Mechanisms for the Modulation of Native Glycine Receptor Channels by Ethanol

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Eggers, Erika D. and Albert J. Berger. Mechanisms for the modulation of native glycine receptor channels by ethanol. J Neurophysiol 91: 2685–2695, 2004. First published February 4, 2004; 10.1152/jn.00907.2003. Previously, we showed that ethanol increases synaptic glycine currents, an effect that depends on ethanol concentration and developmental age of the preparation. Glycine receptor (GlyR) subunits undergo a shift from α2β to α1β from neonate to juvenile ages, with synaptic glycine currents from neonate hypoglossal motoneurons (HMs) being less sensitive to ethanol than those from juvenile HMs. Here we investigate whether these dose and developmental effects are also present in excised membrane patches containing GlyRs and if ethanol changes response kinetics. We excised outside-out patches from rat HM somata and applied glycine using either a picospritzer or piezo stack translator. Ethanol (100 mM) increased the response to glycine (200 μM) of patches from neonate and juvenile HMs. However, 30 mM ethanol increased the response from only juvenile HM patches. Using a lower concentration of glycine (30 μM) to observe single channel openings, we found that 100 mM ethanol increased the response of GlyRs that open in response to glycine and decreased first latency to channel opening. To investigate GlyR kinetic properties, we rapidly applied 1 mM glycine for 1 ms and found that glycine currents were increased by ethanol (100 mM) at both ages. For patches from juvenile HMs, ethanol consistently decreased response rise-time and increased response decay time. Using kinetic modeling, we determined that ethanol’s potentiation of the glycine response arises from an increase in the glycine association (kα) and a decrease in the dissociation (kαd) rate constants, resulting in increased glycine affinity of the GlyR.

INTRODUCTION

Previously, we showed that ethanol potentiates native synaptic glycine receptors (GlyRs) from hypoglossal motoneurons (HMs) in a dose-dependent and subunit-dependent manner (Eggers et al. 2000). This extended earlier studies showing ethanol potentiating glycine-activated currents in different types of cultured cells (Aguayo and Pancetti 1994; Aguayo et al. 1996; Celentano et al. 1988; Engblom and Akerman 1991; Mascia et al. 1996). In our previous study, we extended these results to native synaptic GlyRs, which are the heteromeric αβ form and found that neonatal α2β GlyRs are less sensitive to ethanol than juvenile α1β GlyRs (Eggers et al. 2000). These studies, showing modulation of ethanol potentiation of glycine currents solely due to changes in GlyR composition, suggest that ethanol is having a direct effect on the GlyR and that isolated α2β GlyRs should be less sensitive to ethanol than α1β GlyRs.

Few data are available on the effects of ethanol on the kinetics of glycine responses. A recent study on ventral tegmental area neurons found that ethanol increased the glycine responses to low concentrations of glycine in a proportion of cells and decreased the EC50 of the GlyR for glycine. This study also found that ethanol decreased the rise-time and increased the decay time of these responses for long duration glycine applications at low glycine concentrations (Ye et al. 2001). Thus further investigation is required to determine the specific kinetic parameters of GlyR function that are changing in response to ethanol.

The purpose of this study was to determine if ethanol can directly modulate native GlyRs, and if so, what properties of GlyR function are changing. We also wanted to determine if the change in sensitivity between α2β and α1β GlyRs is present in isolated GlyRs. To investigate ethanol modulation of GlyR function, we used GlyR recordings from HMs.

HMs control muscles of the tongue play an important role in respiration by regulating airway patency (Lowe 1980), and excessive inhibition of HMs can contribute to obstructive sleep apnea (OSA) (Remmers et al. 1980; Wiegand et al. 1991). OSA severity has been shown to be decreased by blocking GlyRs with strychnine (Remmers et al. 1980). These results suggest that glycine-mediated synaptic transmission has important physiologic and pathologic roles in this system. Ethanol modulation of glycine receptor activity is also potentially important. In human studies, OSA severity has also been shown to be increased by
ethanol (Issa and Sullivan 1982; Scrima et al. 1982; Taasan et al. 1981). Ethanol has also been shown to decrease the respiratory-related activity of the hypoglossal nerve in vivo and in vitro (Bonora et al. 1984; Di Pasquale et al. 1995; Gibson and Berger 2000). The decrease in HM activity due to ethanol could lead to reduction of contraction of the tongue muscles and thereby promote OSA.

HMs express heteromeric GlyR channels, containing both α and β subunits (Singer and Berger 1999; Singer et al. 1998). A previous study has shown that the α subunit in HMs undergoes a developmental shift from being primarily α2 at neonate ages (P0–3) to primarily α1 at juvenile ages (P10–18) (Singer et al. 1998). We used this developmental change in receptor subtype to study the differences in ethanol potentiation with changing GlyR subunit composition. To determine if GlyRs are directly modulated by ethanol, we excised outside-out patches from HMs. We studied the response of these patches to brief focal glycine application to determine whether native GlyR channels are sensitive to ethanol, whether the sensitivity changes with subunit composition during the postnatal period, and what kinetic properties of the GlyR response ethanol are changing.

**Methods**

**Brain stem slice preparation**

These experiments utilized in vitro slice recordings from Sprague-Dawley rat HMs (P1–12). Rats were anesthetized with halothane and decapitated. The medulla was isolated and sliced into 250- to 300-μm-thick sections with a vibratome (Pella) while in ice-cold Ringer solution containing (in mM) 119 NaCl, 2.5 KCl, 1.3 MgSO4, 1 NaH2PO4·H2O, 26.2 NaHCO3, 11 glucose, and 2.5 CaCl2, bubbled with carbogen (95% O2–5% CO2). Slices were incubated at 37°C for 1 h in carbogen bubbled Ringer solution, and stored at room temperature in the same solution.

**Recordings**

Excised patch recordings were made from visualized HMs. Slices were placed in a recording chamber mounted on a fixed-stage microscope (Zeiss) and perfused with carbogen bubbled Ringer solution. The microscope had DIC optics and was illuminated with near-infrared light. Neurons were visualized using a 40 X water immersion lens. The output of the microscope was fed to an infrared-sensitive video camera (Hamamatsu) and sent to a video monitor (Sony). HMs were identified based on location in the slice, near the central canal and ventral to the dorsal motor nucleus of the vagus, and by their size and shape (Umemiya and Berger 1994).

Voltage-clamp recordings were made from outside-out excised patch with electrodes of 2–4 MΩ resistance pulled from borosilicate glass capillary tubes (Warner Instruments). The pipette was filled with (in mM) 140 CsCl, 2 MgCl2, 10 HEPES, 10 EGTA, 2 ATP, and 0.2 GTP. Pipette solution pH was adjusted to be 7.3 with CsOH.

Outside-out recordings were made in the Ringer solution described above, and glycine application and recordings commenced immediately after patch excision. In the experiments where GlyR single-channel openings were recorded, the bath solution also contained TTX (0.5 μM, Aldomone) to block voltage-gated Na+ channels and CdCl2 (100 μM) to block calcium channels, DNQX (10 μM, Research Biochemicals) to block non-N-methyl-d-aspartate (NMDA) glutamate receptors, D(-)-2-amino-5-phosphonopentanoic acid (APV, 25 μM, Tocris) to block NMDA receptors, and bicuculline methiodide (BMI, 5 μM, Sigma) to block GABA_A receptors. This concentration of BMI was shown to block GABA_A responses in HMs (O’Brien and Berger 1999). A recent paper also showed possible nonspecific effects of DNQX on glycine receptors, but the dose used here was reported to have minimal effects on glycine currents from homomeric glycine receptors expressed in *Xenopus* oocytes (Meier and Schmieden 2003).

Voltage-clamp recordings were made in the outside-out patch configuration (Vhold = –40 mV, unless otherwise noted) using the Axopatch 200B amplifier (Axon). Clampex software (Axon, version 8) was used to acquire data and to control the glycine application. Data were filtered at 2 or 5 kHz and acquired at 5 or 10 kHz, respectively. A liquid junction potential of 4–5 mV was measured, and the command potentials were corrected for this potential only for the calculation of single channel conductance.

**Glycine application to excised patches**

Brief applications of glycine were performed in two ways. For experiments employing focal pressure application, glycine was applied from a glass electrode filled with Ringer solution containing 30–200 μM glycine using a Picospritzer II (General Valve). Applications were brief, lasting 20–120 ms. In these experiments, control responses were recorded, ethanol of varying concentrations was added to the bath solution, and responses in the presence of ethanol were recorded.

Glycine was also rapidly applied using a high-voltage piezoelectric stack translator (model P-244.40 with an E-470 power supply, Physik Instrumente). The stimuli were controlled by a stimulator (S88, Grass Instruments). The rapid-application experiments were used to mimic synaptic responses by the application of 1 mM glycine for 1 ms. This has been suggested to simulate the release of a single synaptic vesicle (Clements et al. 1992). Solutions were rapidly exchanged between Ringer solution (control) and Ringer solution + 1 mM glycine (measured exchange time from 200 to 400 μs using the junction potential changes between Ringer and 10% Ringer). This system has been used previously in our laboratory, and details about function and switching time can be found in Singer and Berger (1999). Application pipettes were formed using theta glass (WPI). Ethanol (100 mM) was directly applied to the patches by changing only the control Ringer solution to one containing the control Ringer’s plus ethanol (100 mM). Glycine pulses were applied at a frequency of 0.1 Hz, and the solution flow-rate through the rapid-application pipette was 0.04 ml/min.

**Data analysis**

Data arising from glycine applications were analyzed using Clampfit (version 8, Axon). To test for significant differences in data values between pairs of control and ethanol applications, two-tailed paired t-tests were performed. Differences in averages for groups of patches were tested with a two-tailed unpaired t-test. Values reported are mean ± SE. Decay times of glycine currents were computed by averaging the glycine responses and fitting the decay phase of the average current with a single or double exponential function (Igor Pro, Wavemetrics). When a double exponential was used, the weighted mean decay (τw) is reported. τw was calculated as \( τ_w = (A_1τ_1 + A_2τ_2)/(A_1 + A_2) \).

**Analysis of observed single GlyR channel openings**

We used a low concentration of glycine (30 μM) applied with the Picospritzer system to obtain a few GlyR openings in response to glycine application. These recordings showed clear equally spaced levels that corresponded to one or more GlyR openings. After recording 10–20 control responses, we applied 100 mM ethanol to the bathing solution. We counted the maximum number of channels opening due to glycine for each application by determining the maximum number of current levels. We computed the average maximum number of channel openings in control and ethanol conditions (the latter was determined at 2 min after introducing ethanol to the bath solution). For all patches, the current levels observed were evenly
spaced, and no intervening subconductance states were observed. This has been previously observed for GlyRs from HMs (Singer et al. 1998). For this reason, we assumed all evenly spaced levels were separate channels and not subconductance states of a single channel.

To determine if ethanol application changed the average GlyR open probability, we analyzed the single channel openings and transitions with Fetchan (Axon) and computed the dwell times for each channel state (1 channel open, 2 channels open, etc.). From this, the open probability can be calculated using a formula for having more than one channel in a patch. The formula is as follows

\[ P_o = \frac{1}{T} \sum \frac{j_i}{T_N} \]

where \( N \) is the number of active channels in a patch that is calculated by the maximum number of channels observed, \( T \) is the duration of the recording, and \( j_i \) is the time spent at each current level \( j \) \((0, 1, 2, \ldots)\), which corresponds to 0, 1, 2... open channels) (Spruce et al. 1987). Average \( P_o \) was calculated for each glycine application, and the average in control and ethanol conditions was calculated.

To further analyze these data, we created an all-points histogram of responses to glycine (filtered at 1 kHz for analysis). We fit Gaussian distributions to the closed and open peaks of these histograms to estimate single-channel conductance. These histograms were normalized to enable comparisons of time spent in closed versus open states. From the Gaussian distributions, we computed the increase in the maximum value of the open state peaks as well as the amount of time (area under the peaks) in the open states. Finally, we measured the latency to first channel opening and with these data created a histogram of first latency times for control and ethanol responses. For comparison purposes, we also computed an average first latency in control and ethanol.

**Rapid-application data**

The peak amplitude and 10–90% rise-times of rapid-application glycine responses were computed using the basic statistics program of Clampfit 8 (Axon). Decay times were computed by averaging the glycine responses and fitting the decay phase of the average glycine response with a double exponential function (Igor Pro). A two-component exponential function was required to obtain a sufficient fit.

**GlyR channel modeling**

To model the kinetic parameters of the GlyR, the model developed by Legendre (1998) was used (see Fig. 4). This model was developed using data from rapid-application experiments on heteromeric GlyRs, and therefore the modeled currents should be similar to those we have obtained here. To fit the model to the data and determine the appropriate kinetic parameters, we used the modeling program, Axograph 4.6 (Axon). \( K_A \) was calculated as \( k_{off}/k_{on} \).

**RESULTS**

**Ethanol increases responses of excised patches to glycine in a dose-dependent and subunit-dependent manner**

In a previous study measuring ethanol potentiation of miniature inhibitory postsynaptic currents (mIPSCs), we found that ethanol increased mIPSC amplitude in a dose-dependent and GlyR subunit-dependent manner (Eggers et al. 2000). We found that 30 mM ethanol increased the amplitude of mIPSCs from juvenile HMs that contain \( \alpha 1 / \beta \) GlyRs but at this concentration had no effect on mIPSCs from neonate HMs that contain \( \alpha 2 / \beta \) GlyRs. Ethanol at 100 mM increased the mIPSC amplitude from both age groups. To determine if the ethanol dose-dependence and subunit-dependence was present in patches where GlyRs are directly affected, we tested the effects of two different concentrations of ethanol (30 and 100 mM) on glycine responses from excised patches from neonate and juvenile HMs.

We first wanted to confirm that ethanol is capable of directly modulating GlyRs in excised patches. We found that both neonate (\( \alpha 2 / \beta \)) glycine receptors and juvenile (\( \alpha 1 / \beta \)) glycine receptors increased their response to pressure applied glycine (200 µM) in the presence of 100 mM ethanol (Fig. 1B). In neonate HMs, 100 mM ethanol increased the response to glycine by 41 ± 9% (\( n = 5 \), paired \( t \)-test \( P < 0.05 \)), and in juvenile HMs, 100 mM ethanol increased the response to glycine by 40 ± 6% (\( n = 5 \), \( P < 0.05 \)). This shows that ethanol is capable of directly modulating GlyRs in excised patches.

We next wanted to see if decreasing the ethanol concentration decreased the potentiation of glycine responses by ethanol and if this potentiation depended on the GlyR subunits expressed. We thus tested the effect of 30 mM ethanol on glycine responses from patches where GlyRs are directly affected, tested the effects of two different concentrations of ethanol (30 and 100 mM) on glycine responses from excised patches from neonate and juvenile HMs.

![Graphical representation of the data](http://www.jn.org/)

**FIG. 1.** Ethanol increases the glycine-induced current responses of excised patches from hypoglossal motoneurons (HMs) in a dose-dependent and subunit-dependent manner. A: average responses to glycine for an outside-out patch from the neonate (A1) and juvenile (A2) age groups. For the neonate patch shown in A1, 30 mM ethanol had no effect on the glycine response (-2.0%, \( P = 3, V_o = -40 \) mV). For the juvenile patch shown in A2, 30 mM ethanol increased the response to glycine by 14.8% (\( P = 9, V_o = -40 \) mV). B: average responses to glycine for an outside-out patch from the neonate (B1) and juvenile (B2) age groups. For the neonate patch shown in B1, 100 mM ethanol increased the response by 49% (\( P = 2, V_o = -25 \) mV). For the juvenile patch shown in B2, 100 mM ethanol increased the response to glycine by 49% (\( P = 11, V_o = -25 \) mV). C: average percent increases of glycine-induced current responses from excised patches in response to ethanol; 30 mM ethanol increased glycine responses in patches from juvenile HMs by an average of 18.3 ± 3.9% (\( n = 6 \), paired \( t \)-test \( P < 0.05 \)) but had no effect on responses from neonates (1.7 ± 1.6%; \( n = 5, P = 0.4 \)). One hundred micromolar ethanol increased responses from both neonate (41 ± 9%, \( n = 5, P < 0.05 \)) and juvenile (40 ± 6%, \( n = 5, P < 0.05 \)) patches. Change in peak current amplitude due to 100 mM ethanol was significantly greater than change due to 30 mM ethanol for both neonate and juvenile HMs (*unpaired \( t \)-test, \( P < 0.05 \)). Error bars = SE.
responses from excised patches (Fig. 1A). We found that, while 30 mM ethanol potentiated glycine responses of patches from juvenile HMs by 18.3 ± 3.9% (n = 6, P < 0.05), it had no effect on patches from neonate HMs (1.7 ± 1.6%, n = 5, P = 0.4) as shown in Fig. 1A. These results show that 30 mM ethanol had a smaller effect on both juvenile and neonate glycine responses in excised patches. Therefore the dose-dependence observed in mIPSCs is also present in excised patches (unpaired t-test, P < 0.05, Fig. 1C). Furthermore, 30 mM ethanol potentiated glycine responses in patches from juvenile (α1/β), but not neonate (α2/β), HMs (unpaired t-test, P < 0.05, Fig. 1C). Thus the subunit-dependence seen in mIPSCs is also present in excised patches.

**Ethanol increases the number of GlyRs opening in response to glycine**

To investigate what properties of the GlyR are changed due to ethanol we made recordings of GlyRs where single channel openings could be observed. To do this, we excised outside-out patches from HMs and pressure-applied low concentration pulses of glycine (30 μM) to excised patches of GlyRs from HMs that caused one to two channels to open. We looked at the difference in the response of these channels with and without ethanol. These brief duration pulses allow us to see individual channel openings without the desensitization of the GlyRs that occurs in response to long duration glycine pulses (Singer and Berger 1999).

Examples of GlyR responses to short 30-μM glycine applications for patches from a neonate (Fig. 2A) and a juvenile (Fig. 2B) HM are shown in Fig. 2. The current levels corresponding to open channels are marked by the dotted lines. For each patch, we counted the maximum number of channels that were simultaneously open. The bath application of ethanol (100 mM) caused more GlyRs to open in response to ethanol. Ethanol significantly increased the average number of open channels in neonate HMs from 1.8 ± 0.2 to 2.9 ± 0.2 (n = 11, P < 0.001, Fig. 3A1) and in juvenile HMs from 1.6 ± 0.2 to 2.6 ± 0.4 (n = 12, P < 0.001, Fig. 3A2). The average P_o was increased by 160.3 ± 6.1% (P < 0.01 paired t-test) for neonates and by 270.2 ± 150.1% (P < 0.01 paired t-test) for juveniles. Ethanol increased the number of open channels by 63 ± 13% for patches from neonate HMs and 57 ± 7% for patches from juvenile HMs, and there was no significant difference in the change due to ethanol between age groups (P = 0.7).

However, as shown in Fig. 2, there was no change in GlyR single channel conductance due to ethanol. The average conductance in control conditions was 51.8 ± 1.9 pS and ethanol was 50.5 ± 1.9 pS for patches from neonate HMs and 46.1 ± 3.0 pS in control and 49.7 ± 1.6 pS in ethanol for patches from juvenile HMs. Note that there was no change in single channel conductance over development. This was expected since previous work from our laboratory showed that hypoglossal motoneuron glycine receptors were heteromeric at both age groups (Singer et al. 1998) and showed no change in single channel conductance with postnatal development (Eggers et al. 2000; Singer et al. 1998).

To further investigate the increase in channel openings due to ethanol, we plotted a normalized all-points histogram for each patch in control (Fig. 2, C1 and D1) and ethanol (Fig. 2, C2 and D2) conditions. Ethanol decreases the number of counts in the closed state (0 pA) and increases the number of counts when one channel opens (2.5 pA) and when two channels are open (5 pA). The histograms were fit by two (control) and three (ethanol) component Gaussian curves (thick black line). The individual component Gaussians that make up these fits are plotted with dotted lines. The sum of the peaks of the open level Gaussian components was increased by 57.5 ± 21.2% for neonates and 108.7 ± 61.2% for juveniles. The area of the open level Gaussian

**FIG. 2.** Ethanol increases the number of glycine receptors (GlyRs) opening in response to glycine in neonate and juvenile patches. A and B: outside-out patch records from a neonate HM (A, P = 2) and a juvenile HM (B, P = 9) in response to short applications of glycine (30 μM, applied at the arrow). Records in A1 and B1 are control conditions and A2 and B2 are in ethanol (100 mM). Ethanol increases the number of channels that open in response to glycine. Current levels corresponding to closed and open channels are marked by dotted lines. C and D: normalized all-points histograms for the patches in A and B in control (C1 and D1) and ethanol (C2 and D2) conditions. The histograms are fit by 2 (control) and 3 (ethanol) component Gaussian curves (thick line). Individual component Gaussians that make up these fits are plotted with dotted lines.
components was increased by 63.2 ± 20.2% for neonates and 83.9 ± 45.5% for juveniles.

Apparent affinity and changes in latency to first channel opening

A model for the GlyR from Legendre (1998) is shown in Fig. 4. In this model, two molecules of ligand are required to bind before channel opening, and the channel goes through three distinct states before the channel is opened. An increase in $k_{on}$ or $\beta$ or decrease in $k_{off}$ or $\alpha$ could all increase the probability of opening of the channel. Therefore an increase in current at a particular ligand concentration could be due to changes in any or all of these rate constants (Colquhoun 1998).

The affinity of the receptor for ligand is determined by $k_{on}$ and $k_{off}$ and the efficacy of the receptor is determined by $\beta$ and $\alpha$. It is difficult to differentiate between them because the open state of the channel is the only one that can be measured as a current, and this state depends on all of the previous states. We therefore refer to an increase in current at a certain glycine concentration as an increase in the apparent affinity, combining both the binding and opening steps. Thus an increase in apparent affinity should occur because of an increase in the forward rate constants, a decrease in the backward rate constants, or a combination of both of these effects. Changes in the rate constants $k_{on}$, $k_{off}$, and $\beta$ would also change the speed of channel opening, so an increase in apparent affinity would lead to a decrease in the latency to first channel opening.

Ethanol decreases the first latency of GlyRs opening in response to glycine

The results reported above showing that ethanol increases the number of GlyRs that open in response to glycine suggest that ethanol is increasing the apparent affinity of the GlyR for glycine because more GlyR channels open for the same concentration of glycine. The latency to first channel opening is also a measure of apparent affinity of channels, and this was measured for the GlyR responses studied. Shown in Fig. 3, $B1$ and $B2$, are the cumulative probability distributions for latency to first channel opening for a patch from neonate and juvenile HMs. The control and ethanol first latency distributions were fit with a cumulative probability exponential, and the $\tau$ values from these fits were used to compare the data. Cumulative probability distributions were plotted and exponential fits were done in patches that had 20 or more glycine applications in control conditions. Ethanol (100 mM) decreased the latency to first opening for both age groups (Fig. 3B). Ethanol decreases the first latency of GlyRs opening in response to glycine.
Although not statistically significant, the rise-time decreases for all patches by 18.7 ± 7.3% (P = 0.06, n = 4), and the τw increases for all patches by 22.2 ± 10.6% (P = 0.14, n = 4). For each individual glycine response, the decay time to 37% of peak was also measured. In all of the individual patches, these decay values in ethanol were significantly greater than those in control (t-test, P < 0.05, n = 4).

**Ethanol increases currents in response to rapid application of glycine**

In the preceding experiments using pressure application, we found that ethanol is capable of directly modulating GlyRs in excised patches. Next, we used rapid application to these excised patches to simulate synaptic responses with 1 mM glycine applied for 1 ms. With these experiments, we can determine if these responses to a high concentration of glycine are modulated by ethanol as was seen with synaptic responses in mIPSCs and evoked eIPSCs. Also, because rapid application allows fine time distinctions to be made, we can also determine how kinetic parameters of responses such as rise-time and decay time are affected by ethanol application.

Glycine (1 mM) was applied to patches from neonate and juvenile HMs for 1 ms using the piezoelectric stack translator to switch from Ringer to Ringer + glycine solutions. Shown in Fig. 5A are examples of rapidly applied glycine responses from a neonate HM in control (Ringer) and ethanol (Ringer + 100 mM ethanol) conditions. Ethanol increased the amplitude of responses from neonate patches by 20.5 ± 2.8% (P < 0.05, Fig. 5B1). Figure 5A, inset, shows traces normalized to the peak amplitude. This inset shows that, in ethanol, the decay time increases. The averages of the rise-time and τw changes for five patches are shown in Fig. 5, B2 and B3, respectively. Although not statistically significant when all patches are combined, on average, the rise-time increases by 10.3 ± 5.3% (P = 0.08, n = 5) and the τw increases by 18.9 ± 10.5% (P = 0.23, n = 5). For each individual glycine response, the decay time to 37% of peak was also measured. In four of five individual patches, these decay time values measured in ethanol were significantly greater than those in control (t-test, P < 0.05).

Shown in Fig. 5C are examples of rapidly applied glycine responses from a juvenile HM in control (Ringer) and ethanol (Ringer + 100 mM ethanol) conditions. Ethanol increased the amplitude of responses from juvenile patches by 31.9 ± 7.4% (P < 0.05, Fig. 5D1). Figure 5C, inset, shows that the traces normalized to the peak amplitude and that, in ethanol, the decay time increases. The averages of the rise-time and decay-time changes for four patches are shown in Fig. 5, D2 and D3. Although not statistically significant, the rise-time decreases for all patches by 18.7 ± 7.3% (P = 0.06, n = 4), and the τw increases for all patches by 22.2 ± 10.6% (P = 0.14, n = 4). For each individual glycine response, the decay time to 37% of peak was also measured. In all of the individual patches, these decay values in ethanol were significantly greater than those in control (t-test, P < 0.05, n = 4).

**Increasing glycine concentration decreases the effect of ethanol**

As discussed previously and shown in the model in Fig. 4, the opening of ligand-gated ion channels depends on the concentration of ligand, through dependence of koff on ligand concentration. Thus increasing ligand concentration causes the channel to be more maximally opened. When a saturating dose of glycine is used, the binding steps have been activated maximally, and the opening of the channel depends solely on the opening and closing rate constants (β and α). Thus one way of determining what effect a modulator is having is on a ligand-gated ion channel is to determine how the effect changes with increasing ligand concentration.

In the experiments described in this study we recorded the ethanol (100 mM) modulation of glycine currents due to the application of 0.03, 0.2, 10 (pressure applied), and 1 mM (rapidly applied) glycine to excised patches. GlyRs have been shown previously to have no desensitization on short time scales (Singer and Berger 1999), so the data from brief pressure application and 1-ms rapid application have been pooled. To look at the effect of increasing glycine concentration on ethanol potentiation of the glycine response, we have plotted the percentage increase in the glycine of current due to ethanol (100 mM) versus the applied glycine concentration (Fig. 6). As shown in Fig. 6, increasing the applied glycine concentration decreases the potentiating effect of ethanol on the glycine-evoked currents.

Previous experiments have shown that application of 10 mM glycine saturates GlyRs. At this glycine concentration, there is no further increase in current if the time of application is increased (Singer and Berger 1999). As shown in Fig. 6, when 10 mM glycine was pressure applied to excised patches from HMs, ethanol (100 mM, a concentration that affects both age groups) caused no increase in currents for either neonate (2.0 ± 2.0% change, n = 4, NS) or juvenile HMs (0.1 ± 0.6% change, n = 4, NS). These results show that ethanol does not increase the absolute magnitude of the glycine induced current, although it does increase the magnitude of currents at nonsaturating doses of glycine. This suggests that ethanol is affecting the binding steps of the GlyR response and not the opening steps, since ethanol had no effect at saturating doses of glycine where presumably binding is already maximally activated.

**GlyR channel modeling to determine rate constants that are changed by ethanol**

A previous study developed a kinetic model for GlyR function from zebrafish Mauthner cells (Legendre 1998). This

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**Fig. 4.** Kinetic model of GlyR function (Legendre 1998). Boxes represent separate states of the channel, and arrows represent transitions between them, with corresponding rate constants indicated. Boxes outlined in wider lines signify channel open states. States inside dotted box are the reluctant gating mode. k_{on}, association constant and is dependent on glycine concentration; k_{off}, dissociation constant; β, opening constant; α, closing constant; d, transition to reluctant gating; r, transition from reluctant gating.
model (Fig. 4) is based on a typical ligand-gated ion channel model and contains two binding steps, corresponding to the binding of two glycine molecules, and a separate opening step. Legendre (1998) also found that a reluctant open state that has a lower opening probability, outlined in Fig. 4, was required to adequately fit the data for GlyR currents. The latter adds another open state to the model to replicate the observation of open times of single GlyRs, we found in five patches that the open time distributions were best fit by two exponentials with $\tau_{\text{decay}}$ of 7.5 ± 0.3 and 0.96 ± 0.08 ms. The inverse of these $\tau_{\text{decay}}$ values gives two closing constants of 134.2 ± 4.9 s$^{-1}$ and 1,064.1 ± 86.8 s$^{-1}$, which we used as the starting points in our modeling for $\alpha_1$ and $\alpha_2$, respectively.

The effects of ethanol on GlyR kinetics were most consistent for patches from juvenile HMs, so we determined how these observed effects compare with effects predicted with changes in rate constants. The main effects of ethanol on patches of juvenile GlyRs were that ethanol increased the peak amplitude, decreased the rise-time, and increased the decay time. Ethanol also had no effect at saturating pressure-applied glycine concentrations. As discussed previously, this suggests that ethanol affects the binding steps because it has no effect when the binding is maximally activated by saturating glycine concentrations.
To determine which steps of GlyR channel opening are affected by ethanol, we changed the parameters of the GlyR model (Fig. 4) developed by Legendre (1998), one at a time, to determine the effects on rise-time, amplitude, and decay time. Shown in Fig. 7A1 is the fitted data trace from a juvenile HM (1 mM glycine application), labeled as control, and the simulated traces with increased $k_{on}$, decreased $k_{off}$, and increased $\beta_1$. If we increase $k_{on}$, the amplitude of the current is increased with no change in the decay time. If we decrease the $k_{off}$, the amplitude increases and $\tau_w$ increases. If we increase $\beta$, the amplitude also increases, and the $\tau_w$ increases. Because changing both $k_{off}$ and $\beta$ mimics the effects of ethanol on the decay time of GlyR currents and changing all of the parameters mimics the increase in amplitude due to ethanol, it is difficult to choose between these options with a 1-mM glycine application.

However, if we simulate the response to saturating 10 mM glycine, we can discriminate between these options. Shown in Fig. 7A2 is the model response to a pulse of 10 mM glycine. The amplitude and decay time are increased, and the rise-time is decreased by increasing the glycine concentration from 1 to 10 mM. If we increase $k_{on}$, there is no significant change in the amplitude, rise-time, or $\tau_w$ of the current. If we decrease $k_{off}$,

FIG. 6. Increasing glycine concentration decreases ethanol potentiation. Average potentiation of glycine-evoked currents by ethanol (100 mM) at 4 concentrations of glycine (0.03, 0.2, 1, and 10 mM) for patches from neonate and juvenile HMs. Potentiation at 0.03, 0.2, and 1 mM was significantly different from 10 mM at both ages ($P < 0.01$). For neonate patches, potentiation at 0.03 and 0.2 mM was significantly different from potentiation at 1 mM ($P < 0.05$).

FIG. 7. Modeled GlyR responses to pulses of glycine. A1: simulated synaptic responses in response to a 1-ms pulse of 1 mM glycine, using the values for the GlyR fit to a control juvenile patch response (Fig. 5). Trace with starting values is black; other traces show effects of multiplying values for $k_{on}$ and $\beta$ by 2 and dividing value for $k_{off}$ by 2, respectively. A2: simulated synaptic responses in response to a 1-ms pulse of 10 mM glycine, using values for GlyR function fit to a control juvenile patch response. Trace with starting values is black; other traces show effects of multiplying values for $k_{on}$ and $\beta$ by 2 and dividing value for $k_{off}$ by 2, respectively. B: examples from a juvenile HM patch in control and ethanol were fit with the reluctant gating model. Ethanol increased amplitude and decay time and decreased rise-time of the glycine response. An excellent fit to the effects of ethanol in experimental data (black traces) was achieved in the model (red traces) by increasing $k_{on}$ and decreasing $k_{off}$.
the amplitude is also unchanged, the rise-time increases, and the $\tau_o$ increases. On the other hand, if we increase $\beta$, the amplitude and $\tau_o$ increase and the rise-time decreases. Based on our observation that ethanol causes no change in the peak response amplitude at saturating glycine concentrations (Fig. 6), it is unlikely that ethanol’s effect on GlyRs is due to an increase in the opening rate constant ($\beta$). More likely, ethanol’s effect on GlyRs is due to a decrease in the dissociation constant ($k_{off}$) and possibly an increase in the association constant ($k_{on}$). The changes that occur when the dissociation rate is changed in GlyR simulations mimic the effects of ethanol on kinetic parameters and depend on glycine concentration in the same way. We used this model to estimate the rate constants from our data in control and ethanol conditions.

An example of experimental data (1 mM glycine rapid application) and a fitted model from a juvenile patch are shown in Fig. 7B. The kinetic parameters for the model fit in control conditions are as follows: $k_{on} = 3.3 \mu M \times s^{-1}$, $k_{off} = 335 s^{-1}$, $\beta_1 = 1.434 s^{-1}$, $\alpha_1 = 165 s^{-1}$, $d = 259 s^{-1}$, $r = 80 s^{-1}$, $\beta_2 = 2.675 s^{-1}$, and $\alpha_2 = 1.075 s^{-1}$. Figure 7B also shows data and model fit from ethanol conditions. The kinetic parameters, $k_{on}$ and $k_{off}$ were changed to the following values: $k_{on} = 225 \mu M \times s^{-1}$, $k_{off} = 224 s^{-1}$. To obtain a good fit to the data in ethanol, it was necessary to change both $k_{on}$ and $k_{off}$. This gives a change of $K_\alpha$ from 0.01 to 1. As shown in Fig. 7B, ethanol increased the peak amplitude, decreased the rise-time, and increased the decay time of glycine currents from juvenile HMs. This corresponded to an increase in $k_{on}$ and a decrease in $k_{off}$.

### Comparison of excised patch data with previous mIPSC data

In our previous study on ethanol modulation of GlyRs (Eggers et al. 2000), we showed that ethanol potentiates synaptic GlyRs and that $\alpha1/\beta$ GlyRs are more sensitive to ethanol than $\alpha2/\beta$ GlyRs. Here we have found similar results with excised GlyRs. However, there are differences in the synaptic and excised patch data. First, responses of excised patches to rapidly applied glycine have a longer decay time than synaptic mIPSCs, as shown by our laboratory previously observed (Singer and Berger 1991), and required a biexponential distribution to obtain an adequate fit, whereas mIPSC decay time could be well fit by a single exponent (Fig. 8). The average mIPSC decay for neonate was $10.5 \pm 1.5$ ms and for juvenile was $7.4 \pm 0.5$ ms, while the $\tau_o$ for excised patch data was $26.8 \pm 8.8$ ms for neonate and $20.8 \pm 2.4$ ms for juvenile.

Second, in keeping with this difference in decay time between mIPSCs and excised patch responses, ethanol also had a different effect on the decay time. Ethanol (100 mM) has no effect on mIPSC decay time, averaging $10.5 \pm 1.5$ (control) to $11.3 \pm 1.5$ ms (ethanol) for neonates ($n = 13$) and $7.4 \pm 0.5$ (control) to $7.4 \pm 0.6$ ms (ethanol) for juveniles ($n = 9$). Ethanol increased the decay time of the glycine response of excised patches from neonate from $26.8 \pm 8.8$ to $30.2 \pm 7.7$ ms ($n = 5$) and from juvenile from $20.8 \pm 2.4$ to $25.2 \pm 3.2$ ms ($n = 4$). This could be due to differences in the channel behavior in these two different recording configurations.

### DISCUSSION

We have shown that the responses of excised GlyRs from HM s to glycine are augmented by ethanol in four ways. First,
We also investigated the specific kinetic parameters that are changed in GlyR current responses with ethanol. We found that, in juvenile HMs, the 10–90% rise-time was decreased, and the decay time of rapid glycine responses was increased due to ethanol. Both of these effects were consistent between control and ethanol responses in four patches. Using the Legendre GlyR kinetic model, we estimated that this change was due to an increase in $k_{\text{on}}$ and a decrease in the $k_{\text{off}}$ rate constants of glycine.

A few previous studies have been done on the modulation of glycine kinetics, and our results are generally in agreement with these studies. A study on acutely dissociated ventral segmental area neurons found that ethanol increased the glycine responses, decreased the EC$_{50}$, decreased the rise-time, and increased the decay of these responses (Ye et al. 2001). They interpreted these results as both increasing the $k_{\text{on}}$ and decreasing the $k_{\text{off}}$ rate of glycine on the GlyR.

In addition to ethanol, several studies that have focused on the mechanism(s) by which Zn$^{2+}$ potentiates GlyRs. Laube et al. (2000) showed that low concentrations of Zn$^{2+}$ increased the probability of channel opening in α1 homeric outside-out patches, an effect we also saw in our native heteromeric GlyRs in the presence of ethanol. Another study found that Zn$^{2+}$ had no effect on glycine responses to outside-out patches at saturating glycine concentrations and caused a decrease of the glycine dissociation rate constant and increased the amplitude and decay of mIPSCs (Suwa et al. 2001).

Comparison with mIPSC data

In our previous study on ethanol modulation of GlyRs (Eggers et al. 2000), we studied the effects of ethanol on synaptic GlyRs by recording mIPSCs. In that study, we found that ethanol increased the amplitude of mIPSCs and that the degree of potentiation was dependent on the subunits expressed. These results are echoed in the data reported here for excised patches containing GlyRs.

However, there are differences in the synaptic and excised patch data. First, responses to rapidly applied glycine responses had a longer decay time than synaptic mIPSCs and required a biexponential distribution to adequately fit the trace, whereas mIPSC decay time could be well fit by a single exponential. This discrepancy has been noted in previous studies from our laboratory (Singer and Berger 1999) and also for other studies on GlyRs (Legendre 1998) and GABA$_\alpha$ receptor currents (Galarreta and Hestrin 1997; Mellor and Randall 1997). Legendre (1998) saw no statistical difference between individual parameters of decay time ($\tau_{\text{fast}}, \tau_{\text{slow}},$ and relative amplitude contributions), but when these values are combined as a weighted decay time, $\tau_w$, a significant difference emerges, similar to our observations.

There are several possible reasons for these differences between mIPSCs and excised patch data. Synaptic responses come from GlyRs arranged at active zones in the synapse and clustered by gephyrin and microtubules (Kuhse et al. 1995). Excised patches are taken from the soma, and in all likelihood, contain extrasynaptic GlyRs that are not attached to gephyrin and clustered. In a previous study, Nusser et al. (1998) found that synaptic and extrasynaptic GABA$_\alpha$ receptors had different properties in cerebellar granule cells. Therefore structural factors could have a role in modulation of GlyRs, as has been shown previously in several systems. Lewis et al. (1990) found that neurons in culture did not respond to glycine in the whole cell or on-cell configurations, but showed a response when the patch was excised, disrupting structural elements. Delon and Legendre (1995) observed desensitization of whole cell glycine responses during long glycine application that was increased when microtubules were depolymerized and decreased if the microtubules were stabilized. Also, a recent paper found that depolymerizing microtubules caused a decrease in glycineric mIPSCs (van Zundert et al. 2002).

It is also possible that there are effects of phosphorylation or other intracellular modulators on synaptic GlyRs that are removed when patches are excised. Several groups have demonstrated modulation of GlyRs by PKC phosphorylation (Mascia et al.1998; Ruiz-Gomez et al. 1991; Vaello et al. 1994) found that blocking PKC activation decreased the potentiation of glycine currents by ethanol. G protein systems have also been implicated in glycine receptor function. Aguayo et al. (1996) showed that adding GTP-γS (a constitutively active GTP analogue) to the intracellular solution increased the effect of ethanol while adding GDP-BS (a nonhydrolyzable analogue) decreased the effect of ethanol on glycine induced currents.

In keeping with this difference in decay time between mIPSCs and excised patch responses, there is also a different effect of ethanol on the decay time. Ethanol has no effect on mIPSC decay time, although it increased the decay time of glycine responses from excised patches. This could be due to differences in the channel behavior in these two different recording configurations. Also, as we noted above, the decay of mIPSCs is well fit by a single exponential, while the decay of excised patch responses requires a biexponential distribution. The channel model proposed by Legendre (1998) achieves a biexponential current response by the addition of a reluctant gating mode to a simple ligand-gated ion channel model. It is possible that this model does not hold for GlyRs at synaptic sites and that this is reflected by differences in current decay.

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