate a concentration-response relationship for VTA glycine receptors, all neurons were exposed to 1 mM glycine and two to three lower concentrations (0.003–0.3 mM). For each concentration, four to six responses for a given neuron were normalized to the peak current amplitude in response to 1 mM glycine. The normalized values from three to five neurons at each concentration were normalized. These averages were then fit using a Simplex algorithm (Sigma Plot; Jandel Scientific) with the Hill equation: $I = (I_{\text{max}} \times C^n)/(C^n + EC_{50}^n)$, where I , I_{max} , C , EC_{50} , and n are I_{Gly} , maximal I_{Gly} , glycine concentration, the concentration for 50% of maximum, and the Hill coefficient, respectively.

Results

EtOH Enhances Glycine-Induced Depolarization and Neuronal Excitability. EtOH's effect on glycine response was first studied under current-clamp conditions with

the gramicidin-perforated patch technique. In agreement with our previous report (Ye, 2000), glycine elicited depolarization and, in some cases, action potentials in VTA neurons from neonatal rats (Fig. 1). This depolarization is explained by a reversal potential for glycine's action (E_{Gly}) that is much more positive (near -25 mV in neonatal neurons) than the resting potential (-68 ± 2.5 mV, $n = 5$).

EtOH (10 mM) applied alone could also cause depolarization (Ye, 1999); but even when it did not alter the membrane potential, its coapplication with glycine enhanced both the amplitude of the depolarization and the number of action potentials elicited by $10 \mu\text{M}$ glycine (Fig. 1). On average, the depolarization induced by $10 \mu\text{M}$ glycine was 13 ± 3 mV in the absence and 23 ± 3 mV in the presence of 10 mM EtOH; these values are significantly different ($P < 0.01$, $n = 8$). Moreover, $10 \mu\text{M}$ glycine evoked a significantly greater number of spikes in the presence of 10 mM EtOH (5 ± 1) than in its absence (2 ± 1 , $n = 7$, $P < 0.01$). In the absence of glycine, 10 mM EtOH did not increase firing elicited by a depolarizing stimulus (2 ± 1 versus 2 ± 1 spikes in the absence and presence of 10 mM EtOH; $P > 0.05$, $n = 4$).

EtOH Potentiates I_{Gly} . Our previous findings on neonatal VTA neurons recorded under voltage clamp (Ye, 2000) can be summarized as follows. Most VTA neurons (82%) are sensitive to glycine. When elicited by a near-threshold concentration of glycine ($3\text{--}5 \mu\text{M}$), in the majority of cases (64%) I_{Gly} decays; with higher glycine concentrations, I_{Gly} always decays to a lower steady-state value. Peak I_{Gly} increases sigmoidally with the concentration of glycine, with an EC_{50} of $37 \pm 8 \mu\text{M}$ and a Hill coefficient of 1.5. When internal and external $[\text{Cl}^-]$ are equal, I_{Gly} reverses near 0 mV. In recordings made with the gramicidin-perforated patch technique, E_{Gly} was -29 mV.

In the present experiments, acute applications of 0.1 to 40 mM EtOH markedly enhanced I_{Gly} . Figure 2A shows typical examples of I_{Gly} evoked by $3 \mu\text{M}$ glycine alone (A, a) and in the presence of 1 and 10 mM EtOH (A, b and c, respectively); I_{Gly} recovered to control level after washout of EtOH (A, d). Such potentiation of I_{Gly} by very low concentrations of EtOH occurred in 35% (180 of 474) of the neurons tested. On average, 1, 10, and 40 mM EtOH enhanced the peak I_{Gly} induced by $10 \mu\text{M}$ glycine to $120 \pm 3\%$ ($n = 45$), $148 \pm 9\%$ ($n = 123$), and $196 \pm 20\%$ ($n = 64$) of control, respectively. The histogram in Fig. 2C illustrates the marked variability of the enhancement of peak I_{Gly} (evoked by $3 \mu\text{M}$ glycine) by 10 mM EtOH in different neurons.

At an even higher concentration (100 mM), EtOH enhanced I_{Gly} to only $131 \pm 6\%$ ($n = 90$), significantly less than the effect of 40 mM EtOH ($P < 0.01$). This result is consistent

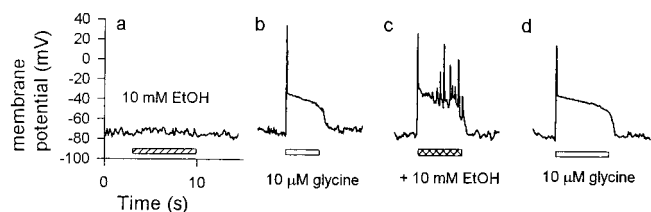


Fig. 1. EtOH enhances membrane depolarization and action potential firing elicited by glycine in current-clamped VTA neurons of a 5-day-old rat. Gramicidin-perforated patch recordings show voltage responses to 10 mM EtOH alone (a) and $10 \mu\text{M}$ glycine in the absence (b, d) and presence of 10 mM EtOH (c). Initial membrane potential was -75 mV.

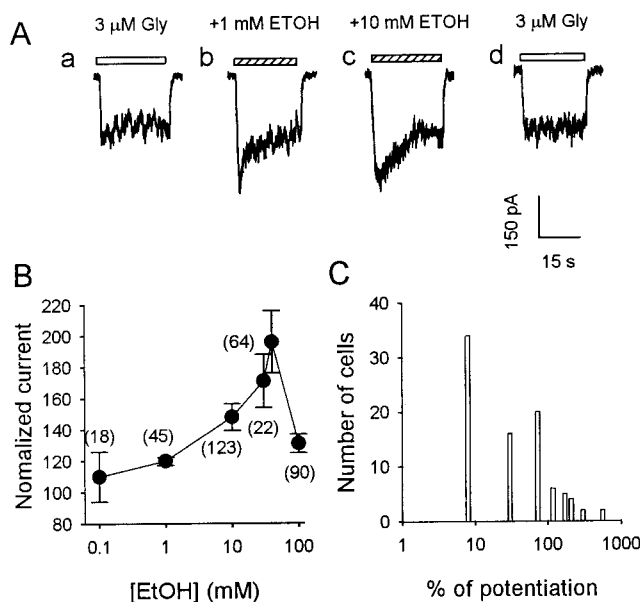


Fig. 2. EtOH enhances peak amplitude of glycine-activated currents (I_{Gly}). A, current traces were obtained from a voltage-clamped VTA neuron of a 5-day-old rat by conventional whole-cell recording. I_{Gly} was elicited by $3 \mu\text{M}$ glycine alone (open horizontal bars) or together with 1 mM (b) or 10 mM (c) EtOH (hatched bars). The V_{H} was -50 mV. B, concentration-response relationship for the effects of EtOH (0.1–100 mM) on peak I_{Gly} (normalized to peak currents recorded in absence of EtOH). Vertical bars indicate \pm S.E.M. and numbers in parentheses indicate the number of cells recorded at each concentration. C, histograms show the variability of 10 mM EtOH-induced enhancement of I_{Gly} evoked by $3 \mu\text{M}$ glycine.

with EtOH's effect on GABA_A -induced ^{36}Cl uptake in PA3 cells transfected with receptor subunits (Harris et al., 1995; but see Aguayo et al., 1996). Responses to glycine in the absence and presence of 40 mM EtOH were completely blocked by 500 nM STR. This suggests the involvement of an immature form of the GlyR because the STR IC_{50} for the adult GlyR is only 12 nM compared with 110 nM for the neonatal GlyR (Ye, 2000). This observation also indicates that potentiated current resulted from an increase in GlyR function and not from an additional effect of the agonist in combination with EtOH.

EtOH-Mediated Enhancement of I_{Gly} 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 looked for evidence regarding the possibility that EtOH's potentiation of I_{Gly} is also voltage-dependent. As illustrated in Fig. 3, A and B, the current-voltage curves obtained with a voltage-ramp were linear and EtOH enhanced I_{Gly} to a similar extent at all voltages between $+60$ and -60 mV. Thus, EtOH's effect on I_{Gly} is independent of voltage.

Furthermore, in the presence of EtOH I_{Gly} remained selectively permeable to Cl^- because E_{Gly} remained close to the calculated Nernst potential for Cl^- (-1 mV in our experimental conditions). Because voltage dependence could have a time-dependent component that is slower than the slew rate of the voltage ramp, we also studied the effect of EtOH while holding the membrane potential constant for >3 min. As illustrated in Fig. 3C, I_{Gly} decays during a continuous application of glycine. The decay of I_{Gly} could be fit with a single exponential function (Fig. 3C), and appeared to be voltage-

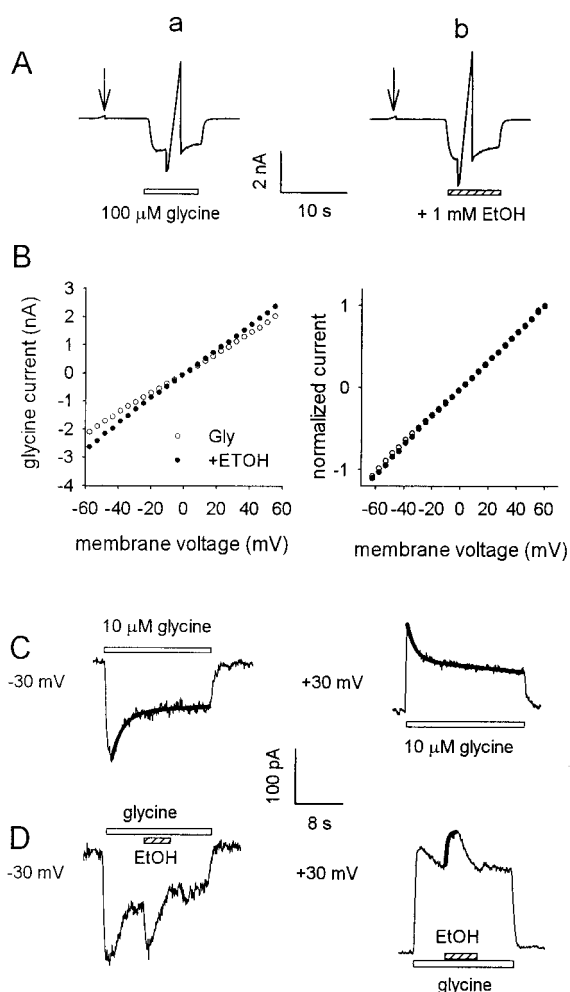


Fig. 3. EtOH-induced potentiation of I_{Gly} is independent of membrane voltage. EtOH's effect on current-voltage relationship of I_{Gly} was studied with a pair of voltage ramps (from -60 to $+60$ mV), applied at a rate of 1 mV/10 ms. Drugs were applied as indicated. Traces obtained from the first voltage ramp (indicated by an arrow in A, a and b) measured background/leakage current; The current-voltage curve was obtained by subtracting the trace from the first ramp from that of the second ramp. A, typical I_{Gly} recorded from neuron exposed to 100 μM glycine alone and in combination with 10 mM EtOH. B, current-voltage curves derived from A show that EtOH enhanced I_{Gly} equally at all potentials, without changing the apparent reversal potential. Similar data were obtained from three other cells. B, right, shows normalized current-voltage relations for the same data (all currents were normalized to values at $+60$ mV). This figure shows that EtOH's effect is not voltage-dependent. C, voltage dependence of I_{Gly} decay. I_{Gly} recorded at a V_{H} of -30 mV (left) decayed faster compared with that of $+30$ mV (right). D, brief pulses of EtOH (40 mM) produced similar potentiation of inward and outward I_{Gly} (obtained at V_{H} -30 and $+30$ mV) elicited by 10 μM glycine. The onset of EtOH's effect could be fit by a single exponential, $\tau = 264$ ms), as shown by the continuous line.

dependent. The decay time constant increases with depolarization: it was 23 ± 5 s for -30 mV and 55 ± 5 s for $+30$ mV, respectively ($n = 4$, $P < 0.01$).

When a brief pulse of 10 mM EtOH was applied during a longer lasting pulse of 10 μM glycine, I_{Gly} was immediately enhanced. After the EtOH pulse ended, I_{Gly} returned to the control level. The percentage potentiation of I_{Gly} was not significantly different at membrane potentials of -30 and $+30$ mV (126 ± 8 and $124 \pm 6\%$, respectively, $P > 0.1$). The onset of the EtOH effect could be fit by a single exponential (smooth line, Fig. 3D, right). The time constant of onset of

EtOH's action was 305 ± 10 ms ($n = 5$). However, the offset time constant could not be readily estimated because the effect of a sustained application of EtOH decreased with time (Fig. 3D).

EtOH-Mediated Enhancement of I_{Gly} Depends on Glycine Concentration. EtOH might augment I_{Gly} by increasing the affinity of the receptor for glycine, by increasing the efficacy of glycine at the receptor, or both. In an attempt to explore these possibilities, EtOH was tested on currents induced by 3 to 1000 μM glycine. Typical I_{Gly} records, obtained in the absence and presence of 40 mM EtOH, are shown in Fig. 4A. Although EtOH strongly potentiated I_{Gly} induced by 10 μM glycine (a–c), it had no appreciable effect on I_{Gly} induced by 1 mM glycine (d–f). On average, 40 mM EtOH potentiated the peak I_{Gly} activated by 10 , 30 , and 1000 μM glycine to $160 \pm 2\%$ ($n = 6$), $133 \pm 3\%$ ($n = 7$), and $98 \pm 4\%$ ($n = 5$) of control, respectively. Figure 4B presents the glycine concentration-response curves for data obtained from neurons in control solution and in the presence of 40 mM EtOH. The EC_{50} and Hill coefficient were 25 ± 4 μM and 1.5 ± 0.2 , respectively, in the absence of EtOH and 14 ± 3 μM and 1.9 ± 0.3 in the presence of 40 mM EtOH. In contrast, the maximum response was not affected by 40 mM EtOH, being 1.0 in its absence and 0.98 in its presence ($P > 0.5$).

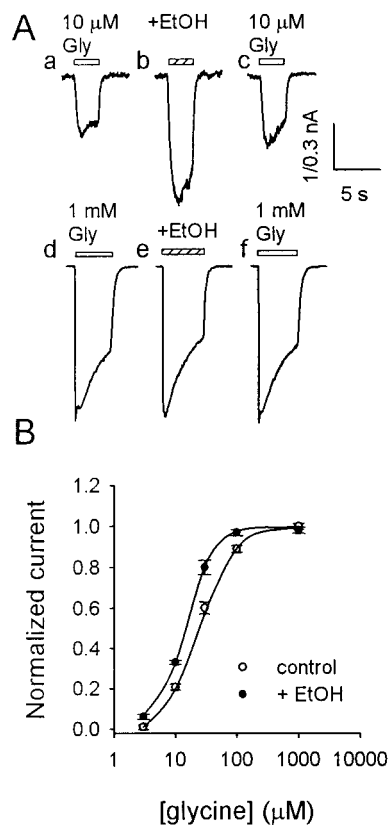


Fig. 4. Averaged concentration-response curves. A, examples of currents induced by low (b and c) and high (d–f) glycine concentrations in the absence and presence of 40 mM EtOH (at V_{H} -50 mV). Calibration: a–c, 0.5 nA; d–f, 1 nA. B, averaged data (four to six neurons) for glycine alone (\circ) or glycine plus 40 mM EtOH (\bullet). Before pooling, amplitudes of the peak I_{Gly} were first normalized to a near maximum response elicited by a concentration of glycine (1 mM) that was common to all sets. For individual neurons, three-point concentration-response curves to glycine were determined first; similar responses were then obtained in the presence of EtOH (see *Materials and Methods* for details). The continuous line is the fit of the Hill equation.

EtOH Effects on the Kinetics of I_{Gly} . Because changes in either agonist affinity or channel opening efficacy can alter the EC_{50} values of agonists (Colquhoun, 1998), we analyzed the channel kinetics, including activation, deactivation, and desensitization of GlyR in the absence and presence of EtOH. To allow accurate measurement of time constants within the limits of the fast perfusion system (time constant of around 10 ms), we applied glycine at concentrations of 30 μM or lower. To ensure that the measurement of the rates of activation and deactivation was not influenced by the rates of onset and offset of EtOH's action, we applied EtOH for 5 s before and after the application of glycine.

The following simple model was assumed for activation of a receptor:



AR and AR* represent a closed channel with all binding sites occupied or an open channel, respectively. To further simplify the model, it was assumed that glycine binding was faster than the experimental time scale (i.e., the unbound and bound states were kinetically indistinguishable) so the binding step was not included in the model. Assuming equilibration of binding to be fast, the activation rate constant ($1/\tau_{\text{on}}$) of a response is as follows: $1/\tau_{\text{on}} = \alpha + \beta \times ([\text{agonist}]^n / ([\text{agonist}]^n + \text{EC}_{50}^n))$, where α is the closing rate constant, β is the opening rate constant, $[\text{agonist}]$ is the agonist concentration, n is the number of binding sites, and EC_{50} is the concentration of agonist that gave 50% of the maximum possible β .

In agreement with previous observations (Akaike and Kaneda, 1989; Harty and Manis, 1998), after a brief latent period, the onset of the inward current after glycine concentration jumps could be fit by a single exponential function (Fig. 5A). The relationship between $1/\tau_{\text{on}}$ and the concentration of glycine was approximately linear (Fig. 5B). The slope of these curves gave an estimated value for the rate of association of glycine (k_{on}). They are $1.1 \times 10^{-7} \text{ mol}^{-1} \text{ s}^{-1}$, $1.75 \times 10^{-7} \text{ mol}^{-1} \text{ s}^{-1}$, and $1.95 \times 10^{-7} \text{ mol}^{-1} \text{ s}^{-1}$ for 0, 10, and 40 mM EtOH, respectively. The y-intercept of these plots was used to estimate the dissociation rate (k_{off}), which was substantially increased by EtOH. The estimated value of this rate was 1.36, 3.25, and 4.14 s^{-1} in the presence of 0, 10, and 40 mM EtOH, respectively.

We also determined the deactivation time constant (τ_{off}) for glycine from the time course of responses when the added glycine was rapidly washed from the external medium. In agreement with previous work (Harty and Manis, 1998), the decreases in I_{Gly} after glycine concentration jumps could also be fit by single exponential functions. The τ_{off} did not change significantly with glycine concentration (Fig. 5C). In contrast, EtOH increased the τ_{off} of I_{Gly} evoked by 10 μM glycine from a control value of 140 ± 25 ms in the absence of EtOH to 210 ± 20 ms in 10 mM EtOH and 300 ± 30 ms in 40 mM EtOH. Thus, the deactivation time constant was highly dependent upon EtOH's concentration (ANOVA, $P < 0.01$, $n = 6$), but was independent of glycine's concentration (ANOVA, $P > 0.25$, $n = 5$).

Effects of EtOH on Receptor Desensitization. EtOH potentiation of I_{Gly} could result from a decrease in the rate of

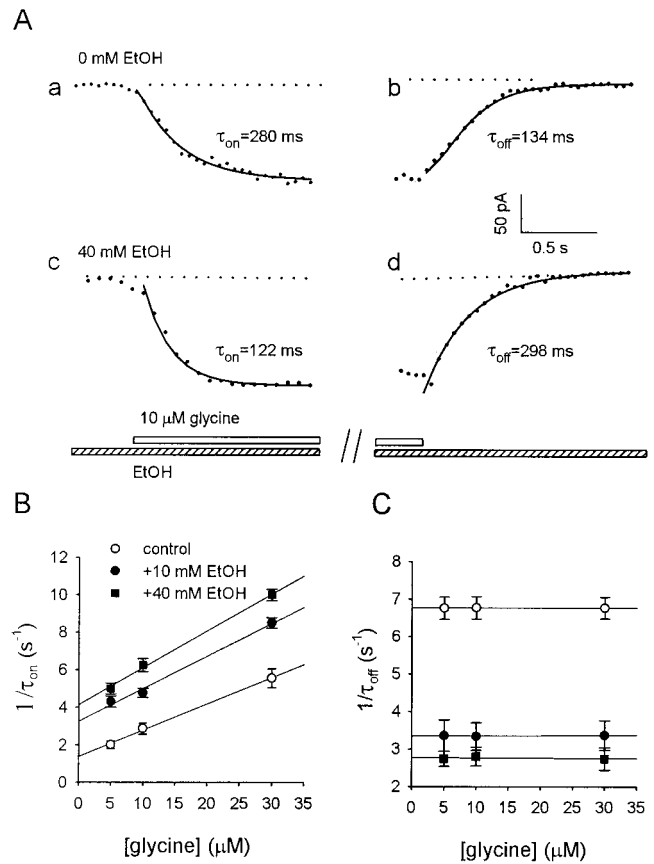


Fig. 5. Effect of EtOH on the kinetics of I_{Gly} . A, whole-cell recordings of currents activated by 10 μM glycine in the absence and presence of 40 mM EtOH (at $V_{\text{H}} = -50$ mV). Both activation and deactivation curves were well fit by a single exponential function (continuous curves). To eliminate possible complicating effect of slower onset and offset of EtOH's action, EtOH application started 5 s before and ended after that of glycine. EtOH (40 mM) decreased the activation time constant (τ_{on}), and increased the deactivation time constant (τ_{off}). Time calibration indicates 0.5 s for A, a, b, and d, and 0.35 s for A, c. B, graph of mean $1/\tau_{\text{on}}$ values as function of glycine concentration in the absence (\circ), and presence of 10 (\bullet) and 40 mM (\blacksquare) EtOH. The data demonstrated an approximately linear relationship between $1/\tau_{\text{on}}$ versus the concentrations of glycine. The slope of these curves gave an estimated value of the forward rate of the glycine response. The y-intercepts of these curves yielded estimates of the reverse rate (k_{off}). C, graph plotting mean $1/\tau_{\text{off}}$ values versus concentration of glycine in absence (\circ) and presence of 10 (\bullet) and 40 mM (\blacksquare) EtOH. Mean $1/\tau_{\text{off}}$ for I_{Gly} was sensitive to the concentration of EtOH (ANOVA, $P < 0.05$; $n = 5$), but not to that of glycine (ANOVA, $P > 0.25$, $n = 5$).

receptor desensitization. Indeed, previous experiments suggested that slowing of desensitization contributes to alcohol potentiation of the 5-hydroxytryptamine₃ receptor (Zhou et al., 1998). To test this possibility, we studied the desensitization of I_{Gly} in the absence and presence of EtOH. As shown in Fig. 6, the differences between peak and late currents, the decay rate of current activated by 3 μM glycine increased, rather than decreased, by application of 40 mM EtOH. The ratio of the decay time constants ($\tau_{\text{EtOH}}:\tau_{\text{control}}$) in Fig. 6 is 0.45. For six neurons, 40 mM EtOH significantly decreased the time constant of desensitization (Student's t test, $P < 0.05$). This is in agreement with EtOH acceleration of current decay induced by extended application of GABA (Nakahiro et al., 1991). Desensitization has previously been studied with whole-cell recording (Akaike and Kaneda, 1989; Harty and Manis, 1998) and the desensitization time constant decreased when glycine concentration was increased, whereas

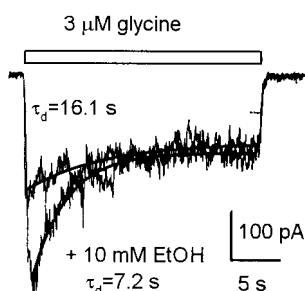


Fig. 6. EtOH accelerates I_{Gly} desensitization. Superimposed traces illustrate the decay of I_{Gly} (elicited by long application of $3 \mu\text{M}$ glycine in the absence and presence of 10 mM EtOH). As suggested by the indicated time-constants of decay (τ_d), the average values of τ_d for currents activated by $3 \mu\text{M}$ glycine differed significantly in the absence and presence of 10 mM EtOH (18 ± 2.5 and 7.1 ± 1.7 s, respectively (t test, $P < 0.01$, $n = 5$). V_H was -50 mV .

the amount of desensitized current increased (Akaike and Kaneda, 1989). Therefore, the increase in response amplitude associated with an increased desensitization evoked by EtOH (Fig. 6) might result from multiple distinct changes in GlyRs kinetics. Figure 7 illustrates that a further increase in glycine concentration resulted in a response similar to that obtained with a lower glycine concentration in the presence of EtOH, indicating that EtOH might decrease the microscopic k_d for glycine.

Discussion

The observations reported here indicate that at pharmacologically relevant concentrations EtOH potentiates the excitatory action of glycine on neonatal mammalian VTA neurons. This is the first report of EtOH effects on the GlyRs of native neonatal central neurons. Our study confirms and expands upon previous findings obtained from recombinant expression systems or native preparations via electrophysiological recording and neurochemical methods.

For the rats studied, the VTA cells are clearly immature and glycine induces neuronal depolarization and excitation. Glycine receptor/channels, together with other ligand-gated channels, mediate fast excitatory and inhibitory synaptic transmission in the developing CNS. These receptors/channels have been shown to have roles in neuronal proliferation,

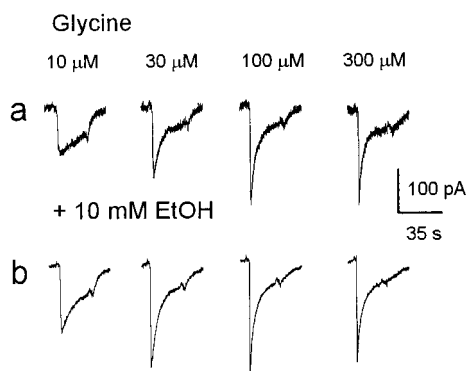


Fig. 7. EtOH enhances I_{Gly} . Current traces elicited by several concentrations of glycine (as indicated) in the absence (a) and presence of 10 mM EtOH (b). This figure shows that a further increase in glycine concentration results in response similar to that obtained with a lower glycine concentration in the presence of EtOH. $V_H = -50 \text{ mV}$. Note that the potentiation of I_{Gly} by EtOH gradually diminished as glycine approached a saturating concentration.

differentiation, and programmed cell death (Costa et al., 2000). Accumulating evidence indicates that prenatal and/or early postnatal EtOH exposure affects neurotransmitter-gated ion channels.

There are several possible mechanisms by which EtOH might act on glycine-gated ion channels. For example, EtOH may alter their ionic permeability. However, E_{Gly} was not significantly altered by EtOH, indicating that EtOH does not alter the ion selectivity of the channel. A related question concerned whether EtOH's effects are voltage-dependent. Because EtOH is not charged at physiological pH, any voltage dependence would result from an EtOH-induced conformational change in the glycine receptor channel that affected its voltage sensitivity. More significant is the fact that in the presence of EtOH, the glycine concentration-response curve shifted to the left in a parallel manner without appearing to alter the maximal value. Thus, the ability of EtOH to enhance I_{Gly} may be at least in part attributable to a slowing of agonist dissociation.

In addition, we demonstrated that EtOH increased the activation rate. This observation suggests that EtOH enhances glycine response by accelerating glycine association to its receptor at the binding site, a mechanism that has been demonstrated for potentiation of GABA_A receptor function by diazepam in expressed $\alpha 2\beta 1\gamma 2$ receptors (Lavoie and Twyman, 1996). However, caution must be taken in interpreting the present data. In the proposed model, the rate of offset of the response is simply $1/\alpha$, or the reciprocal of the closing rate constant, i.e., the mean open time of the channel. This shows that we cannot ignore the gating constants in the interpretation of a deactivation rate, even in the simplest case. In addition, we must consider the contribution of EtOH-induced change of agonist efficacy, as demonstrated for potentiation of 5-hydroxytryptamine₃ receptor channel function by EtOH in NCB-20 neuroblastoma cells (Lovinger et al., 2000). Theoretically, for agonists with high intrinsic activity at ligand-gated ion channels, alteration of apparent agonist affinity may result from changes in agonist efficacy (Colquhoun, 1998). Although we did not observe an increase in the maximum response to glycine in the presence of EtOH, we cannot exclude an effect of EtOH on efficacy for the following reason. In a variety of mechanistic models for receptor activation, the maximum response approximates to $I_{\text{max}} = E/(E + 1)$, where E is the efficacy of the agonist (β/α). At high values of E , increases in E do not result in marked increases in maximum response (which would be supported by the increase in Hill slope; Colquhoun, 1998). However, whether this involves a change in the true agonist binding affinity, or a change in gating properties of the channel (or a combination of both), remains to be seen. Single-channel analysis is necessary because changes in affinity can be separated from alterations in channel gating by examination of burst patterns of single ligand-gated ion channels (Colquhoun and Hawkes, 1995).

Our observation that the glycine-activated current was heterogeneous with respect to modulation by EtOH in different neurons is consistent with studies from other laboratories (Aguayo and Pancetti, 1994; Aguayo et al., 1996). This may reflect different subunit compositions of glycine receptor/channels with different functional properties. Alternatively, the insensitivity of a subset of the glycine-gated channels to EtOH could indicate regulation of the channel protein by a process such as phosphorylation (Mascia

et al., 1998; Swope et al., 1999). This explanation agrees with a previous finding that EtOH's effect on glycine-evoked responses seems to depend in part on the phosphorylation state of GlyRs (Mascia et al., 1998). It is also supported by our observation that EtOH failed to potentiate glycine in all six neurons recorded with pipettes containing no added ATP (J. H. Ye and L. Tao, unpublished data).

Glycine-induced membrane depolarization could result in the activation of voltage-gated Ca^{2+} channels and *N*-methyl-D-aspartate receptor channels, thus increasing intracellular Ca^{2+} . Cytoplasmic Ca^{2+} is an important second messenger and plays critical roles in many neuronal functions, including the regulation of development. Glycine-induced increases of intracellular Ca^{2+} may, therefore, mediate the trophic function of GlyRs at early stages of neuronal development (Cherubini et al., 1991; Reichling et al., 1994). On the other hand, high levels of intracellular Ca^{2+} are toxic. In the present study, pharmacologically relevant concentrations (0.1–40 mM) of EtOH potentiated glycine-induced depolarization in VTA neurons of neonatal rats. If glycine does in fact increase intracellular Ca^{2+} , EtOH's potentiating effect on glycine action may raise cytoplasmic Ca^{2+} concentrations to neurotoxic levels. Hence, the observed change in GlyRs activity may be responsible for abnormal CNS development, as in fetal alcohol syndrome. The relevance of our current finding with fetal alcohol effects and/or fetal alcohol syndrome is clear. Currently, the mechanisms underlying fetal alcohol effects/fetal alcohol syndrome are not well understood. It is thought that the brain is particularly sensitive to the neurotoxic effects of EtOH during the period of synaptogenesis, or the brain growth spurt period, which occurs postnatally in rats but prenatally (during the last trimester of gestation) in humans. Very recently Olney and colleagues (Ikonomidou et al., 2000) demonstrated that during this period, transient EtOH exposure can delete millions of neurons from the developing brain. However, how EtOH kills the neurons is still not well understood. EtOH potentiation of glycine-induced responses in neurons of neonatal rats may contribute to the neurotoxic effects of EtOH.

In summary, our experiments on neonatal VTA neurons show that EtOH enhances both the depolarization and the firing elicited by glycine. The actions of EtOH may be due to EtOH-induced acceleration of glycine binding to its receptor and increases of the apparent agonist affinity. This study may shed light on the role of the GlyR in the neurotoxic effects of alcohol observed in fetal alcohol syndrome.

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