Glycine-Gated Chloride Channels Depress Synaptic Transmission in Rat Hippocampus

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Glycine-gated chloride channels depress synaptic transmission in rat hippocampus. J Neurophysiol 95: 2366–2379, 2006. First published December 28, 2005; doi:10.1152/jn.00386.2005. An inhibitory role for strychnine-sensitive glycine-gated chloride channels (GlyRs) in mature hippocampus is beginning to be appreciated. We have reported previously that CA1 pyramidal cells and GABAergic interneurons recorded in 3- to 4-wk-old rat hippocampal slices express functional GlyRs, dispelling previous misconceptions that GlyR expression ceases in early development. However, the effect of GlyR activation on cell excitability and synaptic circuits in hippocampus has not been fully explored. Using whole cell current-clamp recordings, we show that activation of strychnine-sensitive GlyRs through exogenous glycine application causes a significant decrease in input resistance and chloride ion driving force in recorded cells. Furthermore, GlyR activation depresses the synaptic network by reducing suprathreshold excitatory postsynaptic potentials (EPSPs) to subthreshold events in both cell types. Blockade of postsynaptic GlyRs with the chloride channel blocker 4,4'-diisothiocyanato stilbene-2,2'-disulfonic acid (DIDS) or altering the chloride ion driving force in recorded cells attenuates the synaptic depression, strongly indicating that a postsynaptic mechanism is responsible. Increasing the local glycine concentration by blocking reuptake causes a strychnine-sensitive synaptic depression in interneuron recordings, suggesting that alterations in extracellular glycine will impact excitability in hippocampal circuits. Finally, using immunohistochemical methods, we show that glycine and the glycine transporter GlyT2 are co-localized selectively in GABAergic interneurons, indicating that interneurons contain both inhibitory neurotransmitters. Thus we report a novel mechanism whereby activation of postsynaptic GlyRs can function to depress activity in the synaptic network in hippocampus. Moreover, the co-localization of glycine and GABA in hippocampal interneurons, similar to spinal cord, brain stem, and cerebellum, suggests that this property is likely to be a general characteristic of inhibitory interneurons throughout the CNS.

INTRODUCTION

Effective neuronal inhibition is required for normal brain function. This is particularly true for highly excitable and seizure prone brain regions such as hippocampus, where damage to inhibitory GABAergic interneurons renders the excitatory principal (pyramidal) cells pathologically active (Scharfman and Schwartzkroin 1990; Sloviter 1987; Zhao and Leung 1991). In spinal cord and brain stem, strychnine-sensitive glycine-gated chloride channels (GlyRs), along with GABA-gated receptors (GABAARs), mediate potent inhibition. Legant electrophysiological studies show that glycine and GABA are coreleased from interneuron terminals in both spinal cord and brain stem and simultaneously activate postsynaptic GlyRs and GABAARs (Chery and de Koninck 1999; Jonas et al. 1998; O’Brien and Berger 1999). Until recently, a potential role for inhibitory GlyRs in regions of the forebrain, including hippocampus, received little attention by investigators interested in inhibitory mechanisms, most likely because of the misconception that functional GlyR expression ceases early in development (Ito and Cherubini 1991; Malosio et al. 1991) and to studies showing that fast synaptic inhibition is completely abolished by GABAAR antagonists (Ito and Cherubini 1991; Mody 1998). It is now clear that GlyRs are functionally expressed in many brain regions including hippocampus, hypothalamus, amygdala, striatum, ventral tegmental area, substantia nigra, inferior colliculus, cerebellum, and cortex (Chattipakorn and McMahon 2002, 2003; Dumoulin et al. 2001; Hussy et al. 2001; Karkar et al. 2004; Kawa 2003; Mangin et al. 2002; McCool 2001; Sergeeva and Haas 2001; Xu et al. 2004; Ye et al. 1997, 1999, 2001), suggesting that neuronal inhibition in many, if not all, brain regions is mediated by both GABA and glycine-gated chloride channels.

The precise function of GlyRs among these brain regions is not yet clearly defined, and in many regions, GlyRs are believed to be located only at extrasynaptic sites (Deleuze et al. 2005; Flint et al. 1998; Mangin et al. 2002; Wang et al. 2005) where they could be important mediators of tonic inhibition similarly to the function of extrasynaptic GABAARs (Rarrant and Nusser 2005; Mori et al. 2002; Petrini et al. 2004) and provide critical neuroprotection under pathological conditions when extracellular glycine and taurine levels are elevated (Baker et al. 1991; Saransaari and Oja 1997, 1999; Zhao et al. 2005). In hippocampus, glycinerergic synapses have been morphologically, but not functionally, defined at a small subset of synapses in dissociated hippocampal cultures and in slices (Danglot et al. 2004; Levi et al. 2004; Meier and Grantyn 2004). However, GlyRs have been shown to mediate fast synaptic transmission synapses in cerebellum, where glycine and GABA are coreleased from Golgi cells and activate postsynaptic GlyRs and GABAARs in either separate or mixed synapses depending on the target (Dugue et al. 2005; Dumoulin et al. 2001). More recently, inhibitory synaptic transmission in thalamus has been shown to be partially mediated by GlyRs (Ghavanini et al. 2005).

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Previously, we have reported that CA1 pyramidal cells and GABAergic interneurons in stratum radiatum, as well as granule cells and hilar interneurons in the dentate gyrus, express functional GlyRs in mature hippocampal slices (Chattipakorn and McMahon 2002, 2003). However, little is known regarding the effects of GlyR activation on hippocampal excitability and synaptic circuits. In this study, we report that activation of GlyRs limits activity in the synaptic network by depressing suprathreshold excitatory postsynaptic potentials (EPSPs) to subthreshold events in recordings from both CA1 pyramidal cells and interneurons. Blockade of postsynaptic GlyRs or decreasing the driving force for chloride ions in the postsynaptic cell attenuates the depression. These findings suggest a postsynaptic locus for the depression, which likely involves a current shunt caused by the large GlyR-mediated decrease in postsynaptic input resistance ($R_{in}$). This GlyR-mediated depression of synaptic transmission we report here provides an additional inhibitory mechanism in hippocampus that will work together with other well-known GABAergic inhibitory mechanisms to control neuronal excitability. Using double immunohistochemical labeling, we show that GABAergic interneurons are immunopositive for the amino acid glycine as well as the glycine transporter, GlyT2, strongly suggesting that these cells are a source of glycine. The co-localization of glycine and GABA in hippocampal interneurons raises the interesting possibility that these neurons use both neurotransmitters, similar to interneurons in spinal cord, brain stem, and thalamus and golgi cells in cerebellum (Chery and de Koninck 1998; O’Brien and Berger 1999), to provide critical inhibition by their electrical properties (e.g., input resistance, firing pattern). Evoked synaptic events were elicited at 0.1 Hz through a bipolar stimulating electrode placed in s. radiatum. In addition, neurons were filled with 0.4% biocytin and processed to allow for post hoc neuronal identification (McMahon and Kauer 1997). No recorded interneurons were identified as radiatum giants (Maccacferri and McBain 1996).

**Methods**

All procedures carried out in this study were approved by the University of Alabama Institutional Animal Care and Use Committee and follow guidelines defined by the National Institutes of Health for the use and treatment of laboratory animals.

**Brain slice preparation**

Experiments were performed on acutely prepared hippocampal slices (400 $\mu$m) obtained from 3- to 4-wk-old Sprague-Dawley rats. Slices were cut into ice-cold high sucrose and low calcium artificial cerebral spinal fluid (ACSF; in mM): 85 NaCl, 25 NaHCO$_3$, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 0.5 CaCl$_2$, 4 MgSO$_4$, 25 glucose, 0.5 ascorbate, 75 sucrose, and 2 kynurenic acid; saturated with 95% O$_2$, 5% CO$_2$, pH 7.4. Slices were incubated for 30 min in a submersion chamber before being transferred to standard ACSF that included 1 mM kynurenic acid (in mM): 119 NaCl, 26 NaHCO$_3$, 2.5 KCl, 1.0 NaH$_2$PO$_4$, 2.5 CaCl$_2$, 1.3 MgSO$_4$, and 10 glucose, saturated with 95% O$_2$, 5% CO$_2$, pH 7.4. Slices were rested for an additional 30 min before being used in experiments. ACSF with or without kynurenic acid (as mentioned) was used for recordings. All experiments were performed in a submersion recording chamber with a constant flow rate of 4–5 ml/min and ACSF warmed to 27–28°C.

**Electrophysiological recordings**

Whole cell voltage- and current-clamp recordings of visually identified CA1 pyramidal cells and GABAergic interneurons in s. radiatum were obtained using IR-DIC microscopy and standard recording techniques (Chattipakorn and McMahon 2002). An Axoclamp 2A or Axopatch 200 amplifier (Molecular Devices, Sunnyvale, CA) was used to amplify recorded signals, and responses were monitored on an oscilloscope and computer monitor. Patch electrodes had resistances between 4 and 6 MΩ when filled with (in mM) 110 cesium chloride or cesium gluconate, 0.6 EGTA, 5 MgCl$_2$, 2 ATP-Na, 3 GTP-Na, 40 HEPES, and 0.4% biocytin, pH 7.2, 260–270 mOsm. For current-clamp recordings, 110 mM potassium gluconate was substituted for cesium gluconate. In some recordings, 5 mM QX 314 was added to the internal solution to block voltage-dependent Na$^+$ channels and to enhance space-clamp. In some experiments, the nonselective chloride channel blocker 4,4′-disothiocyanatostilbene-2,2′-disulfonic acid (DIDS) was added to the pipette solution to block postsynaptic GlyRs. DIDS has been shown to block GlyRs (Leewanich et al. 1998) and has been used previously in the pipette solution to block postsynaptic GABA$_A$Rs in hippocampal slices (Albertson et al. 1996; Hollrigel et al. 1998). Recorded neurons were identified as pyramidal or interneuron by their electrical properties (e.g., input resistance, firing pattern). Evoked synaptic events were elicited at 0.1 Hz through a bipolar stimulating electrode placed in s. radiatum. In addition, neurons were filled with 0.4% biocytin and processed to allow for post hoc neuronal identification (McMahon and Kauer 1997). No recorded interneurons were identified as radiatum giants (Maccacferri and McBain 1996).

**Drug delivery**

Stock solutions of all agonists and antagonists were prepared in deionized distilled water with the exception of the GlyT1 inhibitor, N-[3-(4′-fluorophenyl)-3-(4′-phenylphenoxoy)propyl]sarcosine (NFPS), which was dissolved in DMSO. All drugs were diluted to appropriate concentrations in the bathing solution. The glycine concentration (300 $\mu$M) used in this study was chosen because it is near the apparent glycine EC$_{50}$ for these receptors in slices (Chattipakorn and McMahon 2002). In experiments examining the effects of GlyR activation on evoked synaptic transmission, glycine was applied by bath perfusion of a fixed concentration where complete bath exchange occurred within 1.5 min. During bath application of glycine, GlyR currents do not completely desensitize, even during prolonged agonist application, but reach a steady-state current (59 ± 10% of peak current in pyramidal cells; 47 ± 19% of the peak current in interneurons; Chattipakorn and McMahon 2002). In some experiments where the effects of antagonists or the interactions between currents mediated by GlyRs and other ligand-gated channels were being assessed, a small volume of agonist (glycine, GABA, and glutamate) was applied directly to the recorded cell through a glass pipette placed within 50 $\mu$m of the recorded cell. The agonists were applied for 3–30 s through rapid gravity flow using a multichannel valve or by pressure ejection using a picospritzer. This method allowed agonists to be applied repeatedly to a given cell and at short intervals.

**Immunohistochemistry**

Three- to 4-wk-old rats ($n = 8$) were anesthetized with pentobarbital sodium and perfused with ACSF followed by perfusion with cold 4% paraformaldehyde. Brains were postfixed in 4% paraformaldehyde for 2 h (except in the case of GlyR staining), cryoprotected in 30% sucrose, and resectioned (40 $\mu$m). In experiments using anti-GlyR antibodies, sections were permeabilized with methanol and washed with 0.1 M TBS-TX buffers. Primary antibodies used in this study

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RESULTS

We have reported previously that CA1 pyramidal cells and GABAergic interneurons in s. radiatum functionally express GlyRs in hippocampal slices prepared from 3- to 4-wk-old rats (Chattipakorn and McMahon 2002). In all experiments in this study, we used 300 μM glycine to activate GlyRs because this concentration is near to the EC50 for GlyRs expressed by both pyramidal cells and interneurons recorded in hippocampal slices (Chattipakorn and McMahon 2002). Here we show representative GlyR-mediated currents recorded in whole cell voltage-clamp conditions from a CA1 pyramidal cell (Fig. 1A, top) and a s. radiatum interneuron (Fig. 1A, bottom) after brief application of glycine (30 s, ECl = 0 mV) through a drug pipette positioned within 50 μm of the recorded cell. The currents only partially desensitize and rapidly recover to baseline after termination of agonist application. These glycine-induced currents are mediated by GlyRs because they are relatively unaffected by the GABA_A receptor (GABA_A,R) antagonist bicuculline (10 μM; although bicuculline has been shown to block GlyRs; Wang and Slaughter 2005), but are reversibly blocked by the GlyR antagonist strychnine (1 μM; IC50 20–40 nM; see Chattipakorn and McMahon 2002). With repeated glycine applications (30 s, 5-min intervals), GlyR-mediated current amplitude remains stable, indicating that GlyR channel function does not rundown during whole cell recordings (Fig. 1B). GlyR-mediated responses were observed in all recorded pyramidal cells and interneurons, but consistent with our previous report (Chattipakorn and McMahon 2002), the mean current amplitude and decrease in cell input resistance (Rin, measured at the peak of the current) are significantly larger in interneurons (1351 ± 138 pA and 77 ± 4% decrease in Rin, n = 7) than in pyramidal cells (820 ± 173 pA and 65 ±
3% decrease in $R_{in}$, $n = 5$; Fig. 1, C1 and C2; $P < 0.05$, pyramidal cells vs. interneurons). Comparison of GlyR current density (pA/pF) versus percent decrease in $R_{in}$ shows, as expected, that larger currents are accompanied by larger changes in $R_{in}$ (Fig. 1D), and furthermore, there is an overall larger current density measured in interneurons.

**GlyR activation depresses action potentials in pyramidal cells and interneurons initiated by somatic injection of depolarizing current**

GlyRs are well-characterized mediators of neuronal inhibition in spinal cord and brain stem. Therefore we next tested, using whole cell current-clamp recordings from both pyramidal cells and interneurons, whether GlyR activation can depress somatically induced action potentials after direct depolarizing current injection through the patch pipette. In these experiments, we injected hyperpolarizing, subthreshold, threshold, and suprathreshold depolarizing current pulses in the absence and presence of glycine to determine GlyR-mediated effects on $R_{in}$ and somatically generated action potential firing. Glycine was bath applied so that the effects of GlyR activation on action potential generation could be assessed after the current reached steady state. Although GlyR currents partially desensitize, there is a significant steady-state current (>50% of peak amplitude in both pyramidal cells and interneurons) maintained for the duration of glycine application (at least ≤10 min, the longest we have measured; Chattipakorn and McMahon 2002). Importantly, there is no statistical difference in the amount of desensitization between pyramidal cells and interneurons (Chattipakorn and McMahon 2002).

Bath application of glycine (300 μM) elicited a modest depolarization, rather than hyperpolarization, of the membrane potential from the resting potential (pyramidal cells: resting potential, $69 \pm 1$ mV; GlyR-induced depolarization, $2 \pm 1$ mV, $n = 5$; interneurons: resting potential $70 \pm 2$ mV; GlyR-induced depolarization, $6 \pm 1$ mV, $n = 6$, $P < 0.05$ pyramidal cells vs. interneurons) that was accompanied by a significant decrease in $R_{in}$ (measured during steady state rather than at the peak as in Fig. 1) in both cell types [Fig. 2, A and B, cf. amplitude of the downward deflection (blue traces) in left and middle; $33 \pm 4\%$ decrease in pyramidal cells, $n = 5$; $48 \pm 5\%$ decrease in interneurons, $n = 6$, $P < 0.05$ pyramidal cells vs. interneurons]. The glycine-induced depolarization of the membrane potential indicates that the chloride reversal potential is more depolarized than the resting potential, which is often the case for GABA$_A$R-mediated currents (Jackson et al. 1999; Staley and Mody 1992). After washout, the change in membrane potential and $R_{in}$ recovered to their original values. Similar to our findings in the voltage-clamp recordings, there is a larger decrease in $R_{in}$ (and membrane potential depolarization) in interneurons compared with pyramidal cells.

When examining the effect of GlyR activation on action potential generation, we found that glycine application completely prevented somatically induced action potentials elicited at a threshold stimulus in both cell types (Fig. 2, A and B, cf. red traces in left and middle; pyramidal cells, $n = 5$; interneurons, $n = 5$; Fig. 2, C1 and C2; $P < 0.05$). Interestingly, under control conditions, the remaining 2 interneurons, number of action potentials was significantly reduced (C, right). D: bar chart shows average decrease in $R_{in}$ for pyramidal cells ($33 \pm 4\%$, $n = 5$) and interneurons ($48 \pm 5\%$, $n = 6$) during glycine application ($P < 0.05$, pyramidal cells vs. interneurons). For cells shown in A and B, calculated $R_{in}$ decreased from $160 \pm 23$ MΩ in control to $110 \pm 21$ MΩ during glycine application in the pyramidal cell recording and from $215 \pm 23$ MΩ in the interneuron recording. All cells were recorded with K$^+$ gluconate pipette solution and held at their resting membrane potential (pyramidal cells: $69 \pm 1$ mV, $n = 5$; interneurons: $70 \pm 2$ mV, $n = 6$).
rons, \( n = 6 \)). However, when suprathreshold depolarizing pulses were applied, GlyR activation completely blocked action potential generation in four of six recorded interneurons and in three of five recorded pyramidal cells (Fig. 2C, also cf. black traces in left and middle of A and B), but in the remaining cells, the number of action potentials was decreased but not completely eliminated.

**GlyR activation depresses synaptically generated action potentials**

Regulation of excitatory circuits is critical to normal hippocampal function. Therefore we next sought to determine whether GlyR activation could depress synaptically generated action potentials elicited by stimulation of excitatory synapses. We tested this idea by recording in whole cell current-clamp suprathreshold EPSPs generated through electrical stimulation of schaffer collateral afferents located in s. radiatum (0.1 Hz). In these experiments, we set our stimulus intensity to a minimum (threshold) value so that only one synaptically generated suprathreshold EPSP was evoked in the majority of trials.

Within 1–2 min, bath perfusion of glycine (300 \( \mu M \)) reversibly depressed suprathreshold EPSPs to subthreshold events in both pyramidal cells (\( n = 9/10 \)) and interneurons (\( n = 10/10 \); Fig. 3, A and B). This effect of glycine was prevented in the presence of 1 \( \mu M \) strychnine. Although suprathreshold EPSPs were depressed to subthreshold events in nearly all cells recorded (1 pyramidal cell had no effect), the magnitude of the depression was variable from cell to cell (Fig. 3, A1 and B1).

In pyramidal cells, the percentage of trials generating suprathreshold EPSPs during glycine application was decreased by \( 22 \pm 8% \) (\( n = 4 \)) and by \( 27 \pm 8% \) in interneuron recordings (\( n = 4 \); \( P > 0.37 \), pyramidal cells vs. interneurons). Because glycine application increases N-methyl-D-aspartate receptor (NMDAR)-mediated currents (Bergeron et al. 1998; Wilcox et al. 1996), we wondered whether a potential increase in NMDAR transmission was limiting the synaptic depression mediated by GlyRs. Therefore we tested whether the percentage of trials generating suprathreshold EPSPs would be further decreased in the presence of APV (100 \( \mu M \)) to block NMDARs. In pyramidal cells, the percentage of trials generating suprathreshold EPSPs was decreased by \( 32 \pm 6\% \) (\( n = 6 \)). Although there is a trend toward a greater amount of depression when NMDARs are blocked, the values are not significantly different (\( P > 0.08 \)). Similarly, in interneurons, the percentage of trials generating suprathreshold EPSPs was also not significantly different during blockade of NMDARs with APV compared with without the antagonist (33 \( \pm 5\% \) decrease in \( \alpha \)-APV, \( n = 6 \), vs. 27 \( \pm 8\% \) without \( \alpha \)-APV, \( n = 4 \), \( P > 0.25 \)).

In sum, our findings indicate that GlyR activation depresses synaptically driven action potentials in both pyramidal cells and interneurons.

**FIG. 3.** Synaptically evoked suprathreshold excitatory postsynaptic potentials (EPSPs) are reduced to subthreshold events by GlyR activation. A1 and B1: representative traces show suprathreshold EPSPs recorded from a pyramidal cell and an interneuron evoked by electrical stimulation of schaffer collaterals (0.1 Hz). Stimulus intensity was set near threshold to generate a single suprathreshold EPSP in the majority of trials. Bath application of glycine (300 \( \mu M \)) to block NMDARs. In pyramidal cells, the percentage of trials generating suprathreshold EPSPs was decreased by 32 \( \pm 6\% \) (\( n = 6 \)). Although there is a trend toward a greater amount of depression when NMDARs are blocked, the values are not significantly different (\( P > 0.08 \)). Similarly, in interneurons, the percentage of trials generating suprathreshold EPSPs was also not significantly different during blockade of NMDARs with APV compared with without the antagonist (33 \( \pm 5\% \) decrease in \( \alpha \)-APV, \( n = 6 \), vs. 27 \( \pm 8\% \) without \( \alpha \)-APV, \( n = 4 \), \( P > 0.25 \)). In sum, our findings indicate that GlyR activation depresses synaptically driven action potentials in both pyramidal cells and interneurons.

A2: summary plots show percentage of trials that elicit suprathreshold EPSPs during control conditions, bath application of glycine (300 \( \mu M \)), and washout for each recorded cell; \( \geq 60 \) trials per condition were analyzed, and number of suprathreshold EPSPs for each cell in control conditions was normalized to 100% for ease of comparison. For each cell type, 4 cells were recorded in absence of N-methyl-D-aspartate receptor (NMDAR) antagonist \( \alpha \)-APV (as in A1 and B1 above, A1 and B1 and 6 cells were recorded in 100 \( \mu M \) \( \alpha \)-APV (\( \bullet \)) to block potential glycine-mediated increase in NMDAR currents. The percentage of trials generating suprathreshold EPSPs in pyramidal cells during glycine application was decreased by 22 \( \pm 8\% \) in the absence of NMDAR blockade (\( n = 4 \)) and by 32 \( \pm 6\% \) when NMDARs were blocked by \( \alpha \)-APV (\( n = 6 \), \( P > 0.08 \) vs. the presence of \( \alpha \)-APV). In interneurons, percentage of trials generating suprathreshold EPSPs was decreased by 27 \( \pm 8\% \) (\( n = 4 \)) in the absence and 33 \( \pm 5\% \) in the presence of NMDAR blockade with \( \alpha \)-APV (100 \( \mu M \), \( n = 6 \), \( P > 0.25 \) in the absence vs. presence of \( \alpha \)-APV). Magnitude of depression observed in pyramidal cells is not significantly different from that in interneurons (\( P > 0.05 \)). All cells were recorded with K\(^+\) gluconate pipette solution and were held at their resting membrane potential (pyramidal cells: 67 \( \pm 1\) mV, \( n = 10 \); interneurons: 68 \( \pm 1\) mV, \( n = 10 \)).
rons, indicating the involvement of a general mechanism that affects both major cell types in hippocampus.

We also noted in a few cells that during strychnine perfusion, a second EPSP after the synaptically generated action potential can be recruited (Fig. 3A, arrows), and in one case, the second EPSP reached threshold (interneuron shown in Fig. 3B, arrows). The increase in excitability is not a result of strychnine-induced reduction of GABA<sub>R</sub>-mediated inhibition. Rather, the amplitude of evoked GABA<sub>R</sub>-mediated inhibitory postsynaptic currents (IPSCs; recorded in 10 μM DNQX and 50 μM d-APV) is significantly increased (129 ± 3%, P < 0.01, n = 4, data not shown). Interestingly, this was also the case for the evoked glutamatergic excitatory postsynaptic current (EPSC) amplitude (recorded in 10 μM bicuculline; 192 ± 20%, P < 0.01, n = 5, data not shown) where the effect was greater. The increase in amplitude of the synaptic currents could be explained by better space clamp because of blockade of tonically active GlyRs that are open during basal conditions (Mori et al. 2002). Thus, the strychnine-mediated increase in excitability observed in the current-clamp recordings is likely caused by inhibition of GlyRs that are tonically open under basal conditions, although we cannot completely exclude some nonspecific effects of strychnine on other channels (Garcia-Colunga and Miledi 1999; Matsubayashi et al. 1999). We also noted in a few cells that during strychnine perfusion, GlyR-mediated depression of IPSC amplitude recorded from pyramidal cells and interneurons (GABA<sub>A</sub>R-mediated IPSCs) was blocked by 1 mM strychnine, indicating involvement of GlyRs. Note that in the presence of strychnine, amplitude of EPSC and IPSC is increased compared with control (arrows). Bicuculline (10 μM) and d-APV (100 μM) or kynurenic acid (2 mM) were included in the bath solution to isolate EPSCs (mediated by AMPARs only) and IPSCs, respectively. All traces shown are averages of 20 consecutive events. C and E: plots are a schematic representation of experiments involving cells shown in A and B showing GlyR-induced depression of EPSC (C) and IPSC (E) amplitude. D: summary plot shows normalized average of all experiments examining the GlyR-induced depression of EPSCs recorded from pyramidal cells and interneurons. Mean EPSC amplitude was depressed to 48 ± 7% and 52 ± 7% of control in pyramidal cells (n = 5) and interneurons (n = 7), respectively (values not significantly different, ANOVA, P ≥ 0.36). F: summary plot shows normalized average of all experiments examining the GlyR-induced depression of IPSC amplitude recorded from pyramidal cells and interneurons. Mean IPSC amplitude was depressed to 56 ± 2% and 66 ± 7% of control from pyramidal cells (n = 4) and interneurons (n = 9), respectively (values not significantly different, ANOVA, P ≥ 0.31). All recordings were performed with a Cs<sup>+</sup> chloride pipette solution and cells were voltage clamped at −70 mV. Points plotted in D and F represent mean ± SE.

GlyR activation depresses the amplitude of evoked monosynaptic currents

There are several potential mechanisms that could underlie the GlyR-mediated depression of synaptically evoked suprathreshold EPSPs, including depression of glutamate release by presynaptic GlyRs (Turecek and Trussell 2001), facilitation of GABA transmission by presynaptic GlyRs (Ye et al. 2004), or a postsynaptic mechanism resulting from GlyR-mediated changes in membrane potential and R<sub>m</sub> that could shunt incoming synaptic currents, similarly to GABA<sub>A</sub>R activation (Jackson et al. 1999; Staley and Mody 1992). To determine which mechanism(s) are responsible, we performed a series of experiments in voltage clamp where we measured changes in the amplitude of pharmacologically isolated AMPA receptor (AMPAR)-mediated EPSCs and GABA<sub>A</sub>R receptor (GABA<sub>A</sub>R)-mediated IPSCs after GlyR activation. We limited our examination here to EPSCs mediated by AMPARs to simplify data interpretation because applied glycine can enhance NMDAR-mediated currents (Bergeron et al. 1998; Wilcox et al. 1996).

In voltage-clamp recordings, we found that activation of GlyRs by bath application of glycine (300 μM; CsCl pipette solution) elicited a >50% depression of both EPSCs (68 ± 15% n = 5 pyramidal cells; 64 ± 5% n = 10 interneurons) and IPSCs (52 ± 5%, n = 5 pyramidal cells; 64 ± 6%, n = 7 interneurons) that quickly recovered after washout (Fig. 4). The effect of glycine on EPSC and IPSC amplitude was prevented by strychnine (1 μM), showing the involvement of GlyRs. Interestingly, the amount of depression of EPSC and IPSC amplitude recorded from either cell type was not signifi-

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significantly different ($P \geq 0.42$ for EPSC vs. IPSC depression in pyramidal cells and $P \geq 0.23$ for the same comparison in interneurons), indicating that the mechanism causing the depression is not cell type specific and is not specific to a certain set of synapses. Rather, these findings indicate that the mechanism of depression is a general mechanism affecting all synapses on both cell types.

We entertained the possibility that the GlyR-mediated depression of evoked EPSCs in hippocampus is a result of activation of presynaptic GlyRs because these ligand-gated ion channels have been shown to depress glutamate release at synapses in auditory brain stem (Turecek and Trussell 2001; Ye et al. 2004). To determine whether presynaptic GlyRs modulate glutamate release at schaffer collateral synapses, we examined the paired-pulse facilitation (PPF) ratio at CA3 synapses on pyramidal cells before and after bath application of glycine (300 μM). The PPF ratio (determined by dividing the amplitude of the second EPSC by the first when pairs of pulses are given at short intervals, here 50 ms) is an indirect measure of the initial release probability. An increase in this ratio during the course of an experiment is most often interpreted as a decrease in the release probability (Dobrunz and Stevens 1997; Kim and Alger 2001; Zucker 1989). However, we found that GlyR activation causes no change in the PPF ratio at glutamate synapses onto pyramidal cells (PPF ratio 1.9 ± 0.3 during baseline compared with 1.7 ± 0.2 during glycine, $n = 5$, $P > 0.1$, data not shown), indicating that the mechanism underlying the GlyR-mediated synaptic depression is most likely not presynaptic (Dobrunz and Stevens 1997; Kim and Alger 2001; Zucker 1989). Because CA3 synapses onto interneurons have a different release probability than those onto pyramidal cells (there is often paired-pulse depression or no change in transmission; Sun et al. 2005), examination of the paired-pulse ratio at interneuron synapses is not straight forward. Therefore given this issue, together with the lack of an effect on the PPF ratio at pyramidal cell synapses, we did not investigate this further.

Presynaptic GlyRs have been shown to increase, not decrease, GABA$_A$R-mediated transmission (Ye et al. 2004). Therefore depression of the evoked IPSC amplitude is not likely mediated by presynaptic GlyRs. We considered the possibility that an alteration in $E_{\text{Cl}^-}$ during prolonged GlyR activation could be the mechanism. However, we observed no change in the reversal potential of currents mediated by exogenous application of GABA (300 μM, 3 s) in the presence of glycine compared with without glycine ($E_{\text{Cl}^-} = 0$ mV; $E_{\text{GABA}A} = 0.03 \pm 0.06$ mV in the absence and 0.01 ± 0.01 mV in the presence of glycine, $P \geq 0.3$, $n = 10$, data not shown) indicating that $E_{\text{Cl}^-}$ has not been altered and therefore is not responsible for the GlyR-mediated depression of evoked GABA$_A$R-mediated IPSCs.

**GlyR-induced depression of synaptic transmission is mediated through a postsynaptic mechanism**

The lack of a significant effect of GlyR activation on the PPF ratio during a >50% depression of the EPSC amplitude suggests that the GlyR-mediated depression of transmission is most likely not caused by presynaptic depression of neurotransmitter release. Also, because both EPSCs and IPSCs are depressed to the same degree in pyramidal cells and interneurons, we reasoned that the mechanism underlying the depression must be caused by a common postsynaptic mechanism that does not selectively affect excitatory or inhibitory synapses. We therefore investigated whether a postsynaptic locus is involved by performing several types of experiments. Because all of the results we have obtained thus far are common to both pyramidal cells and interneurons, the mechanism underlying the synaptic depression is likely to be the same between the cell types. Therefore to facilitate the study, we focused the next series of experiments on a single cell type and chose to concentrate on interneurons because GlyR responses are larger in these cells. However, as previously mentioned, because all of our findings thus far are the same between cell types, any conclusions reached in these experiments can most likely be extrapolated to pyramidal cells.

First, we reasoned that if the magnitude of the postsynaptic GlyR-mediated current contributed to the amount of depression of the synaptic events, changing the driving force for chloride in the postsynaptic cell should change the magnitude of the depression. We found that the depression of the EPSC amplitude is significantly less when recordings were performed with internal solution containing 10 mM chloride versus 110 mM chloride (Fig. 5, A and B; 17 ± 5% depression, Cs$^+$ gluconate)
solution, \(E_{\text{Cl}^-} = \text{approximately} -60 \text{ mV}, n = 6 \text{ vs. } 51 \pm 4\% \text{ depression}, Cs^+ \text{ chloride solution}, E_{\text{Cl}^-} = 0 \text{ mV}, n = 5\). As expected, the larger magnitude depression correlates with the larger postsynaptic GlyR-mediated current (146 ± 51 pA with \(Cs^+\) gluconate, \(n = 6\), vs. 656 ± 152 pA with CsCl, \(n = 5\); \(P < 0.05\)). Although the magnitude of the depression (and GlyR-depression, \(Cs^+\) gluconate) is much less using 10 mM intracellular chloride (\(Cs^+\) gluconate pipette solution), the amplitude of the EPSC is significantly depressed from baseline during glycine application (\(P < 0.05\)).

The above results indicate that the mechanism of the depression must be postsynaptic because the magnitude of the depression is altered when the chloride driving force in the postsynaptic cell is altered. Therefore, if the GlyR-mediated depression of the evoked EPSC amplitude is indeed postsynaptic, GlyR activation should depress pharmacologically isolated AMPAR- and GABAAR-mediated currents elicited by exogenous application of agonist (which will activate both synaptic and extrasynaptic receptors). Furthermore, blocking postsynaptic GlyRs by including the nonspecific chloride channel blocker DIDS (Deleuze et al. 1998; Saransaari and Oja 1998) in the pipette solution should prevent the effects of glycine on AMPAR currents, both synaptically evoked and elicited through exogenous agonist application. Note that because including DIDS in the pipette solution blocks GABAAR currents (Albertson et al. 1996; Hollrigel et al. 1998), GlyR-mediated effects in these experiments are limited to the examination of AMPAR-mediated responses.

Indeed, we found that pharmacologically isolated AMPAR and GABAAR currents elicited by brief application of glutamate (100 \(\mu M\), 3 s) and GABA (50 \(\mu M\), 3 s), respectively, were depressed (42 ± 5%, \(n = 5\), and 50 ± 4%, \(n = 5\), respectively; Fig. 6, A1 and A2) by bath perfusion of glycine (300 \(\mu M\), CsCl pipette solution) during blockade of synaptic transmission with TTX (1 \(\mu M\)). In the next set of experiments, postsynaptic GlyRs were blocked by including DIDS in the pipette solution to test whether the effects of GlyR activation on the amplitude of AMPAR-mediated currents was prevented. First we confirmed that DIDS was able to block GlyRs and found that within 10–15 min of whole cell recording, GlyR-mediated currents were depressed nearly 80% (Fig. 6B1, \(n = 6\)). The depression of the GlyR currents with DIDS in the pipette solution cannot be explained by a nonspecific rundown of GlyR currents because we showed in Fig. 1 that these currents remain stable after repeated application of agonist during this same time frame. In an additional set of control experiments, we found that intracellular DIDS also blocks GABAAR-mediated currents (49 ± 7% within 20 min of whole cell recording, \(n = 4\), data not shown) as previously reported (Albertson et al. 1996; Hollrigel et al. 1998). As expected, when postsynaptic GlyRs are blocked by DIDS, glycine application no longer was able to depress AMPAR currents elicited by exogenous glutamate application (Fig. 6B2). Moreover, inclusion of DIDS completely inhibited the glycine-induced depression of evoked EPSCs (Fig. 6, C and D; \(n = 7\)), and we observed no GlyR-mediated effects in these experiments. Thus
collectively, these findings provide strong evidence that a postsynaptic locus is the site of action of the GlyR-mediated depression of transmission.

In a final set of experiments, we attempted to test whether a decrease in $R_m$ alone is enough to permit the synaptic depression by holding cells (in current-clamp recordings) near the reversal potential of GlyR currents (approximately $-50$ mV; empirically tested and see Chattiparkorn and McMahon 2002) to minimize net movement of ions. In these experiments, it was necessary to examine subthreshold EPSPs (while blocking action potential generation with QX314 included in the pipette solution), because holding the membrane potential at the GlyR reversal potential is too near action potential threshold, and cells were randomly firing. Additionally, in these experiments, we were limited to an examination of only EPSPs because GABAergic inhibitory postsynaptic potentials (IPSPs) reverse at the same potential as GlyR-mediated currents. Thus, based on Ohms’ Law, a synaptic current evoked when the membrane resistance is decreased (e.g., caused by open GlyR channels) will cause a smaller voltage change than when the resistance is higher (e.g., no open GlyR channels). Accordingly, it is predicted that the EPSP amplitude will be depressed by GlyR activation even if there is little to no net current through the open channels. In 5/10 recorded cells, the membrane potential was unchanged during glycine application but was slightly depolarized (1–3 mV) in the remaining 5 cells. Importantly, however, in all cells recorded, GlyR activation reversibly depressed the EPSP amplitude (Fig. 7; $31 \pm 3\%$ depression, $n = 10$) and the depression was blocked by 1 $\mu M$ strychnine.

**Blocking the glycine transporter GlyT1 depresses synaptic excitability**

We were next interested in determining whether endogenously released glycine could modulate synaptic transmission through activation of GlyRs, similarly to what we have observed after exogenous glycine application. Electrical stimulation in CA1 s. radiatum releases glycine through a mechanism dependent on action potential generation, suggesting that at least some of the glycine released in hippocampus is of neural origin (Klancnik et al. 1992). Interestingly, blockade of GlyT1, the glycine transporter present in astrocytes (Zafra et al. 1995, 1997), potentiates evoked NMDAR-mediated currents in hippocampal slices (Bergeron et al. 1998) by elevating the extracellular glycine concentration in the vicinity of NMDARs. Here we tested whether preventing reuptake of glycine into astrocytes using the a potent GlyT1 transport inhibitor NFPS (Chen et al. 2003; Herdon et al. 2001) would cause accumulation of endogenously released glycine that could activate GlyRs and depress synaptically generated action potentials. In recordings from interneurons ($n = 4$), but not pyramidal cells ($n = 4$), we observed a depression of synaptically evoked suprathereshold EPSPs in the presence of low concentrations of NFPS (1 $\mu M$; Fig. 8). The NFPS-induced depression was antagonized by strychnine, indicating that the effect is mediated by GlyRs and not the result of a nonspecific action of the drug. As mentioned above, blocking GlyT1 with NFPS potentiates NMDAR currents in CA1 pyramidal cells (Bergeron et al. 1998), therefore we considered the possibility that the GlyR-mediated depression of EPSP amplitude was masked by an increase in NMDAR transmission. However, even when we included the NMDAR antagonist APV (100 $\mu M$) in the bath solution together with the NFPS, the EPSP amplitude still was not significantly depressed in pyramidal cells ($5 \pm 4\%$ depression, $n = 4$, $P > 0.05$). It is unclear why the NFPS-induced depression of suprathereshold EPSPs was not observed in recordings from pyramidal cells similar to what was observed in interneurons, but this finding may indicate that GlyT1 on astrocytes are not expressed in the near vicinity of GlyRs on pyramidal cells. The GlyT2 transporter is expressed by neurons; however, pharmacological tools are not yet available commercially to test whether blocking this reuptake mechanism can modulate synaptic transmission recorded from pyramidal cells or interneurons. Nevertheless, these experiments using the GlyT1 antagonist clearly show that extracellular levels of endogenous glycine can be altered through modulation of glycine reuptake, suggesting that changes in the extracellular glycine concentration in vivo could lead to a depression of neuronal activity mediated through GlyR activation.
Inhibitory glycinergic interneurons in spinal cord and brain stem also contain and release GABA (Chery and de Koninck 1999; Jonas et al. 1998; O’Brien and Berger 1999). We therefore considered the possibility that the converse may be true in hippocampus, that GABAergic interneurons also contain glycine. We tested this idea using a highly specific antglycine antibody (Crook and Pow 1997; Pow et al. 1995) (generous gift of Dr. David Pow, University of Queensland) and found that hippocampal interneurons, but not pyramidal cells, are immunopositive for glycine (Fig. 9, A–D, G, and J). Additionally, presumed astrocytes were also observed to be glycine immunopositive (Fig. 9G, *), an expected result because previous studies have shown that these cells express the glycine transporter, GlyT1 (Zafra et al. 1995, 1997). In double-labeling experiments we find that all glycine-positive interneurons are also immunopositive for the neuronal glycine transporter GlyT2, a reliable marker for glycinergic neurons (Jursky et al. 1981; Seiler and Sarhan 1984a,b). This is further supported by another GlyR agonist, depress seizure activity in some animal models of epilepsy, first reported two decades ago (Cherubini et al. 2002) by showing that GlyR activation is capable of depressing activity in the hippocampal synaptic network. This ability of GlyRs could be the mechanism by which glycine and taurine, another GlyR agonist, depress seizure activity in some animal models of epilepsy, first reported two decades ago (Cherubini et al. 1981; Seiler and Sarhan 1984a,b). This is further supported by recent reports in hippocampal slices showing that GlyR activation can decrease hyperexcitability in the dentate gyrus and area CA1 (Chattipakorn and McMahon 2003; Kirchner et al. 2003).

A major finding in our study is that GlyR activation depresses synaptically generated action potentials recorded from both pyramidal cells and interneurons, indicating that these receptors have a general function to limit activity in hippocampal circuits. In addition, we find in some cells strychnine increases excitability, indicating that GlyRs are tonically active and can set the ambient level of neuronal activity, as suggested by others (Mori et al. 2002), although it is worth mentioning that strychnine could have some nonspecific effects (Garcia-Colunga and Miledi 1999; Matsubayashi et al. 1998). Tonic

**GABAergic interneurons are a source of glycine in hippocampus**

These data suggest the interesting possibility that a subset of interneurons may use this inhibitory amino acid exclusively, although much more work is needed to confirm this initial observation. Together our immunohistochemical results support and extend the elegant studies of Danglot et al. (2004), where hippocampal interneurons were shown to express the glycine transporter, GlyT2.

**DISCUSSION**

Despite previous misconceptions, it is now clear that GlyRs are expressed by neurons in mature hippocampus (Chattipakorn and McMahon 2002, 2003; Kirchner et al. 2003). Here, we extend our previous results (Chattipakorn and McMahon 2002) by showing that GlyR activation is capable of depressing activity in the hippocampal synaptic network. This ability of GlyRs could be the mechanism by which glycine and taurine, another GlyR agonist, depress seizure activity in some animal models of epilepsy, first reported two decades ago (Cherubini et al. 1981; Seiler and Sarhan 1984a,b). This is further supported by recent reports in hippocampal slices showing that GlyR activation can decrease hyperexcitability in the dentate gyrus and area CA1 (Chattipakorn and McMahon 2003; Kirchner et al. 2003).
inhibition is provided by extrasynaptic GABA_{A}Rs (Farrant and Nusser 2005; Petrini et al. 2004), thus tonically active GlyRs are also likely to be extrasynaptic. This idea is supported by a recent anatomical study showing that nearly 70% of clustered GlyRs are not opposed by presynaptic terminals and are thus at extrasynaptic sites (Danglot et al. 2004).

GlyRs are expressed both pre- and postsynaptically in many brain regions; however, several findings suggest that the GlyRs responsible for depressing neuronal excitability and synaptic transmission are located at postsynaptic rather than at presynaptic sites. Clearly, depression of somatically induced action potentials through DC injection must be caused by a postsynaptic mechanism because synapses are not involved. The large decrease in $R_{in}$ that accompanies GlyR activation is likely a major contributor to this depression of action potential generation because a given stimulus is much less effective in changing membrane voltage when the resistance is decreased by open channels. This mechanism could also easily explain the depression of synaptically induced action potentials (suprathreshold EPSPs). A postsynaptic mechanism is further supported by the larger depression of the EPSC amplitude when the postsynaptic GlyR current is increased by an increased driving force for chloride and also by the lack of depression of the EPSC amplitude when postsynaptic GlyRs are blocked by including DIDS in the pipette solution. However, a point that should be mentioned regarding the voltage-clamp experiments is that the magnitude of the postsynaptic GlyR current should, provided the recorded cells are well clamped, have no measurable effect on the amplitude of the EPSC, because the voltage-clamp circuit should compensate for the GlyR current that attempts to change membrane voltage. It is well-known that voltage control decreases with distance from the recording site (soma) (Carnevale et al. 1997; Spruston et al. 1993). Thus the larger synaptic depression, which accompanies the larger postsynaptic GlyR current, may be a consequence of inadequate space clamp. If true, this could indicate that the GlyRs responsible for the synaptic depression are “out of the reach” of the clamp, and may be in dendrites near the glutamatergic synapses, because somas are generally under good voltage control. Future studies involving direct recordings from dendrites will be needed to address this issue further. Finally, we cannot rule out some contribution of a
GlyR mediated decrease in presynaptic release probability, even though we find no significant effect of GlyR activation on the PPF ratio (Dobrunz and Stevens 1997; Kim and Alger 2001; Zucker 1989).

Are GlyRs involved in fast inhibitory transmission in hippocampus? It seems possible because our immunohistochemical studies show that hippocampal interneurons co-contain glycine and GABA and also express the neuronal glycine transporter GlyT2 (Borowsky et al. 1993; Danglot et al. 2004). This suggests the interesting possibility that interneurons use both inhibitory neurotransmitters, similarly to interneurons in spinal cord and brain stem and Golgi cells in cerebellum which co-release glycine and GABA at mixed synapses (Chery and de Koninck 1999; Dumoulin et al. 2001; Jones 1993; O’Brien and Berger 1999). However, it seems that the majority of inhibitory synapses in hippocampus are devoid of GlyRs (Danglot et al. 2004; Levi et al. 2004; Meier and Grantyn 2004). Furthermore, data from electron microscopy studies show that when GlyRs are found at synapses, they are expressed at low density and are located near the edge of the postsynaptic density or just outside (Danglot et al. 2004). However, synaptic clustering of GlyRs can be increased when clustering of GABA<sub>R</sub>Rs is prevented by depletion of the gephyrin splice variant responsible for formation of GABA<sub>R</sub> clusters (Meier and Grantyn 2004). This gephyrin isoform, but not the isoforms responsible for clustering GlyRs, is usually highly expressed at hippocampal synapses indicating a predominant synaptic localization of GABA<sub>A</sub>R<sub>s</sub> and a most likely extrasynaptic location of GlyRs (Meier and Grantyn 2004). Taken together, these morphological data suggest that GlyRs may not be involved in fast synaptic inhibition in hippocampus. This potential synaptic GABA<sub>A</sub>R and extrasynaptic GlyR receptor distribution would thus explain why IPSCs at hippocampal synapses are blocked with GABA<sub>A</sub>R but not GlyR antagonists. Therefore the role of GlyRs in hippocampus may not be to mediate fast synaptic inhibition but instead to serve in a neuromodulatory role at extrasynaptic sites where they provide tonic inhibition, similarly to the tonic inhibition provided by extrasynaptic GABA<sub>A</sub>R<sub>s</sub> (Farrant and Nusser 2005; Pettini et al. 2004). Thus functional data clearly supporting the notion that GlyRs mediate fast synaptic inhibition at hippocampal synapses awaits future confirmation.

Our data showing the depression of suprathreshold EPSPs (recorded from interneurons) after blockade of glycine reuptake into astrocytes (i.e., blockade of GlyT1) shows that endogenously released glycine can activate GlyRs and modulate neuronal excitability. The lack of an effect of GlyT1 blockade on evoked EPSPs recorded from pyramidal cells, similarly to that reported by Mori et al. (2002) in recordings from cultured hippocampal slices, could be caused by the co-localization of GlyT1 near glutamate synapses (Cubelos et al. 2005), which would regulate NMDAR activity, rather than being located near sites of GlyR expression on pyramidal cells. Because blockade of GlyT1 will prevent both glycine reuptake into astrocytes as well as glycine release that occurs after astrocyte depolarization, it seems that the source of the glycine that accumulates extracellularly in the presence of NFPS is not likely to be released from astrocytes and suggests that it may be of neural origin, likely from the interneurons through vesicular and/or transporter-mediated (GlyT2) release. Unfortunately development of pharmacological tools directed at GlyT2 lags behind that of GlyT1; therefore resolution of the functional role of GlyT2 awaits further clarification and investigation.

In summary, we propose that GlyRs participate in a novel inhibitory mechanism in hippocampus, modulating neuronal activity and synaptic transmission. The GlyR-mediated depression of synaptic potentials in hippocampus we report here is similar to the GlyR-mediated depression of nicotinic receptor–mediated EPSPs at synapses in the ciliary ganglia (Tsien et al. 2000), suggesting that this role of GlyRs in modulating synaptic efficacy could be a general mechanism applicable to many synapses throughout the autonomic and central nervous systems. Because inhibitory interneurons and excitatory pyramidal cells express GlyRs, we predict the net effect of GlyR activation in vivo is likely to be quite complex because selective activation of GlyRs on interneurons will cause disinhibition, whereas selective activation on pyramidal cells will cause net inhibition. Nevertheless, the findings in this study, together with our previously published work in dentate gyrus, show that globally enhancing GlyR activity throughout the network will cause net inhibition. Thus our findings indicate that therapeutic agents targeted at GlyRs, similarly to those that increase GABA<sub>A</sub> receptor activity, could be beneficial in depressing hyperexcitable hippocampal circuits that ensue in epilepsy, encouraging future studies into the precise location and function of these understudied inhibitory receptors.

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